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Evidence of virulence and antibiotic resistance genes from the microbiome mapping in minimally processed vegetables producing facilities

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ABSTRACT

Daily consumption of fresh vegetables is highly recommended by international health organizations, because of their high content of nutrients. However, fresh vegetables might harbour several pathogenic microorganisms or contribute to spread antibiotic resistance, thus representing a hazard for consumers. In addition, little is known about the transmission routes of the residential microbiome from the food handling environment to vegetables. Therefore, we collected environmental and food samples from three manufactures producing fresh vegetables to estimate the relevance of the built environment microbiome on that of the finished products. Our results show that food contact surfaces sampled after routine cleaning and disinfection procedures host a highly diverse microbiome, including pathogens such as the enterotoxigenic *Bacillus cereus sensu stricto*. In addition, we provide evidence of the presence of a wide range of antibiotic resistance and virulence genes on food contact surfaces associated with multiple taxa, thus supporting the hypothesis that selection of resistant and pathogenic taxa might occur on sanitized surfaces. This study also highlights the potential of microbiome mapping routinely applied in food industries monitoring programs to ensure food safety.

1. Introduction

Fresh vegetables are an essential part of a healthy dietary pattern and have been used for centuries (Randhawa et al., 2015). Indeed, these foods contain high levels of phytochemicals, fiber and minerals (Liu, 2013). International organizations such as the World Health Organization (WHO) suggest a 400 g/day intake of vegetables (World Health Organization (2020), 2020).

Although the consumption of raw vegetables is highly recommended, their use arises concerns about their safety. Indeed, raw vegetables are subjected to limited processing before their arrival to the shelf, that includes selection and (optional) portioning and removal of non-edible parts. In some cases, a rough washing step is applied. Therefore, they might represent a risk for the health of the consumers, since it has been demonstrated that several pathogenic taxa can survive and proliferate on their surfaces (Al-Kharousi et al., 2016; Tatsika et al., 2019; Yin et al., 2022). This evidence, together with the inefficiency of domestic washing procedures to remove microorganisms (Tatsika et al., 2019), should draw the attention of the food industry and consumers on the potential outcome that might derive from the consumption of contaminated products.

Recent reports (Carstens et al., 2019) indicate that a large part of foodborne outbreaks can be linked to the consumption of minimally processed vegetables such as sprouts, lettuce, cucumbers and spinaches, with a wide range of associated symptoms, including bloody diarrhoea and gastroenteritis. Most of these outbreaks are attributed to well-known pathogens conveyed by fresh vegetables, such as *Salmonella enterica* and *Escherichia coli* O157:H7 (Carstens et al., 2019), although the range of hazardous microorganisms that could survive and replicate in fresh vegetables is wider, also including *B. cereus* and *Pseudomonas aeruginosa* (Afolabi et al., 2011; Fiedler et al., 2019; Rosenquist et al., 2005; Yu et al., 2019). In addition, several opportunistic pathogens, such as *Pantoea agglomerans*, *Klebsiella pneumoniae* and *Rahnella aquatilis* have been also reported (Al-Kharousi et al., 2016).

Also, contamination of fresh vegetables might occur at multiple points from farm to fork. The soil is the primary source of pathogenic

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microorganisms, since minimally processed vegetables grow within or near the ground, although irrigation water, fertilizers and insects may also carry hazardous microbes (Carstens et al., 2019). However, post-harvesting and processing of vegetables also contribute to contamination, due to the contact with transportation vehicles, operators and equipment inhabited by pathogens (Carstens et al., 2019).

Multiple studies have shown that some pathogenic/commensal bacteria associated with food and its production environment may carry out Antibiotic Resistance Genes (ARGs) in their genomes, which might be transferred to other microorganisms through Mobile Genetic Elements (MGEs) and represent a potential hazard (Oniciuc et al., 2019). According to WHO, antibiotic resistance (AR) is one of the most important public concerns, since the overuse of antibiotics in all fields (e.g., agriculture, farming and individual medications) has led to the selection of resistant strains (Ventola, 2015; World Health Organization (2020), 2020). Indeed, farm soils have been addressed as a "hot spot" of resistant microorganisms (Founou et al., 2016).

Food processing environments are an important reservoir of microorganisms that may be easily transferred to the product. Indeed, microbial consortia might adapt to the specific microclimatic conditions of the food processing plant and establish on the surfaces by forming biofilms (De Filippis et al., 2021). In such circumstances, bacteria might resist to cleaning and disinfection procedures, becoming resident in the food processing environment. For example, *Salmonella* and *Acinetobacter* isolated from vegetables can produce biofilms on various types of surfaces (Bae et al., 2014; Isoken, 2015). Indeed, the combination of AR and biofilm formation represents a successful microbial strategy to promote the survival under environmental stress conditions (Carter & Brandl, 2015; Xu et al., 2021) and enhance the long-term colonization of environmental surfaces associated to food production.

Few investigations about the colonization of the fresh vegetables handling environment by bacteria and on the assessment of their resistance to antimicrobials are available. This topic needs attention and proper investigation, since AR microorganisms embedded into biofilms on industrial surfaces might end up on the vegetables, which are often consumed without prior cooking, spreading ARGs and representing a safety hazard.

The purpose of this work is to assess the taxonomic composition, the antimicrobial resistance and virulence potential (including genes involved in biofilm formation) of the microbiome residing in the environment of three facilities producing minimally processed vegetables in order to ascertain the relevance of the environmental microbiome on the safety of the end products.

2. Materials and methods

2.1. Samples collection, DNA extraction and whole metagenome sequencing

Three facilities from Southern Italy producing minimally-processed vegetables (named G, J, P) were visited (February–October 2020) after the completion of the routinary cleaning procedures. Facility G produced spinaches (*Spinacia oleracea*), whereas facilities J and P produced endive (*Cichorium endivia*) and arugula (*Eruca vesicaria*), respectively. Raw vegetables were not subjected to prior washing, but the process included three steps: separation of soil particles from leaves (by vibration/optical sorting), portioning and packing (Supplementary Fig. 1). Prior to the sampling, details about the cleaning and sanitation procedures were recorded (Table 1).

Food contact (FC) and non-food contact (NFC) surfaces from the facilities were sampled using Whirl-Pak Hydrated PolyProbe swabs (Whirl-Pak, Madison, Wisconsin, US), covering an area of about 1 m^2, or a sampling unit (e.g., one knife, one box). In addition, swabs were collected from hands/aprons of employees working on the sampled production line. Five swabs from each sampling point were collected and pooled before DNA extraction. A total of 32 pooled composite samples were available from the three facilities, including vegetables (about 100 g) at the beginning (n = 6) and at the end of the processing (n = 6), environmental FC swabs (n = 12) and NFC swabs (n = 5), and swabs from hands/aprons of employees (n = 3).

Table 1
Description of the sanitation procedure adopted by each facility. DFC = Disinfectant concentration; CT = Contact time; SF = Sanitation frequency; R1 and R2 = Rinsing; NA = Information not provided.

Facility	Detergent	R1	Disinfectant	DFC – CT (min)	R2	SF
G	Pressurized air/water	H_2O 60 °C	Sodium hypochlorite	25 mL/L – 10 min	H_2O 65 °C	Weekly
J	Pressurized air	NA	Sodium hypochlorite	NA	H_2O 25 °C	Weekly
P	Pressurized air	NA	Sodium hypochlorite	10 mL/L – 5 min	H_2O 25 °C	Weekly

All the samples were stored at 4° C and transported to the laboratory, where they were pre-processed within 2 h.

In the laboratory, the 5 swabs from each surface were pooled together, and 10 mL of Phosphate Buffered Saline (PBS) 1X were added. In addition, the surfaces of the raw materials and final products were swabbed with 5 swabs/sample in sterile conditions and the five samples per sample were pooled together and processed following the same procedures as for the environmental swabs. Microbial cells were detached from the pools of swabs using a Stomacher (300 rpm × 30 s), then the supernatant was collected and aliquoted in 5 mL sterile tubes (Eppendorf, Hamburg, Germany). The tubes were centrifuged at 14.000 × g for 2 min, then the cellular pellet was washed twice with 2 mL of sterile PBS. The cellular pellets were stored at −80 °C until further processing.

DNA extraction was performed from the pellets using the PowerSoil Pro Kit, adopting a modified version of the standard protocol previously validated to increase the total microbial DNA yield from food processing environments (Barcenilla et al., under review). Briefly, these modifications were the use of Qiagen's UCP MinElute Spin Columns instead of the standard spin columns; addition of 600 μl 100 % isopropanol to the silica columns during DNA binding step; addition of 40 % EtOH (100 %) to solution C5 on wash step; and perform the final elution in a volume of 20 μl. Then, the concentration of extracted DNA was quantified using the Qubit HS Assay (Thermo Fisher Scientific, Waltham, Massachusetts, United States).

Metagenomic libraries were prepared using the Nextera XT Index Kit v2 (Illumina, San Diego, California, United States), then whole metagenome sequencing was performed on an Illumina NovaSeq platform, leading to 2 × 150 bp reads.

2.2. Bioinformatic and statistical analysis

Reads were quality-checked by PRINSEQ lite (version 0.20.4; (Schmieder & Edwards, 2011) using parameters "-trim_qual_right 5" and "-min_len 60", then taxonomic profiles were obtained using Kraken2 (Wood et al., 2019), jointly with the "maxikraken2" database (available at https://lomanlab.github.io/mockcommunity/mc_databases.html), using default parameters. Bacterial counts were extracted from each profile and merged in one file using an in-house script, then the proportion of reads mapping to each taxon was computed. In addition, SourceTracker2 (Knights et al., 2011) was used on the bacterial counts, with the options "–beta 0", "–source_rarefaction_depth 1000", "–sink_rarfaction_depth 1000" and "–burnin 500". For this analysis, the initial product and the surfaces were defined as "source", whereas final products were labelled as "sinks".

For each sample, reads were independently assembled into contigs using MEGAHIT (version 1.2.2; Li et al., 2016), filtering out contigs shorter than 1,000 bp. Then the reads from each sample were mapped to the corresponding sample contigs using bowtie2 (version 2.2.9; Langmead & Salzberg, 2012), with parameters "–very-sensitive-local" and "–no-unal". The *jgi_summarize_bam_contig_depths* script, from MetaBAT v2.12.1 (Kang et al., 2015), was used to calculate contigs depth values from the sam files obtained by bowtie2 alignment, mandatory for per-sample contig binning by MetaBAT in order to reconstruct Metagenome-Assembled Genomes (MAGs). Only contigs longer than 1,500 bp were binned.

The CheckM "lineage_wf" workflow (version 1.0.13, Parks et al., 2015), was used to assess the quality of MAGs, and only those with completeness $\geq$ 50 % and contamination $<$ 5 % (i.e., medium/high quality MAGs, with high quality MAGs being those with completeness $>$ 90 %; Pasolli et al., 2019) were retained for further analyses.

Pairwise Mash distances (version 2.0; option "-s 10000"; Ondov et al., 2016) were computed between the MAGs, and a 5 % dissimilarity threshold was used to assign MAGs to a Species-level Genome Bin (SGB), as previously suggested (Pasolli et al., 2019). Taxonomy was inferred by comparing the most complete and less contaminated MAG from each SGB to the MetaRefSGB database (December 2020 release; Pasolli et al., 2019), selecting 5 %, 15 % and 30 % dissimilarity threshold for species, genus and family level, respectively.

In addition, phylogeny of MAGs was inferred with the tool GT-DBTk (version 0.3.3; Chaumeil et al., 2020) using the "classify_wf" and "infer" commands, and the resulting tree was visualized in iTol (version 6.5.3; Letunic & Bork, 2021).

In order to assess the pathogenetic potential of 4 MAGs taxonomically assigned to *B. cereus sensu stricto*, we manually downloaded the sequences of *hblCDA*, *nheABC*, *cytK* and *entFM* operons from the NCBI GenBank database (Supplementary File 1, sheet 2). These genes are responsible for the secretion of *B. cereus* enterotoxins (Senesi & Ghelardi, 2010). Genes were predicted from MAGs using Prokka (version 1.11; Seemann, 2014), then they were mapped to the previously collected sequences using blastn (version 2.2.30; options "-evalue 0.00001", "-perc_identity 50" and "-word_size 7").

Metagenome assemblies were screened for AR and Virulence Factor (VF) genes using TORMES (version 1.3.0, Quijada et al., 2019). Only contigs matching with identity and coverage $\geq$ 80 % were retained for further analyses. Contigs were taxonomically classified with Kraken2 as previously described, then Platon (Schwengers et al., 2020) and PlasFlow ("threshold 0.8"; Krawczyk et al., 2018) were used to assess whether ARG-associated contigs were part of plasmids or chromosomes. In addition, reads per kilobase per million reads (RPKM) abundance of both AR and VF contigs was estimated by multiplying the number of reads mapping to each gene for 10^9 and normalizing for gene length and total number of bacterial reads in the metagenome.

Data visualization and statistical analysis were performed in R environment (version 4.1.3; https://www.r-project.org). Mean values for each group were compared using the Wilcoxon rank sum test ("wilcox.test" from "base" package), with a 0.05 p-value threshold for significant results (unless otherwise stated). The functions "vegdist" and "diversity" from the "vegan" package were used to compute Bray-Curtis distances and alpha diversity indices, respectively, whereas "geom_point" from "ggplot2" plotted the first two Principal Coordinates. Barplots figures were produced using "geom_col" from the "ggplot2" package.

2.3. Data availability

Raw reads are available on the Sequence Read Archive of the National Center of Biotechnology Information (NCBI) under the accession number PRJNA897099.

3. Results

3.1. Taxonomic composition of the microbiome of raw materials, end products and environments and SourceTracker analysis

Pseudomonas was the most abundant taxon in both vegetables and surfaces, with a mean percentage of reads of 16.44 $\pm$ 10.14 % and 8.02 $\pm$ 20.16 %, respectively, followed by *Bacillus* (7.53 $\pm$ 22.58 % and 5.29 $\pm$ 13.27 %). Other abundant genera were *Kocuria* and *Acinetobacter*, which reached 4.77 $\pm$ 5.28 % and 4.55 $\pm$ 14.63 % on surfaces, respectively (Supplementary Fig. 2). In addition, remarkable differences in taxonomic composition were observed between FC/NFC surfaces and food products, as showed by a PCoA based on the Bray-Curtis distance (adonis $p < 0.001$, Fig. 1). This separation might be partially explained by *Pantoea*, *Pseudomonas*, *Enterococcus* and *Escherichia*, that were significantly more abundant on vegetables (both at the beginning and at the end of the processing), as well as *Paracoccus* and *Actinomyces*, that were more abundant on surfaces (both FC and NFC; Supplementary Fig. 3). However, no clear separation of FC and NFC surfaces was observed (Fig. 1). No significant differences were found in alpha diversity parameters among the sample groups (Supplementary Fig. 4).

The analysis with SourceTracker2 identified the initial vegetables as the major source contributor to the microbial composition of the final products. However, FC/NFC surfaces in the production area also had a leading role for all the three facilities, with the overall contribution ranging between 10.0 and 39.2 % (Fig. 2). Moreover, there was a high contribution estimated from unknown sources (i.e., potential sources of contamination that we did not sample), which ranged between 21.2 and 34.7 % in the different facilities (Fig. 2).

3.2. Mags reconstruction and phylogenetic analysis

Overall, a total of 290 medium/high quality bins were reconstructed from the metagenomes. Of these, 181 were included into SGBs with $>$ 1 MAG. From the phylogenetic analysis of MAGs, a separation between foods and surfaces emerged (Fig. 3): in particular, vegetables were dominated by *Proteobacteria*, with genomes assigned to *Pantoea* (n = 9), *Xanthomonas* (n = 4), *Psychrobacter* (n = 5), *Pseudomonas* (n = 7) and *Acinetobacter* (n = 7), whereas *Actinobacteria* (*Kocuria*, n = 27; *Glutamicibacter*, n = 6) and *Bacillota* (*Bacillus*, n = 8; *Staphylococcus*, n = 5) were more prevalent on surfaces.

In addition, 4 out of 8 genomes assigned to the *Bacillus* genus were highly similar to *B. cereus*, a well characterized human pathogen (Fig. 4). Three of these MAGs were reconstructed from 3 FC surfaces from facility "G", whereas 1 was from the operator's hands from facility "J". The alignment of genes predicted from *B. cereus* MAGs to the characteristic virulence gene sequences from this taxon (i.e., *hblCDA*, *nheABC*, *cytK* and *entFM*) suggests the presence of the pathogenic operons in the genomes reconstructed from the surfaces (Supplementary File 1, sheet 1).

3.3. Several taxa from environmental surfaces and vegetables carry ARGs

The screening of the metagenome assemblies for the presence of ARGs highlighted that 277 contigs carried at least one ARG. According to the Kraken2 taxonomic assignment, *Bacillus* harboured the highest number of AR related contigs, with 45 contigs carrying ARGs (Supplementary File 2, sheet 1). Of these, 19 were assigned to *B. cereus*, 7 to *B. clausii* and 6 to *B. thuringiensis*. In addition, *Pseudomonas*, *Pantoea* and *Acinetobacter* contributed significantly to AR, with 30, 22 and 20 contigs, respectively. *Bacillus* sshowed a high number of AR genes from the beta-lactams (n = 20), fosfomycin (n = 6) and multidrug (n = 6) antimicrobial classes, and notably, 8 contigs carried genes related to resistance to Critically Important Antibiotics (CIA), as described by the World Health Organization (World Health Organization, 2018). On the opposite, contigs associated with *Pseudomonas* showed multidrug resistance genes (n = 27), but none of them was related to CIA (Supplementary File

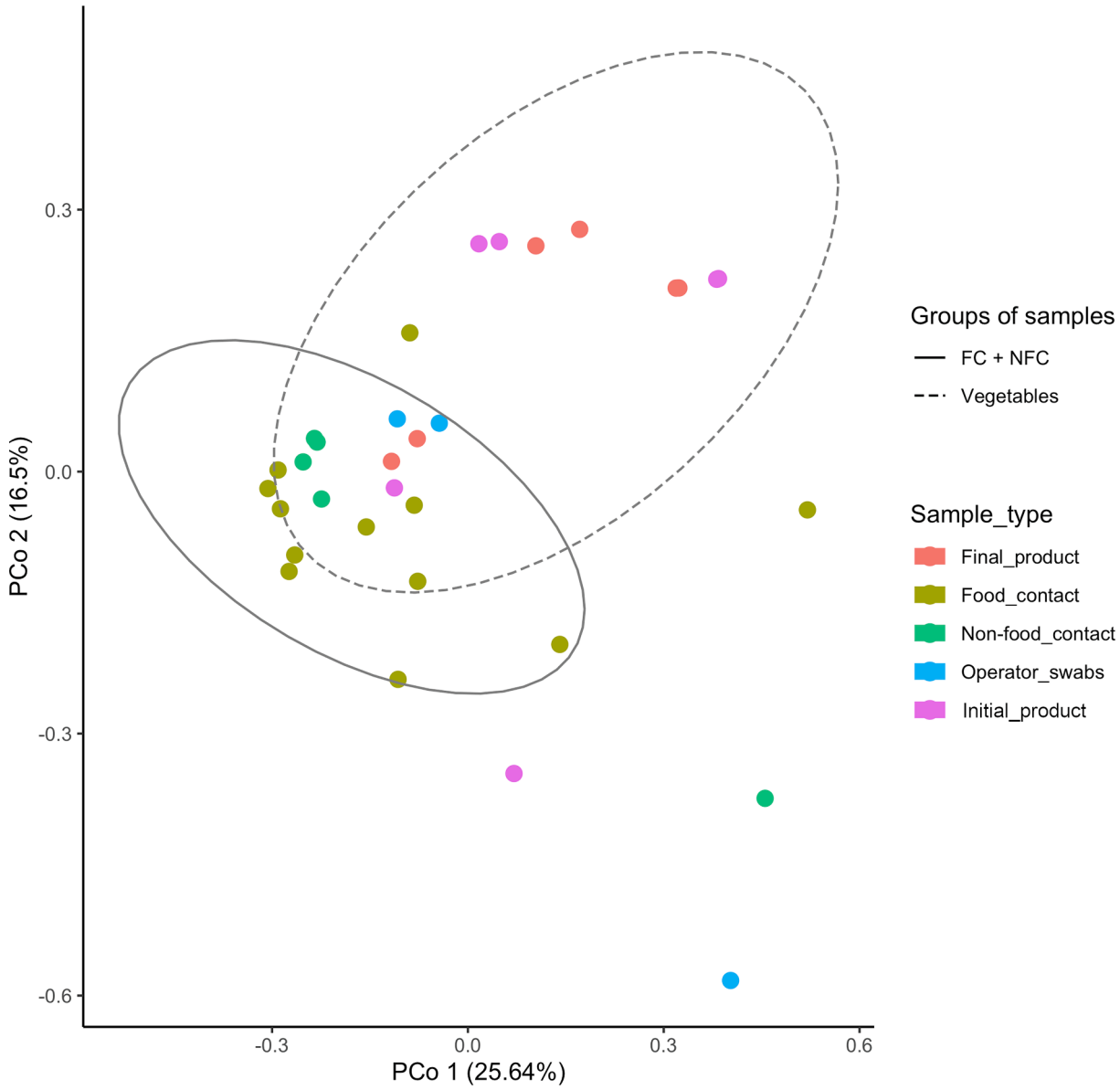

Fig. 1. PCoA based on the Bray-Curtis distance performed on the genus-level bacterial profiles obtained with Kraken2. Points are color-coded according to the sample type. Ellipses are drawn around surfaces (FC + NFC) and Vegetables (Initial + Final products).

2, sheet 1).

Regardless of the taxonomic assignment, FC surfaces hosted the highest number of AR-related contigs, with an average of 11.9 contigs per sample, compared with NFC surfaces (avg. 6 contigs/sample), samples from operators (avg. 6.6 per sample), and vegetables at the starting (avg. 5.6 per sample) and ending point (avg. 8.1 per sample) of the process (Supplementary File 2, sheet 2). In addition, 42 out of 143 AR-associated contigs recovered from FC surfaces might be part of plasmids, which were mainly linked to *Acinetobacter*, *Bacillus* and *Staphylococcus* (Supplementary file 2, sheets 1 and 2).

In addition, the abundance of AR-associated contigs was estimated. Overall, genes showing resistance to tetracyclines were the most abundant, with a mean RPKM value of 122.9 ± 150.3, followed by genes associated with resistance to multiple drugs (96.1 ± 243.4), macrolides (83.3 ± 143.2) and streptomycin (70.7. ± 28.2; Supplementary Fig. 5). Interestingly, 16 out of the 36 most abundant ARGs (i.e., with RPKM > 50) coded for resistance to multiple drugs and were assigned to *Bacillus* sand *Pseudomonas* spp.

Abundance estimation of ARGs further showed that FC/NFC surfaces have a leading role in the potential transfer of ARGs to the products, since no significant differences were observed between surfaces and vegetables (Supplementary Fig. 6A). *Bacillus*, *Acinetobacter*, *Staphylococcus* and *Pseudomonas* contributed the most to AR on surfaces (Fig. 5). Also, FC surfaces hosted a broader range of ARG classes, some of which were totally absent from other sample groups (e.g., streptomycin, streptogramin; Fig. 5). Finally, there were no significant differences in AR abundance among the three facilities (Supplementary Fig. 6B).

3.4. *Pseudomonas* virulence factors are widespread on surfaces and vegetables

We used the same approach to estimate the abundance and assess the taxonomic assignment of genes coding for Virulence Factors (VFs). Overall, 658 contigs carrying VFs were found in the metagenomes, 504 of those were assigned to the genus *Pseudomonas*, while 33, 23 and 11 belonged to *Bacillus*, *Rhizobium* and *Pantoea*, respectively (Supplementary Table 3). In addition, vegetables (both at starting and ending point of the process) reported the highest count of VFs. Contigs related to

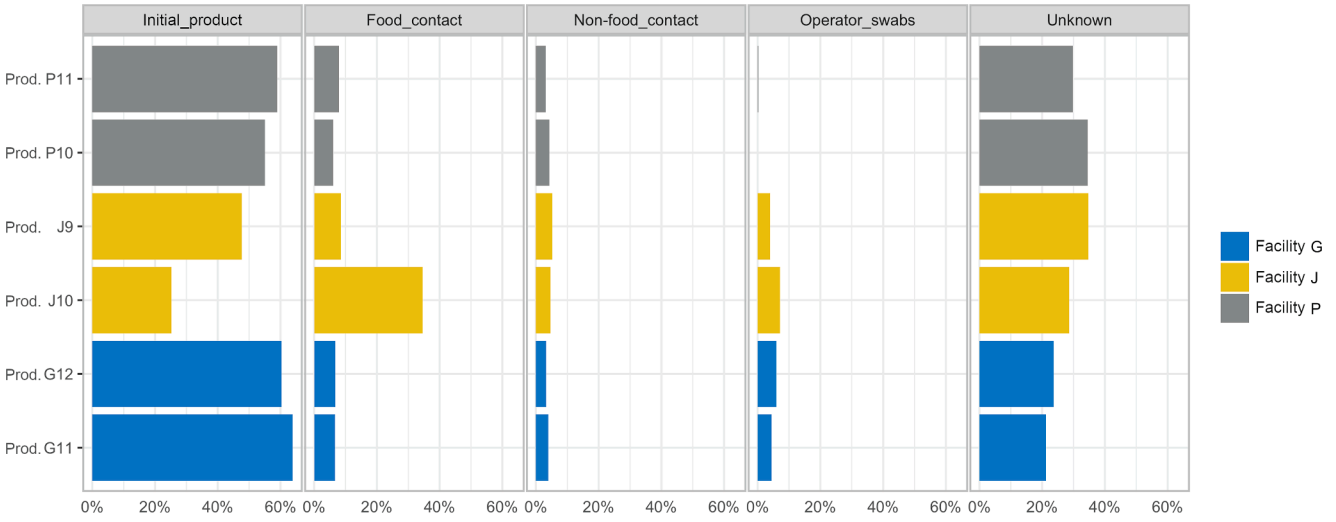

Fig. 2. Barplot showing the percentage contribution (x-axis) of each source of contamination to the taxonomic composition of the final products (reported on the y-axis). The 2 final products from each facility were analysed independently.

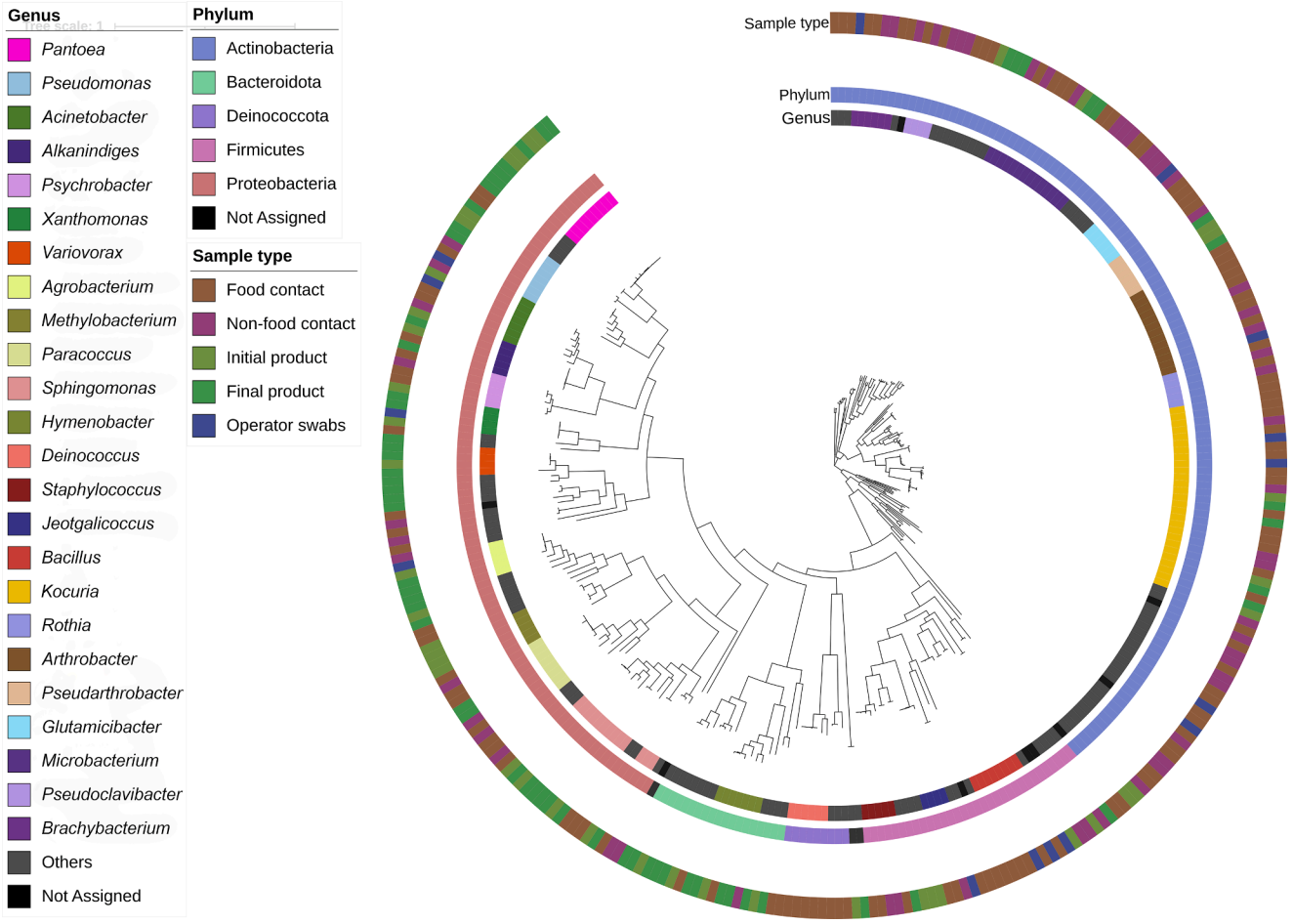

Fig. 3. Phylogenetic tree of all the medium/high quality MAGs reconstructed from the metagenomes.

motility were the most widespread on vegetables, as well as on FC surfaces. On the contrary, 17 and 12 contigs out of 32 associated with exotoxin production were reconstructed from FCS and operator swabs, respectively. Interestingly, all except one of these contigs belonged to *Bacillus*.

Finally, abundance analysis showed that "Biofilm", "Effector delivery system", "Immune modulation" and "LPS" VF classes did not differ significantly between surfaces and vegetables from all the facilities (Fig. 6).

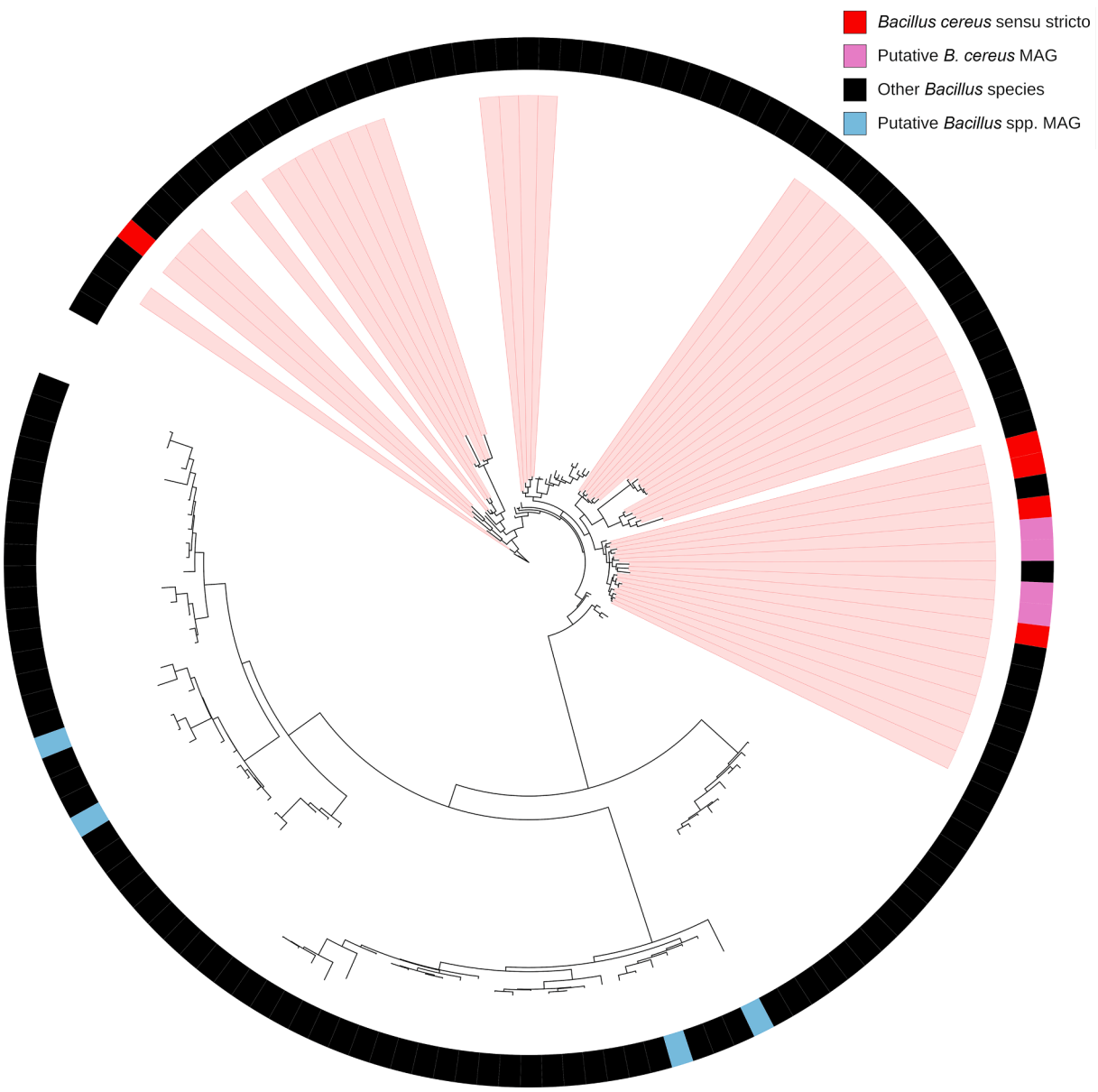

Fig. 4. Phylogenetic tree of a subset of NCBI RefSeq genomes spanning across multiple *Bacillus* species and those MAGs from surfaces and vegetables attributed to *Bacillus* sClades highlighted in red belong to the *Bacillus cereus* group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The environmental microbiome of vegetable processing plants can be an important factor influencing the quality and safety of the final product. Therefore, the taxonomic composition and potential genomic features of the microbiome need in depth investigations. The microbial composition of vegetables was largely consistent with previously published reports. Indeed, *Pseudomonas*, *Bacillus* and *Pantoea* were previously identified as the core microbiota of fruit and green leafy vegetables (Sequino et al., 2022; Soto-Giron et al., 2021; Taffner et al., 2020). Most of the highly abundant taxa identified in this study are common soil inhabitants (Deakin et al., 2018; Jiao et al., 2019; Simonin et al., 2022), mainly belonging to the *Proteobacteria* phylum, which is generally related to carbon, nitrogen and sulphur cycling (Mhete et al., 2020).

In addition, vegetables and surfaces harbour different microbial communities, as detected at read-level analysis, which was further confirmed by the taxonomic identification of MAGs that showed surfaces as dominated by *Bacillota* and *Actinobacteria*, while *Proteobacteria* and *Bacteroidota* were more prevalent on vegetables.

Although varying in composition, both vegetables and surfaces host a high number of microbial taxa. Indeed, alpha diversity indices showed no difference between foods and clean surfaces, suggesting that the stressful environmental conditions (i.e., the sanitation procedure) might not be able to alter the persistence of a highly diverse microbiome on sanitized surfaces, as previously reported (Møretrø & Langsrud, 2017). Also, we observed a range of potential virulence factors, a wide range of molecules and cellular structures produced by pathogenic microorganisms to help overcoming host's defence systems and cause disease (Chen et al., 2005; Leitão, 2020), which mainly belonged to *Pseudomonas* sand were related to bacterial adherence, biofilm production and effector delivery systems, also linked to the production of biofilm in *Pseudomonas* (Chen et al., 2015). Such genes reached a high abundance in the food production environments (Fig. 6). *Pseudomonas* have been widely reported as common inhabitants of food-handling environments (De Filippis et al., 2021; Sequino et al., 2022; Stellato et al., 2016). Their adaptation to environmental stress through the production of biofilms

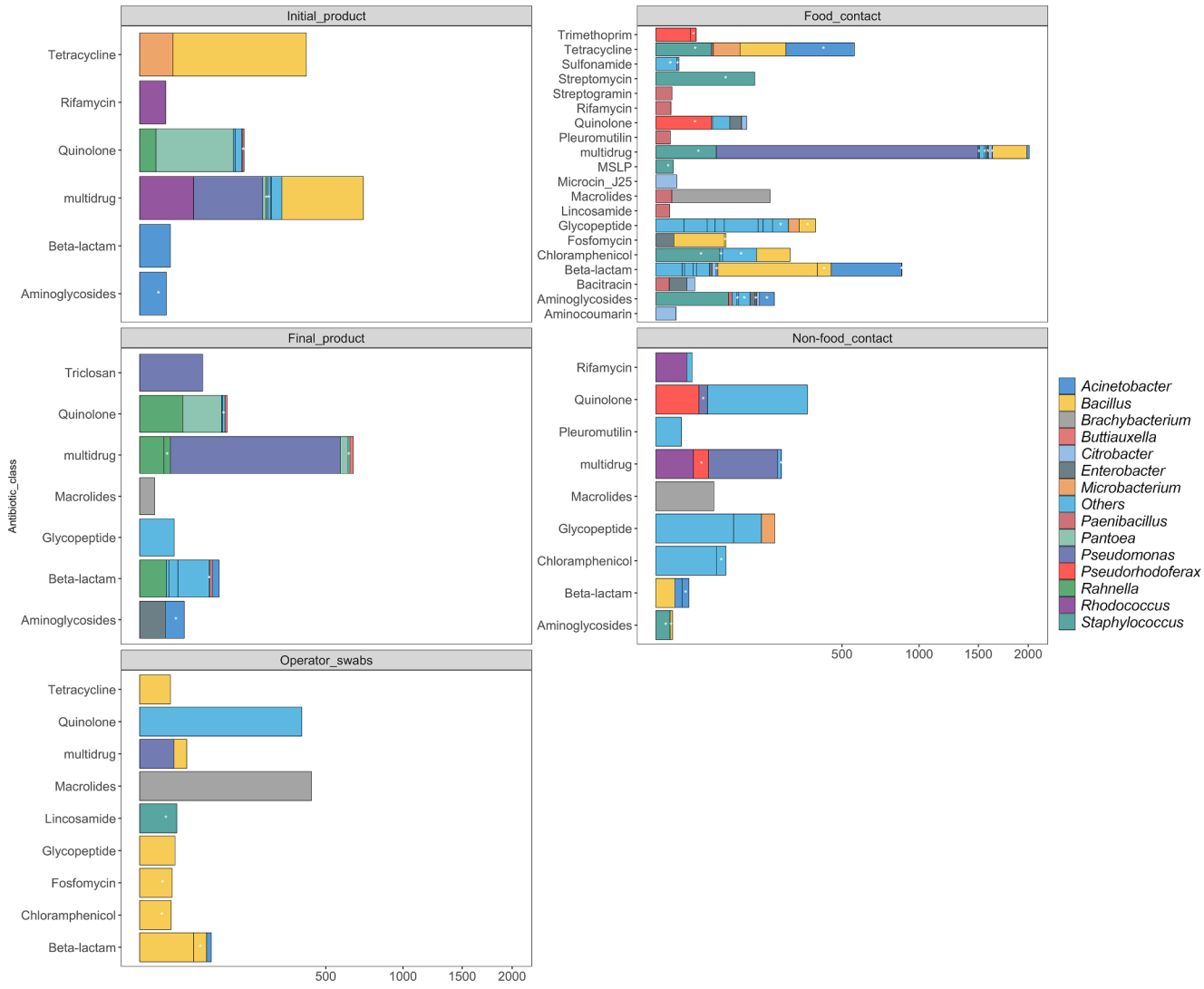

Fig. 5. Barplot showing, for each sample category, the abundance in RPKM of the Antibiotic Resistance Genes classes. Bars are color-coded according to the taxonomic assignment of the ARG-carrying contigs reported by Kraken2. Genes marked with an asterisk (*) are reported to be part of plasmids according to Platon and/or PlasFlow.

has been widely described, especially for *P. aeruginosa* (Pericolini et al., 2018), even though it has been observed that this ability is common within the genus (Fazli et al., 2014; Mann & Wozniak, 2012). In addition, biofilms produced by *Pseudomonas* may potentially entrap pathogenic microbes, thus protecting them from external stress (Guzmán et al., 2020). Evidence suggests that *Pseudomonas* are often present in multi-species biofilms involving pathogenic bacteria (Quintieri et al., 2021), and the non-pathogenic species *P. fluorescens* is able to enhance the adhesion and biofilm formation of *Listeria monocytogenes* (Maggio et al., 2021; Puga et al., 2018).

Moreover, the extracellular polymeric substance (EPS) that protects cells embedded into biofilms, also limits the entry of biocides such as disinfectants, exposing microorganisms to sub-Minimal Inhibitory Concentrations (MIC) of these compounds (Flores-Vargas et al., 2021). It has been shown that exposition of some bacterial strains to sub-MIC of quaternary ammonium compounds and sodium hypochlorite – two of the most used disinfectants in the food industry – might enhance the acquisition of resistance to fluoroquinolone, beta-lactam and aminoglycoside antibiotic classes (Nasr et al., 2018; Oniciuc et al., 2019; Piovesan Pereira et al., 2021), as a result of cellular response mechanisms that strengthen the tolerance of microorganisms to multiple biocide agents (i.e., cross-resistance; Wales & Davies, 2015). This phenomenon, together with the natural AR pattern occurring in soil and vegetables (Wang et al., 2022), might explain the broad diversity and high abundance of ARGs from different taxa (including *Bacillus* and *Acinetobacter*) that we observed on sanitized FC surfaces (Fig. 5), as well as the presence of toxigenic *B. cereus* strains on some of these surfaces.

Sanitation of food processing plants is extremely important to avoid foodborne outbreaks, especially in facilities producing fresh vegetables, where the absence of lethal operation units promotes the survival and growth of pathogens (2008). Nonetheless, the so-called "disinfectant-induced antibiotic resistance" (Chen et al., 2021) might have a negative outcome on the consumer's health (Jin et al., 2020). Stakeholders should seriously address this problem, promoting the use of alternative compounds in order to limit the long-term spread of ARGs (Tarricone et al., 2020).

We were able to reconstruct 9 medium/high quality MAGs belonging to *Pantoea agglomerans* from both initial and final products. According to some reports, this genus was sporadically isolated from nosocomial environments, and may be implicated in infections, specifically in immunocompromised patients (Walterson & Stavrinides, 2015). Also, the biofilm formation ability (Yannarell et al., 2019) and the antibiotic resistance (Guevarra et al., 2021) of *P. agglomerans* have been discussed. Consistently with results from Guevarra et al. (2021), we found a high

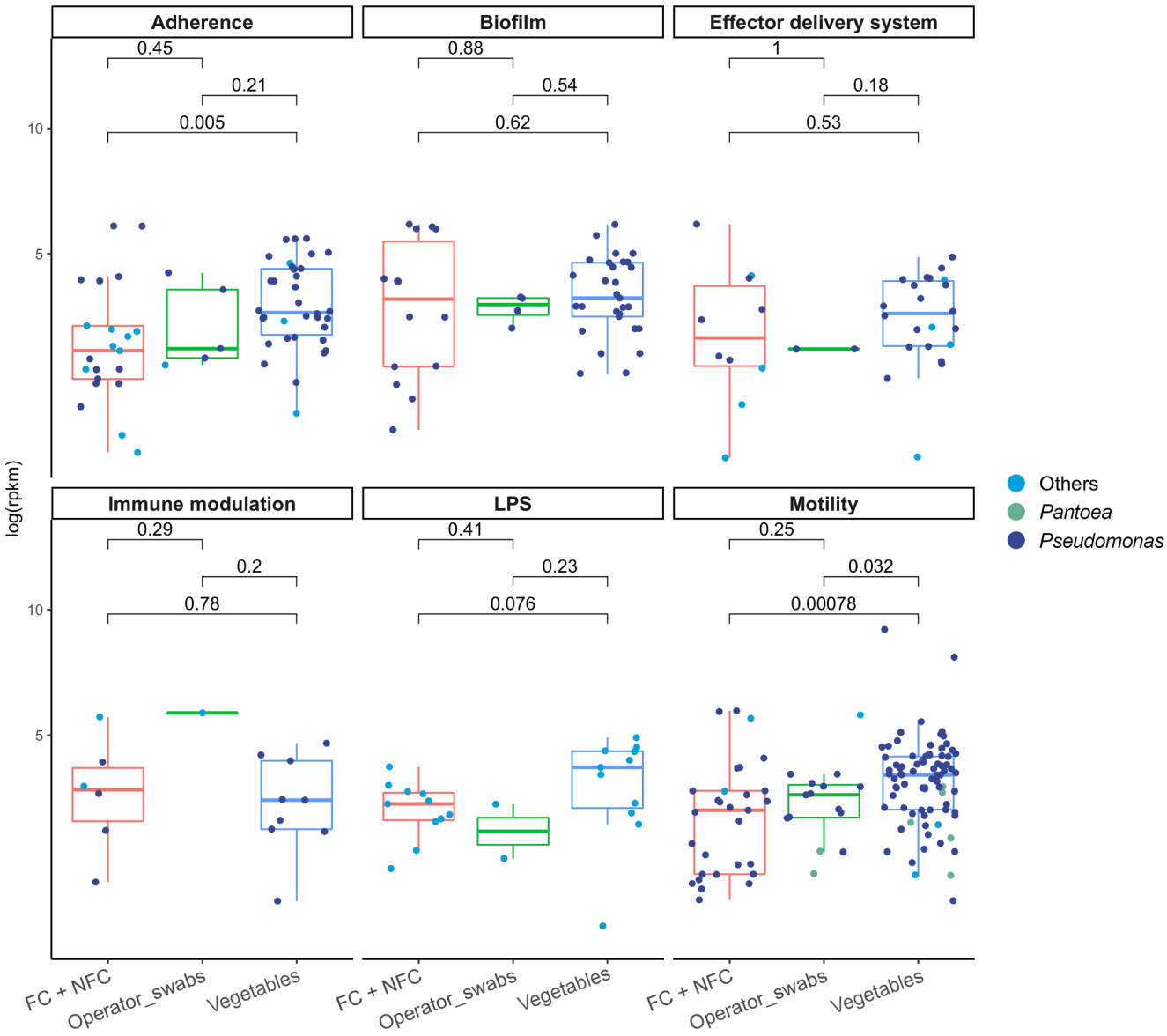

Fig. 6. Boxplot showing the RPKM abundance (in log scale) of several Virulence Factor Genes (VFGs) for each group of samples ("FC + NFC", "Operator swabs" and "Vegetables"). Points are color-coded according to the taxonomic assignment of the VFG-carrying contigs reported by Kraken2.

abundance of *Pantoea* contigs coding for resistance to quinolones and multiple drugs, mainly distributed in vegetables.

We attempted to identify the sources of contamination determining the taxonomic composition of the final product. Results from this analysis suggest that the microbiome of the vegetables at the end of the process mostly reflect that of initial vegetables. This was not surprising, since none of the processing steps strongly influences the structure or the properties of vegetables. However, despite the short contact time of vegetables with surfaces, an important influence of FCS on the microbial composition of the final product was observed in all the three facilities. This suggests that taxa from surfaces might end up in the final product, potentially reaching the gut after ingestion, since this product is commonly consumed raw.

Notably, several of the AR genes that we found across all the samples were associated with mobile elements, hence they might be transmitted to human pathogens. Previous reports already suggested that vegetables and minimally processed foods contribute the most to shape the gut resistome (da Silva et al., 2021), and HGT events involving bacteria from vegetables (mostly *Proteobacteria*) and from the gut microbiome have been documented (Blau et al., 2018; Ghaly et al., 2017).

In conclusion, we showed that sanitation procedures in minimally processed vegetables producing facilities might be ineffective in eradicating hazardous microorganisms (such as *B. cereus*) from FCS, which also show a broad pattern of resistance to antibiotics. On the contrary, our data suggest that the extensive usage of biocides might exacerbate AR selection. Overall, our findings evidence that there is a need to integrate microbiome-mapping in food processing environments into the routine monitoring procedures applied in the food industry to support appropriate strategies for the safety of the products. Integration of microbiome mapping in food manufactures, together with compliance to good hygiene practices in harvesting and processing of vegetables, might help food business operators to ensure safety and quality of foods.

CRediT authorship contribution statement

Vincenzo Valentino: Data curation, Formal analysis, Writing – original draft. **Giuseppina Sequino:** Formal analysis. **José F. Cobo-Díaz:** Formal analysis. **Avelino Alvarez-Ordonez:** Data curation, Funding acquisition, Resources, Supervision, Writing – review & editing. **Francesca De Filippis:** Conceptualization, Data curation, Funding

acquisition, Supervision, Writing – review & editing. **Danilo Ercolini:** Conceptualization, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.112202.

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