

# Microbial contamination pathways in a poultry abattoir provided clues on the distribution and persistence of *Arcobacter* spp.

Cristian Botta,<sup>1</sup> Davide Buzzanca,<sup>1</sup> Elisabetta Chiarini,<sup>1</sup> Francesco Chiesa,<sup>2</sup> Selene Rubiola,<sup>2</sup> Ilario Ferrocino,<sup>1</sup> Edoardo Fontanella,<sup>3</sup> Kalliopi Rantsiou,<sup>1</sup> Kurt Houf,<sup>4</sup> Valentina Alessandria<sup>1</sup>

**AUTHOR AFFILIATIONS** See affiliation list on p. 18.

**ABSTRACT** The consumption of contaminated poultry meat is a significant threat for public health, as it implicates in foodborne pathogen infections, such as those caused by *Arcobacter*. The mitigation of clinical cases requires the understanding of contamination pathways in each food process and the characterization of resident microbiota in the productive environments, so that targeted sanitizing procedures can be effectively implemented. Nowadays these investigations can benefit from the complementary and thoughtful use of culture- and omics-based analyses, although their application *in situ* is still limited. Therefore, the 16S-rRNA gene-based sequencing of total DNA and the targeted isolation of *Arcobacter* spp. through enrichment were performed to reconstruct the environmental contamination pathways within a poultry abattoir, as well as the dynamics and distribution of this emerging pathogen. To that scope, broiler's neck skin and caeca have been sampled during processing, while environmental swabs were collected from surfaces after cleaning and sanitizing. Metataxonomic survey highlighted a negligible impact of fecal contamination and a major role of broiler's skin in determining the composition of the resident abattoir microbiota. The introduction of *Arcobacter* spp. in the environment was mainly conveyed by this source rather than the intestinal content. *Arcobacter butzleri* represented one of the most abundant species and was extensively detected in the abattoir by both metataxonomic and enrichment methods, showing higher prevalence than other more thermophilic Campylobacterota. In particular, *Arcobacter* spp. was recovered viable in the plucking sector with high frequency, despite the adequacy of the sanitizing procedure.

**IMPORTANCE** Our findings have emphasized the persistence of *Arcobacter* spp. in a modern poultry abattoir and its establishment as part of the resident microbiota in specific environmental niches. Although the responses provided here are not conclusive for the identification of the primary source of contamination, this biogeographic assessment underscores the importance of monitoring *Arcobacter* spp. from the early stages of the production chain with the integrative support of metataxonomic analysis. Through such combined detection approaches, the presence of this pathogen could be soon regarded as hallmark indicator of food safety and quality in poultry slaughtering.

**KEYWORDS** poultry slaughtering process, emerging foodborne pathogen, detection, microbiota, *Arcobacter*, metataxonomic

Poultry meat is one of the most consumed foods worldwide, and its market in the European Union (EU) has grown constantly in the last decade, reaching in 2019 an estimated threshold of 13.3 million tonnes produced (1). Italy is the fifth largest producer of poultry meat in the EU and it has shown a steadfast export growth over the past

**Editor** Christopher A. Elkins, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Address correspondence to Davide Buzzanca, [davide.buzzanca@unito.it](mailto:davide.buzzanca@unito.it).

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few years, notwithstanding the adverse impacts of the global pandemic. At the national level, most poultry farms are concentrated in the northern regions, and broilers are slaughtered in few large-scale abattoirs (2–4). Proportionally to the dimension of this food trade, cases of recall related to poultry meat and poultry products are frequent and often associated with foodborne outbreaks in different countries (5, 6).

Contamination along the poultry processing chain is a common event that extends from farm to fork. Each step of this process plays a role in shaping the ultimate microbiota profile of poultry meat, encompassing both spoilage and pathogenic microorganisms (7). It has been documented that the carcass surface microbiota originates from the skin, gut, and processing environment (8). The microbiota of chicken carcasses depends on multiple elements, including the amount of microbial contamination of live birds prior to slaughter that changes composition and abundance over the various production stages, determining the final meat spoilage (8, 9). In particular, the microorganisms found on chicken skin represent the initial contributors to the carcass surface microbiota. Most of the microbial populations harbored on feathers, feet, and carcass surface are eventually concentrated on the neck skin through the percolation of water that occurs in different processing stages, including scalding, plucking, intermediate, and final rinses before chilling (10).

Another processing step influencing the skin microbiome composition is the evisceration, where contamination from the viscera to the carcasses and slaughterhouse equipment might occur if hygienic parameters are not fully accomplished (11). Chicken gut microbiota is dynamic and complex, influenced by rearing conditions, disease status, curative antibiotic interventions, breed, genetics, age, feed type, and additives (12). Apart from the animal conditions and diet, the microbial composition of poultry gut and skin can be altered through the administration of probiotics, prebiotics, and organic acids (13).

Microorganisms colonizing the processing environment during slaughtering cause cross-contamination, with the inevitable transmission of spoilage microorganisms and foodborne pathogens from the product to the consumer. Therefore, an important step in food safety control is to elucidate the contamination routes in the food chain (14). In poultry slaughterhouses, pathogens such as *Campylobacter*, *Salmonella*, and *Listeria monocytogenes* have been reported as commonly present (15, 16). Noteworthy, in the last 2 years, *Salmonella* has been the responsible pathogen for more than 94% of alert notifications in the European poultry market followed by *L. monocytogenes* (4%) and *Campylobacter* spp (5). The gastrointestinal tract of chickens has been identified as reservoir of several foodborne pathogens, with *Campylobacter* spp. and *Salmonella* sp. as the most dominant ones. All have the potential to cause gastroenteritis in humans with often severe impact on public health (17).

In this frame, the transmission routes of *Arcobacter* spp. in slaughterhouses cross-contamination, on both environment and broiler's carcasses, are still poorly understood. *Arcobacter* is part of the Campylobacterota phylum, which contains other two genera sources of human foodborne pathogens, i.e., *Campylobacter* and *Helicobacter* (18, 19). *Arcobacter* (*A.*) *butzleri*, *A. cryaerophilus*, and *A. skirrowii* are the species most associated with human clinical cases of gastrointestinal disorders (20–22). Particularly relevant is the association of *A. butzleri* with a food outbreak in the US linked to the consumption of contaminated broasted chicken (20). Moreover, *A. butzleri* isolated from poultry and slaughterhouse environments have shown biofilm production abilities (23), which can favor its colonization aptitude (23–25). Several studies have been conducted to assess the importance of *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* as contaminants of specific food products and slaughterhouse environments (21, 26–29). For this purpose, it is essential to determine the pathogen's ecology within the environmental contamination dynamics of the whole microbiome in space and time. Since microbial species actively coexists, mutualistically or competitively, within the communities of ecological niches, it is of pivotal importance a holistic evaluation of the transmission routes of *Arcobacter* spp. in the frame of the entire bacterial population of a poultry slaughterhouse.

The present study aims to assess the extent to which skin and caeca of incoming broilers contribute to the composition of the resident microbiota in a modern slaughterhouse, as well as to elucidate how the environmental persistence and distribution of *Arcobacter* spp. are affected by these cross-contamination patterns. Therefore, neck skins and caeca of 49 poultry flocks were sampled during eight slaughtering processes over 4 months, whereas the microbiota accumulated on the equipment's surfaces of the abattoir was subsequently analyzed after cleaning and sanitizing in two distinct sampling campaigns (Fig. 1). The DNA amplicon-based sequencing of the 16S rRNA gene was applied to characterize bacterial communities and to detect the presence of *Arcobacter* species, which was benchmarked in parallel with selective isolation.

## RESULTS

### Metataxonomic analysis showed distinct bacterial communities in the environment, caeca, and skins

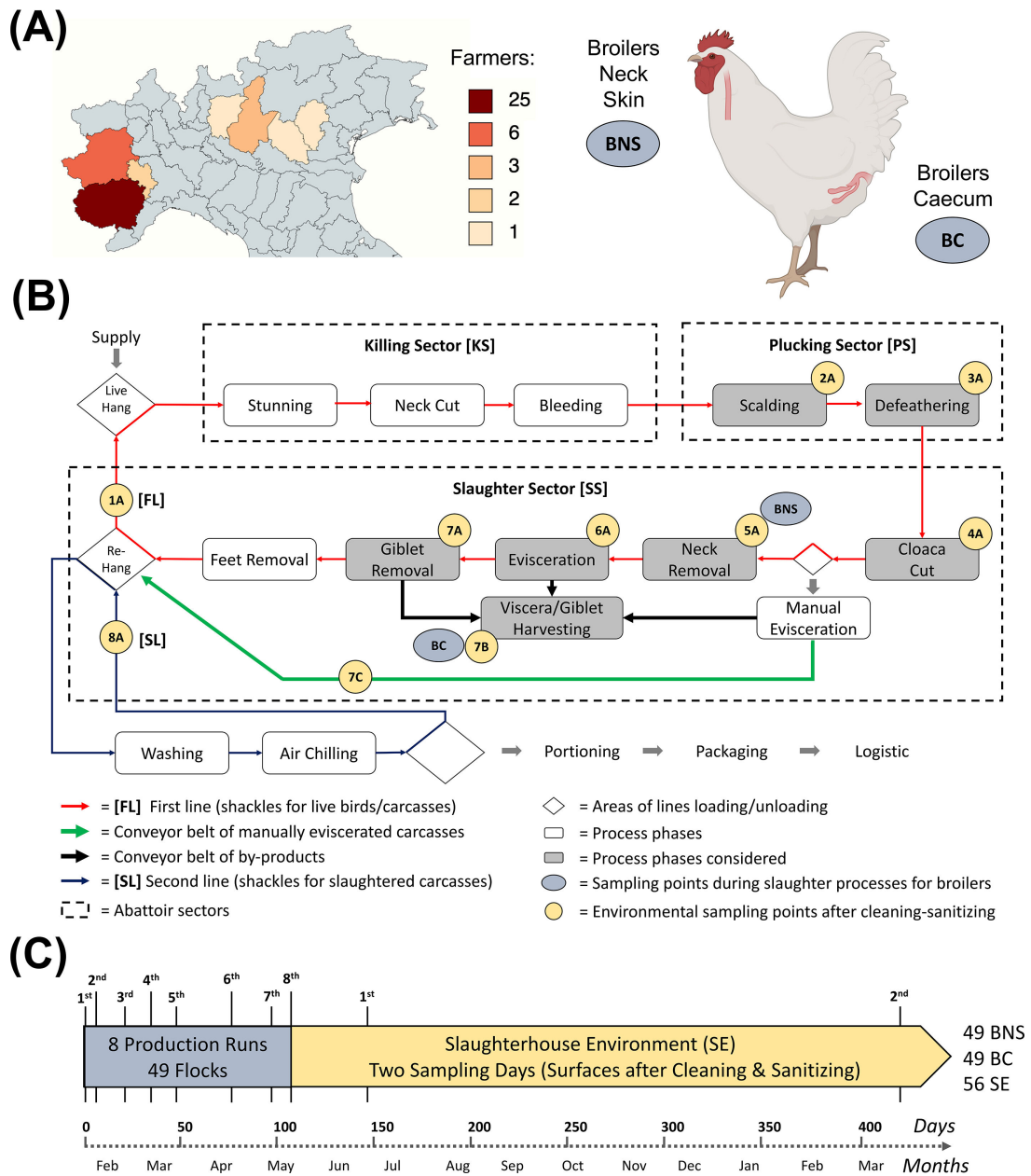
Transfer of microbiota from the broilers neck skin (BNS) and caecum (BC) through the processing steps on surfaces of a slaughtering environment (SE), and the establishment of a resident in-house microbiota, have been assessed in an abattoir localized in the north-western Italy (Fig. 1). Bacterial communities of all three sampling sources (BNS, BC, and SE) were examined through metataxonomic analysis based on amplicon sequence variants (ASVs), and BC and BNS microbiota refer to samples that represent a pool for each slaughtered flock.

The phylogenetic variation of the samples was visualized with a principal coordinate analysis (PCoA) plot based on weighted UniFrac beta-diversity distance (Fig. 2A). Bacterial communities of BC, BNS, and SE were graphically segregated and parametric permutational multivariate analysis of variance (PERMANOVA) confirmed that most of the microbiota variability was explained by these three sampling sources ( $R^2 = 0.50$ ;  $P$  [FDR] <0.001). Bacterial communities of BC were clearly different from BNS and SE, as significantly indicated by both PERMANOVA and pairwise comparison analysis of similarities (ANOSIM) tests, as well as by their marked segregation in the PCoA plot. Although statistical tests identified BNS and SE as two distinct microbiotas, they were partially overlapping in the plot, showing a certain degree of similarity. In addition, the dispersion of bacterial communities in each source was examined by measuring the distance between samples and the centroid (Fig. 2B). The dispersion increased significantly and progressively from BC to BNS and SE. BC showed a compact microbiota with a limited phylogenetic and compositional variation among samples, while more dispersed bacterial communities were observed in BNS and in SE.

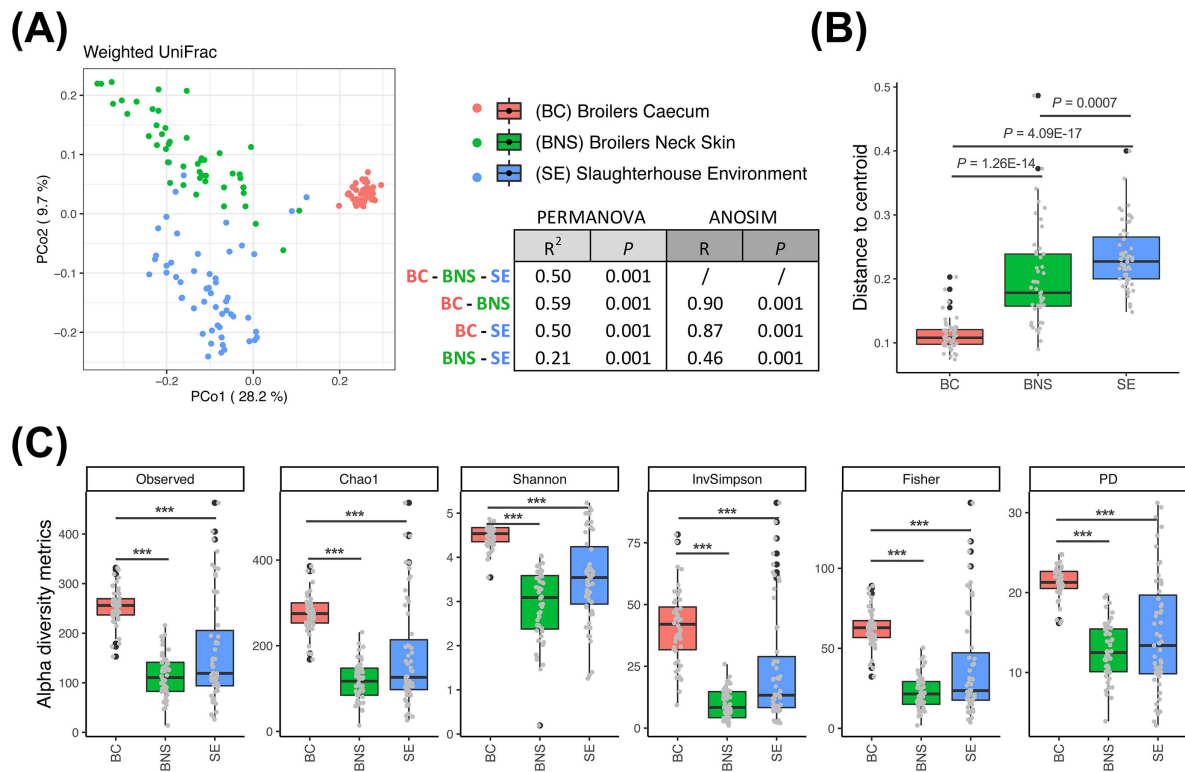
The comparison of alpha-diversity metrics between the three sources showed significantly ( $P$  [FDR] <0.001) higher values for the number of observed taxa, richness (Chao1), evenness estimators (Shannon, Inverse Simpson, and Fisher) and phylogenetic diversity (PD) in BC compared to both BNS and SE (Fig. 2C). Regardless of the sampling day, the phylogenetic diversity was significantly (Wilcoxon's test;  $P$  [FDR] <0.05) higher in the samples collected from the first compared to the second shackles line, and has progressively decreased along processing phases (data not shown). No other significant variations in alpha-diversity metrics were observed in each source as function of the sampling area and surface material (SE) or among broilers' samples (BC and BNS), the flock origin, and processing run.

### Composition and distribution of the microbiota in between and within the three sampling sources

A total of 6,681 unique ASVs were detected in the 151 samples analyzed. After alignment to the Silva's reference database, the majority (70%) of ASVs were assigned to the genus taxonomic rank, while only 7% of the ASVs reached the species-level assignment. Assignment to the species rank have been made only for ASVs that aligned 100% to the reference V3-V4 region of 16S rRNA gene, while for higher taxonomic ranks the



**FIG 1** Experimental design, spatiotemporal organization of samples collection, and process layout. Graphical summary (A) of broiler flocks' origin with localization (North-Italy), number of farmers, and type of samples collected from broilers during slaughter: i.e., caecum (BC) and neck skins (BNS). Detailed information about flocks rearing conditions are provided in Table S1. The map was produced with MapChart. Schematic representation (B) of the abattoir process layout with equipment's surfaces sampled (alphanumeric code) after the routine cleaning-sanitizing. Upon arrival at the slaughterhouse, broilers are unloaded from crates and manually hooked in "head down position" to stainless steel shackles (1A) of the first line [FL], which transports the live birds/carcasses in the establishment through the killing sector ([KS]; electrical stunning, neck cut, bleeding), plucking sector [PS] and slaughter sector [SS]. PS includes: scalding (2A) by submersion in warm water (50–56°C); defeathering (3A) with rubber-fingered pluckers on rotating disks. Once in the SS, the cloaca is cut (4A) by a vent cutter and most of the carcasses processed (>90%) are transported by the FL to the neck cut (5A): collection point of BNS during processing. Following: evisceration by spoon-shaped scoop (6A); giblet removal by rake-like extractor and aspirator (7A); feet removal. Viscera and giblets are collected on a conveyor belt (7B): collection point of BC during processing. Alternatively, from neck cut step onward, the manual evisceration is performed for broilers commercialised with head and feet, which are reunited to the main line with a plastic conveyor belt (7C). At the end of the slaughter, carcasses are moved from FL to plastic shackles (8A) of the second line [SL] and transported through further sectors for washing, chilling (air chilling tunnel), and final portioning/packaging. Detailed information about SE sampling points and cleaning-sanitizing procedures followed are described in Table S2 and Table S3, respectively. Duration of the study (C) with the number of production runs (day slaughter processes) followed, total samples collected from broilers (BC and BNS) and from SE.

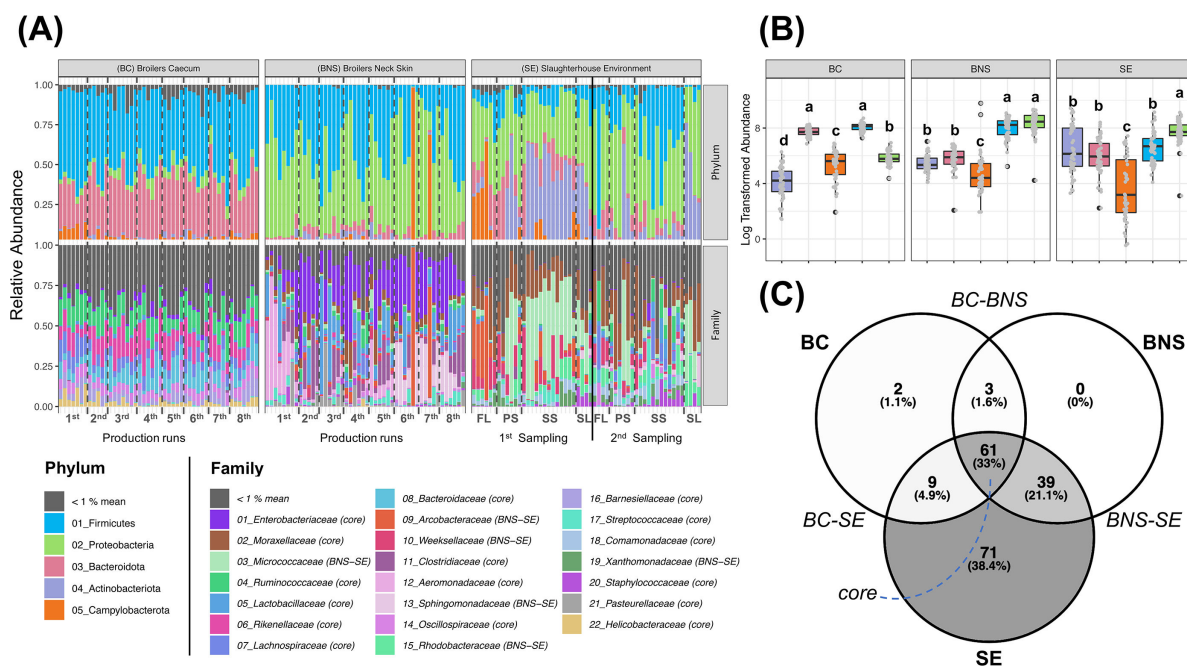


**FIG 2** Beta-diversity and alpha-diversity of the bacteria communities. Principal coordinates analysis (PCoA) plot (A) displaying weighted UniFrac distances matrix ( $\beta$ -diversity): sampling sources are shown by different colors as reported in the color coding key. Variance explained ( $R^2$  value) by each sampling source (BC, BNS, and SE) and pairwise biological dissimilarity ( $R$  value) are quantified by Permutational Analysis of Variance (PERMANOVA) and analysis of similarities (ANOSIM), respectively; since  $P$  values result from a 999 permutations test, they are only reported significant down to 0.001. Box plots illustrating beta-dispersion of the samples from the centroid (B) and alpha-diversity metrics (C) in the three sampling sources: boxes represent the interquartile range (IQR); central line indicates the median; whiskers indicate the furthest point within  $(1.5 \times \text{IQR})$ ; black points beyond whiskers represent outliers; gray points display the samples. Significant differences between sources are highlighted by  $P$  value (Kruskal-Wallis and pairwise Wilcoxon's tests; FDR adjusted) or asterisks ( $P$  value: \* =  $<0.05$ ; \*\* =  $<0.01$ , \*\*\* =  $<0.001$ ).

assignment was based on 99% of similarity. Overall, in comparison to BNS and SE a lower taxonomic resolution was achieved in BC, in which 21% of the reads were only assigned to order and family (Fig. S1).

The phyla Firmicutes, Proteobacteria, Bacteroidota, Actinobacteriota, and Campylobacterota were predominant and ubiquitously distributed, by representing up to 80% of the relative abundance in all samples (Fig. 3A). While in the caeca microbiota predominated Firmicutes and Bacteroidota, the neck skin was characterized by higher abundances of Firmicutes and Proteobacteria (Fig. 3B). Proteobacteria together with Actinobacteria represented the dominant phyla in the SE, and the relative presence of Campylobacterota (formerly in the Proteobacteria phylum) was here significantly lower than in broilers samples (BC and BNS).

At the family level, 21.1% of the taxa were shared between SE and BNS, while 33% of the taxa were included in the core microbiota. The SE harbored 71 source-specific families, while only two families were characteristic of BC, and none were exclusively present in BNS (Fig. 3C). Accordingly, the most abundant families belonged alternatively to the core microbiota and BNS\_SE-specific subgroup (Fig. 3A). Considering the core families in each sampling source, anaerobes like *Ruminococcaceae*, *Rikenellaceae*, *Bacteroidaceae*, and *Lachnospiraceae* accounted for more than 50% of average abundance in BC samples, whereas in BNS the same abundance percentage was represented by *Enterobacteriaceae*, *Lactobacillaceae*, *Clostridiaceae*, and *Aeromonadaceae*. The environmental microbiota was dominated by *Moraxellaceae*, which were rarely detected in caecal samples, and by other families exclusively present in the BNS-SE subgroup,



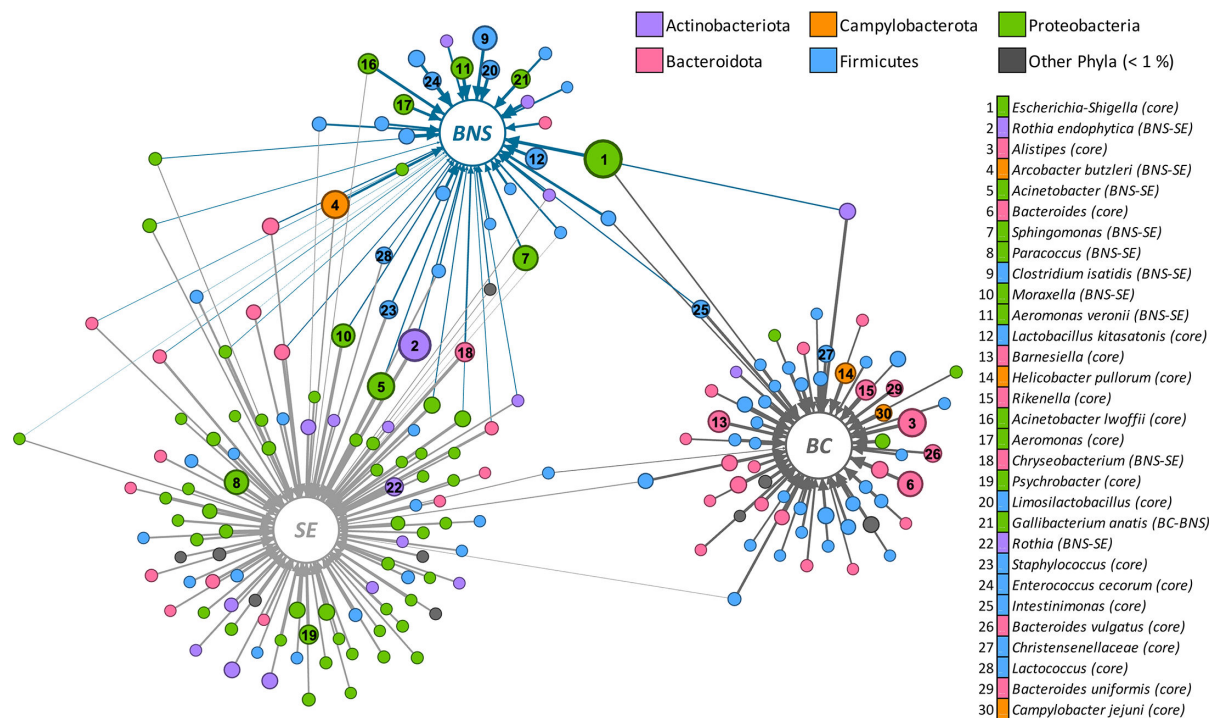
**FIG 3** Overview of microbiota composition and distribution. Stacked bar plots (A) showing microbiota composition (relative abundance) in phylum and family taxa ranks, with color coding keys. Samples are grouped following the temporal sampling order in each sampling source (BC, BNS, and SE), and then according to the flock's slaughtering order and processing phase order BC-BNS and SE, respectively. Abattoir sectors and transport lines are indicated: FL = first line; PS = plucking sector; SS = slaughter sector; SL = second line. Taxa are sorted in the legend from the most to the least abundant (>1% average). Belonging to core microbiota or subgroup is reported for each family. Box plots (B) displaying log-transformed abundances of phyla. Different letters (a, b, c, d) highlight significant differences (ANOVA coupled with Tukey's test;  $P < 0.001$ ). Venn diagram (C) showing the number of shared taxa at the family level among the three sampling sources; only taxa present in more than two samples were considered.

namely *Micrococcaceae*, *Arcobacteraceae*, *Weeksellaceae*, and *Sphingomonadaceae*. While a stable community during the entire monitoring period in BC was observed, a marked succession of dominant families occurred along production runs in BNS. Indeed, apart from the constant presence of *Enterobacteriaceae* overtime, the *Aeromonadaceae* were predominant in the first production run and were thus replaced by *Lactobacillaceae* in the middle productions, while from the sixth run, the *Sphingomonadaceae* took over the dominance. A time course succession of families was observed in SE as well, with *Arcobacteraceae* abundances that decreased significantly from the first to the second sampling day, while in parallel *Lachnospiraceae* and *Sphingomonadaceae* abundances were significantly higher (Wilcoxon's test;  $P$  [FDR] < 0.001) in the second ones (data not shown).

### Microbiota snapshots at the highest taxonomic resolution

At the highest taxonomic resolution, the three sampling sources showed distinct microbiota composition and distribution at the genus or species level. In particular, the proportion of taxa harbored in the BNS-SE subgroup was higher than in the core microbiota (Fig. S2).

To identify genera and species associated with caeca, neck skin, and environment, the indicator species analysis was performed based on point biserial correlation. Out of 568 taxa identified up to genus or species rank level and present in more than two samples, 193 taxa showed significant associations (*multipatt* statistic;  $R > 0.4$ ;  $P < 0.001$ ) with the three sampling sources or their pairwise combinations, which were illustrated with a bipartite network (Fig. 4). The core taxon *Escherichia-Shigella* was the most abundant in the entire data set but appeared mainly associated to BNS and secondly to BC. Core taxa included in the Bacteroidota phylum, like *Alistipes* and *Bacteroides*, were indicators of the caecal microbiota, together with minor members of Firmicutes. *Rothia endophytica* was



**FIG 4** Bipartite network revealing the taxa (genus or species level) associated to the three sampling sources. Taxa (colored nodes) are unidirectionally connected with arrows (edges) to the sampling sources (BC, BNS, and SE) if significant associations have been detected (Indicator Species Analysis: *multipatt* statistics;  $R > 0.4$  and  $P$  value  $< 0.001$ ). Nodes are made proportional to taxa abundances (log-transformed) and colored in relation to the belonging phylum (refer to color coding key). Only the taxa present in more than two samples were considered and most abundant taxa ( $>0.5\%$  in average) are reported in the legend with codifying number (from the most to the least abundant), together with their belonging to core microbiota or subgroups (Fig. S2). Edges thicknesses and length are, respectively, directly and indirectly proportional to the association strength (significance parameters in *multipatt* statistic), while color refers to the associated source. Network layout was constructed using ForceAtlas2 algorithm: distance between node and associated source is proportional to the association strength.

the more abundant Actinobacteria and together with major Proteobacteria members like *Acinetobacter*, *Paracoccus*, and *Psychrobacter*, were the main taxa associated with SE, while *Clostridium isotidis*, *Lactobacillus kitasatonis* and minor Proteobacteria members were the most significant indicators of BNS ecology. Most of the taxa significantly associated with two sources were shared between BNS and SE (28 taxa), while only four and three taxa were indicators of BC-BNS and BC-SE, respectively. Among the main indicator taxa of BNS-SE, *Arcobacter* (*A.*) *butzleri* and *Sphingomonas* tended to be more associated with the broiler's skin than to the environment, in contrast to *Acinetobacter*, *Moraxella* and *Rothia endophytica* were markedly more abundant in the environment. Interestingly, *A. butzleri* was the only Campylobacterota uniquely found and significantly associated with BNS and SE, while *Helicobacter pullorum* and *Campylobacter jejuni* were indicators of BC although they were part of the core microbiota.

Following the previous observations at the family rank, temporal successions of the dominant genera and species occurred in BNS (along production runs) and SE (sampling days), but not in BC. *A. butzleri* was constantly present on broiler's skin along production runs, but significantly more abundant the first sampling day in SE. Considering the SE layout, the genera *Acinetobacter* and *Psychrobacter* were significantly associated to the plucking sector and chilling line, respectively. Looking into BC and BNS samples, no taxa were significantly associated to any extent with the different conditions of flocks' rearing, such as the eventual need of antibiotic treatment or different types of diet (data not shown).

Focusing on *Arcobacter* spp., *A. butzleri* was not detected in the scalding and defeathering tunnel of the plucking sector, in contrast to *A. cryaerophilus* and *A. cibarius* (Table

TABLE 1 Distribution of *Arcobacter*, *Campylobacter*, and *Helicobacter* genera in broilers and environmental samples<sup>a</sup>

Family	Species	ASVs (no.)	Positive samples (no.)	Average abundance (%) in positive samples	Distribution		
					Associated to	SE sectors/lines	Production runs (BC and BNS)
<i>Arcobacteraceae</i>	<i>Arcobacter butzleri</i>	2	72	6.59	BNS-SE	FL; SS; SL	All runs
	<i>Arcobacter cryaerophilus</i>	1	14	0.45	BNS-SE	PS; SS	Runs 1, 2, and 5
	<i>Arcobacter</i> spp.	7	11	0.40	BNS-SE	FL; SS	Runs 5 and 6
	<i>Arcobacter cibarius</i>	1	6	0.29	SE	PS; SS	/
<i>Campylobacteraceae</i>	<i>Campylobacter jejuni</i>	2	74	1.05	Core	FL; PS; SS	All runs
	<i>Campylobacter</i> spp.	22	13	0.08	BC	/	All runs except 4
<i>Helicobacteraceae</i>	<i>Helicobacter pullorum</i>	2	72	2.12	Core	FL; PS; SS	All runs
	<i>Helicobacter</i> spp.	31	20	0.06	BC-BNS	/	All runs except 4

<sup>a</sup> For the SE sector codes (\*) refer to Fig. 1; Table S2: first line [FL] of stainless steel shackles; plucking sector [PS]; slaughter sector [SS]; second line [SL] of plastic shackles.

1). Considering the slaughterhouse layout, *A. butzleri* was the only *Campylobacterota* detected on the surfaces of the plastic shackles in the final chilling line.

### Microbiota structure in the three sampling sources

To explore the structure of microbial communities in BC, BNS, and SE, the Sparse Correlations for Compositional data were computed and significantly positive correlations (*SparCC* algorithm;  $R > 0.4$ ;  $P$  value  $< 0.001$ ) have been displayed in three distinct co-occurrence networks (Fig. 5). Pairwise correlations were calculated within each sampling source to avoid the detection of interactions solely dependent to the compositional distance among the three ecologies. Besides, only taxa present in more than three samples and with  $>0.2\%$  of average abundances were considered.

Overall, the slaughterhouse environment showed the highest proportion of taxa significantly correlated and thus included in the graph. Indeed, SE network showed a greatest number of edges and triangles, as well as the widest diameter and a major connectivity among taxa, referred to as average degree, in comparison to BC and BNS networks (Table 2). However, despite a relatively large number of pairwise co-occurrences, the taxa in SE were not more densely connected and did not show a relatively higher tendency to group in modules than what was observed in BC and BNS. Focusing on the co-occurrences type, the intra-family and mainly the intra-genus pairwise correlations were more frequent in BC and BNS networks than in SE.

To examine whether certain taxa exhibited keystone roles in the microbial ecosystem of each sampling source, the betweenness centrality, which measures the number of shortest paths going through a node and it is directly correlated to the core location of a given node in a network, was considered. Overall, this topological parameter of the node (taxon) was not correlated (Pearson's correlation;  $P > 0.05$ ) to its abundance or occurrence in the data set and showed significantly higher values in the SE network (Pairwise Wilcoxon's test,  $P$  [FDR]  $< 0.001$ ). By applying a cutoff value of threefold the upper interquartile range (IQR), a set of 15 potential keystone taxa in the SE network was identified, which comprised also *Arcobacter butzleri*, and only 4 taxa in both BC and BNS networks. The keystone taxa varied in the three sampling sources, with the exception of *Ruminococcus torques* which represented a central taxon in both SE and BC network structures (Fig. 5; Table S4).

Next, networks were partitioned in group of highly interconnected (co-occurring) nodes, defined as modules (30), which were more numerous and dimensionally larger in the SE network. Modules segregation within the SE network was significantly dependent (Kruskal-Wallis and Pairwise Wilcoxon's tests;  $P$  [FDR]  $< 0.001$ ) to the distribution of the taxa between the two sampling days and along the lines-sectors of the slaughterhouse, i.e., automated line, plucking, evisceration, and chilling line (Table S5). Analyzing the composition of the modules in this network, many of the taxa included in the two largest



TABLE 2 Summary of SparCC-based co-occurrence networks features and topology<sup>a</sup>

	Parameters	Sampling source		
		BC	BNS	SE
General features (counts)	Taxa analyzed	148	163	285
	Nodes (taxa correlated)	47	81	214
	Edges (correlations)	50	128	908
	Triangles	7	81	2.756
	Diameter	8	7	12
Network topology (average value)	Degree	2.128	3.16	8.486
	Density	0.046	0.040	0.040
	Modularity	0.709	0.772	0.664
	Clustering coefficient	0.357	0.474	0.513
	Path length	3.418	2.657	4.793
Co-occurrence type (% on the total)	Intra-phylum	88.0%	66.4%	59.4%
	Intra-family	34.0%	25.0%	12.0%
	Intra-genus	4.0%	7.8%	1.5%

<sup>a</sup>Topological features description: diameter = shortest path length (no. of edges) between the two most peripheric nodes in the network; degree = number of edges per node; density = ratio between edges present and maximum number of edges that the graph can contain; modularity = index of graph sub-division strength in modules; clustering coefficient = abundance of connected triangles in a network; and path length = number of edges along the shortest path for all possible pairs of nodes.

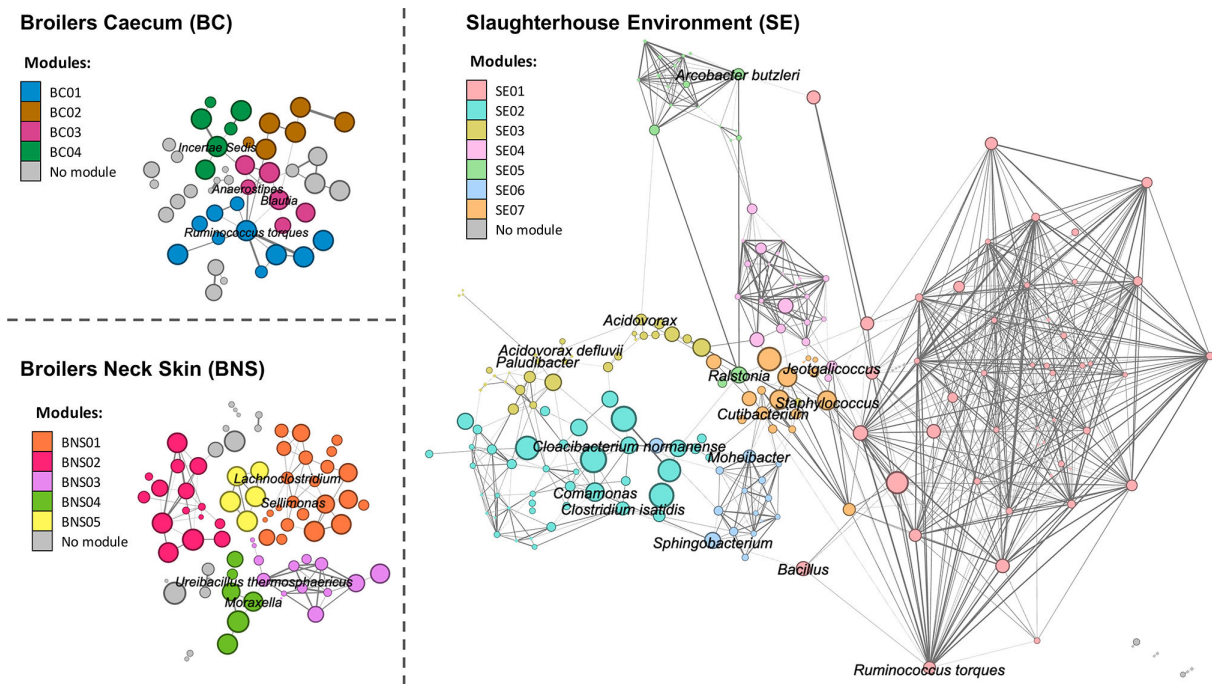


FIG 5 Co-occurrence networks of each sampling source. Taxa (nodes) are connected by lines (edges) in relation to significantly positive pairwise correlation (SparCC algorithm with 100 bootstraps;  $P$  value < 0.001,  $R > 0.4$ ). Nodes are made proportional to taxa occurrences and colored in relation to the co-occurring modules (refer to color coding keys); hub taxa with the highest value of betweenness centrality (>3× of upper IQR) are reported. Edges thicknesses are made proportional to SparCC correlation values and network layout was constructed using ForceAtlas2 algorithm. Detailed information on module composition is reported in Table S4.

modules, here coded as SE01 and SE02, co-occurred in BNS network modules as well (Fig. S3).

More in general, adjacent nodes (taxa directly correlated) in the SE network were also correlated in the BNS one, likely highlighting co-transferring phenomena for some of the taxa between broiler's skins and environment. To better depict this aspect, all pairwise

correlations existing in both SE and BNS sources were extracted and plotted in a network of shared co-occurrences: core taxa like *Faecalibacterium* and *Bacteroides* co-occurred together with minor taxa mostly related to BC microbiota (Fig. S3C).

### Isolation of *Arcobacter* from production runs and specific niches in the slaughterhouse environment

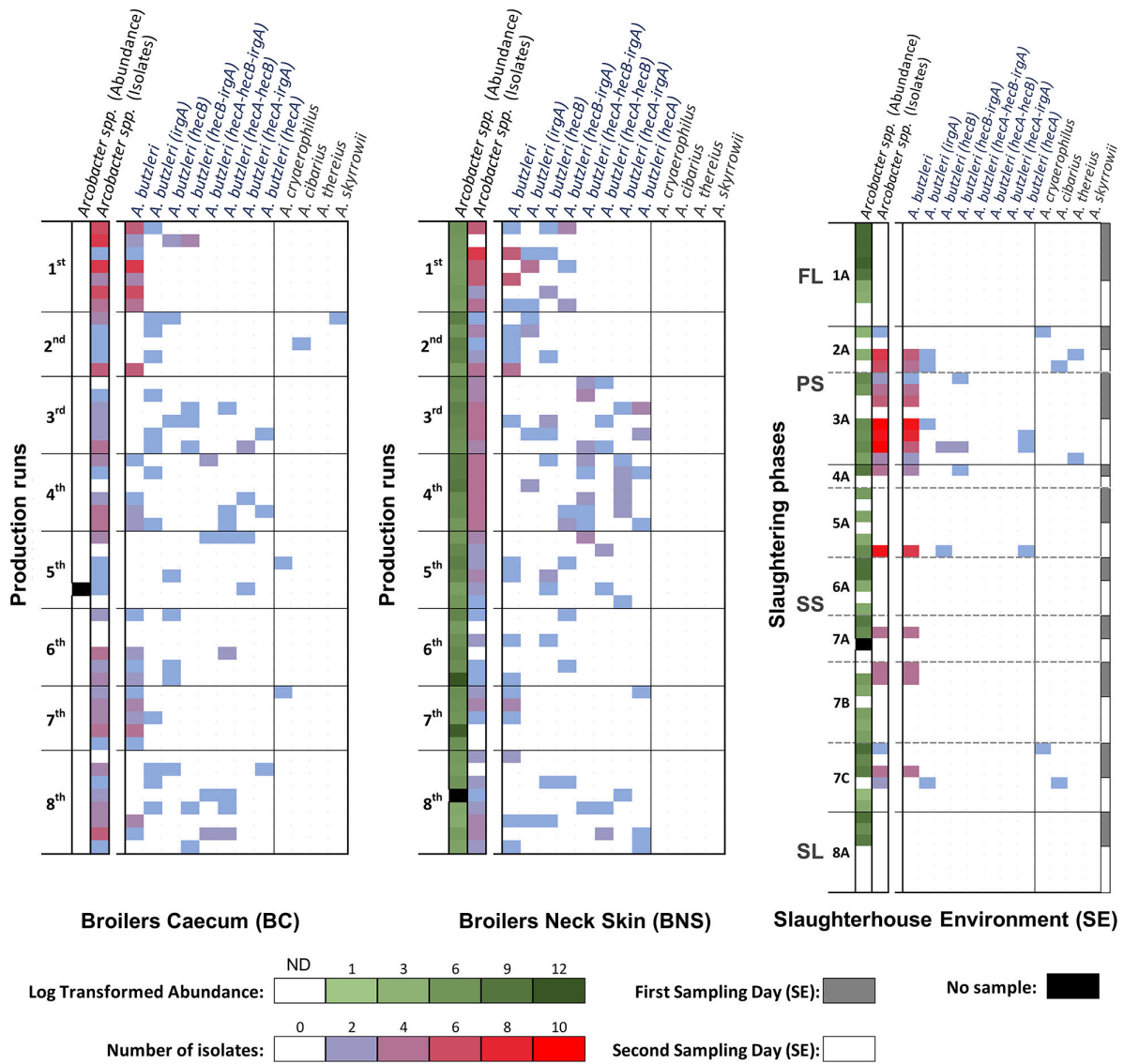
A total of 371 colonies were obtained after selective enrichment and assigned to the *Arcobacter* genus using MALDI-TOF MS (71% of the isolates), while 23% of the isolates could not be identified and 6% were assigned to the genera *Bacillus*, *Listeria*, or *Pseudomonas* (data not shown). After genus- and species-specific PCR, a total of 330 isolates were confirmed as *Arcobacter* spp., of which 320 identified as *A. butzleri* species recovered from all three sampling sources (Fig. 6; Table S6). Besides, *A. cibarius* (three isolates) and *A. cryaerophilus* (four isolates) were detected in the environment and caecum samples, but not on broilers neck skin. One isolate was identified as *A. skirrowii* and two *A. thereius* species were recovered from caeca and environment, respectively. Differentiation among isolates of the same species was performed by considering the presence-absence profiles of three virulence-associated genes, namely *irgA*, *hecA*, and *hecB*, which are genetic elements encompassed in variable regions of *Arcobacter* pangenome (25, 31). The species *A. cibarius*, *A. cryaerophilus*, *A. skirrowii*, *A. thereius*, and 50% of the *A. butzleri* isolates did not possess these three genes. Isolates of *A. butzleri* devoid of these genes and other four genotypes were detected in all three sampling sources, whereas the genotypes *hecA-irgA*, *hecA-hecB*, and *hecA-hecB-irgA* were not recovered in the environment, which therefore harbored a lower number of the *A. butzleri* genotypes.

Overall, *Arcobacter* spp. have been isolated from broilers in all production runs, with 86% and 88% positive samples in caeca and neck skins, respectively, while only one flock resulted *Arcobacter*-free in both BC and BNS pooled samples. In the cleaned and sanitized SE, only 31% of the samples were positive to the presence of *Arcobacter*. At least one positive sample was detected in each processing phase considered in the plucking and slaughtering sectors, except for the automated evisceration ( $n = 5$  samples). No *Arcobacter* was isolated from the shackles of the automated slaughtering line ( $n = 7$ ) and chilling line ( $n = 7$ ). Surfaces of the scalding tank (sampling point 2A) and the rubber-fingers of the defeathering tunnel (sampling point 3A) showed the highest number of positive samples and the greatest biodiversity: i.e., the sites from which more different species and biotypes have been isolated. Besides, species different from *A. butzleri* were only detected in the plucking sector and upon the conveyor belt of manually eviscerated carcasses (Fig. 6).

Comparing the distribution and numbers of isolated *Arcobacter* to the relative abundance of this genus, no significant pairwise correlation was observed in the BC and SE samples (Spearman's correlation;  $P > 0.05$ ), while in BC samples, it was not detected at all by the metataxonomic analysis. Moreover, correlating sample distance matrices generated from isolates and relative abundances, we did not observe meaningful relationships between the microbiota composition and the presence of alive *Arcobacter* spp. (Mantel's test;  $P > 0.05$ ).

## DISCUSSION

The investigation of microbiota distribution, diversity, and dynamic within an ecosystem is the ultimate goal of any biogeographical study. When applied to food processing environments, it becomes pivotal to reconstruct contamination routes of productive processes, which are in turn highly influenced by factors like the premise layout, sanitizing interventions, productive flow chart, and temperature (14, 32–34). DNA-based metataxonomic analysis and parallel targeted isolation of *Arcobacter* spp. have been performed in this study toward that scope. Common microbiological analysis consists of neck skins (representative of the entire carcass microbiota) and caeca sampling during poultry slaughtering (35, 36), where their metataxonomic profiles can provide a reliable



**FIG 6** Pseudo-heatmap summarising the frequency of *Arcobacter* spp. isolation and abundances in the three sampling sources. Species and biotypes of the isolates are reported on the X axis. Samples (Y axis) are ordered by production runs in BC and BNS; while in SE, the order follows the succession of sectors/lines and slaughtering phases (sampling points). Sectors/lines: (FL) first shackles line for live birds and carcasses; (PL) plucking sector; (SS) slaughter sector; (SL) second shackles. Sampling points: (1A) shackles automated line; (2A) scalding tunnel; (3A) defeathering tunnel; (4A) vent cutter; (5A) neck cutter; (6A) spoon-shaped scoop eviscerator; (7A) rake-like extractor and aspirator for giblets; (7B) conveyor belt for viscera and giblets; (7C) conveyor belt for manually eviscerated carcasses; (8A) shackles of chilling line. For the number of isolates and abundances refer to colors coding key (ND = not detected).

picture of the incoming contaminant microbiota. On the other hand, metataxonomic profiles of environmental samples collected after cleaning-sanitizing provide insights on the resident populations and the potential pathogens prevalence during processing (32, 37).

Confirming previous metataxonomic studies, neck skins (BNS) and caecal samples (BC) represented two distinct microbiotas (38, 39). In comparison to BNS microbiota, BC was characterized by higher biodiversity and presence of anaerobes included in Bacteroidota phylum (12). Moreover, BC microbiota showed a lower level of intra-communities' phylogenetic variability ( $\beta$ -dispersion) in comparison with BNS and SE, as well as a stable composition in time among the production runs. On the other hand, BNS microbiota showed a temporal succession of taxa during 3 months, in relation to the production runs and despite the different flock origins.

Microbiota composition of both BNS and BC was not influenced by rearing conditions like the diet type and antibiotic treatments. It has been reported that diet can influence the intestinal and skin microbiota composition of broilers, which however are more dependent to the stocking density and the housing conditions, such as the sharing transport crates (40, 41). Indeed, caecal microbiomes of poultries reared in conventional and antibiotic-free farms showed distinct taxonomic and functional profiles, although this separation was completely lost in the carcasses' microbiome collected downstream of the slaughtering process (42). Another metagenomic investigation highlighted a major impact of packaging and processing environments on chicken breast microbiome in comparison to the antibiotic usage (9). Therefore, in this study, the negligible effect of different diets and antibiotics observed on broilers microbiota can be explained by closeness and contact among animals or carcasses during processing, as well as flock-to-flock contamination conveyed in the same processing day by devices and surfaces. Taking into consideration the new limiting European regulation on the use of veterinary medical products and medicated feed (43, 44), the identification of processing phases in which the microbiota/microbiome analyses can be useful to define a previous use of antibiotics will be fundamental in the near future.

The composition and structure of SE microbiota have shown similarities with BNS. These two sampling sources shared several species including *Arcobacteraceae*, *Weeksellaceae*, and *Sphingomonadaceae* families, which were not detected in BC samples. This aspect indicates that skin, feet, and feathers (here represented by the BNS samples) are the major sources of contamination that determine the resident microbiota in a modern automated abattoir. It suggests that Good Manufacturing Practices (GMPs) were properly followed in the monitored abattoir, but it is also determined by intrinsic characteristics of BC microbiota. Indeed, the none aerophilic and thermophilic taxa harbored in the poultry gut are unlikely to survive and colonize the processing environment (38, 45). Noteworthy, *A. butzleri* represented a predominant and abundant taxon in BNS and SE, but was not detected in BC. In contrast, more thermophilic members of the Campylobacterota phylum, such *H. pullorum* and *C. jejuni*, were significantly associated to BC (46). Despite these two species were distributed in all plucking and slaughter phases, they have not been detected on the shackles of the second line that convey slaughtered carcasses through the washing step and air chilling tunnel.

Aside from the direct association between taxa and sources, co-transferring phenomena have been observed for certain groups of abundant taxa through the network analysis of co-occurrences. In particular, co-transferring from BNS (*Acinetobacter* – *Aeromonas*) and BC (*Faecalibacterium* – *Bacteroides*) to the SE surfaces. Despite positive correlations among abundances that do not always reflect their real ecological interaction in a given habitat (47), the resulting network analysis can help to decipher spatial segregation and contamination routes in food processing environments (32, 48, 49). In this frame, transferring of *A. butzleri* between BNS and SE did not occur together with other abundant taxa associated with BNS, perhaps highlighting a primary origin different from the animal's skin and/or a reciprocal exchange between the two sources (BNS-SE).

A lower  $\alpha$ -diversity and minor number of taxa were observed on the shackles of the second line used for carcasses chilling in comparison to the first line, which crosses plucking and slaughter sectors. The reduction of biodiversity along the poultry slaughtering process is not surprising since several steps can progressively act on carcasses' microbiota composition through mechanical removal, washing, and high temperatures. These phenomena have been already observed in rinsates of broiler carcasses collected after plucking and chilling (50), and are often associated with a parallel reduction of the viable bacterial counts (51). Taking into account that both shackles' lines undergone the same cleaning-sanitizing intervention, this aspect seems to indirectly highlight a temperature-related selective pressure as well. Noteworthy, in cattle slaughterhouses, the minimal differences of temperature ( $\sim 2$ – $3^\circ\text{C}$ ) between

processing rooms have been sufficient to significantly modify the resident microbiota in favor of psychrotrophic taxa (32).

Besides the longitudinal variation along the process, the resident microbiota of SE changed, between the 2 days of sampling, in relation to the different flocks of broilers processed and the routine turnover of sanitizers (32, 52). *Arcobacter* abundance varied significantly between the 2 days and was thus mainly affected by these two variables, whereas it seemed to be minimally affected by processing phases and environmental temperature during slaughtering. Indeed, it was homogeneously distributed in all sectors and phases considered, with high abundances on the shackles of the second line used for carcass chilling. *Arcobacter* is more resistant to cold temperature than other Campylobacterota species (46, 53). This characteristic together with its aerotolerance can increase the probability of final contamination and persistence on broiler carcasses at the retail level (28). However, it should be highlighted that microbiota of broiler carcasses undergoes more changes downstream of slaughtering in relation to selective pressures of packaging type and storage (17, 54). Therefore, the presence of spoilage or pathogenic bacteria detected in a poultry abattoir, such as *Campylobacter* and *Escherichia coli*, does not necessarily indicate a contamination of the product at the retail level (55).

Previous observational studies based on 16S rRNA-amplicon sequencing did not detect *Arcobacter* spp. in poultry slaughterhouses and processed carcasses (8, 28, 38, 56, 57). In other similar studies, this emerging pathogen has been detected at lower abundance levels compared to our outcomes (<3–5%) and only in water samples or on carcasses during defeathering steps (11, 58). Discrepancies in sampling times, type of samples, and slaughtering environments make always difficult direct comparisons among different observational studies. However, the use of a metataxonomic approach based on ASVs instead of operational taxonomic units (OTUs) is probably the technical reason behind the high *Arcobacter* recovering rate here observed, since ASVs generally provide a more reliable assignment at the taxonomic levels of genus and species (59, 60). This approach allowed us to classify all Campylobacterota members at the highest taxonomic rank, which was the species for *Arcobacter*: assigned with 100% of similarity to reference sequences. On the other hand, taxonomic assignment ended above the genus for other pathogens inhabiting the poultry processing environments, such as *Shigella*, *Salmonella*, and *Escherichia* (61). Accordingly, this metataxonomic approach can be used as complementary analysis to the culture-based detection of *Arcobacter* spp., although it usually provides a reliable overview of major taxa, but with a limited capability in detecting the minor ones (32, 62).

The limited capability of detecting minor taxa raises the question whether the absence of *Arcobacter* in the BC metataxonomic profiles was here determined by extremely low and thus undetectable abundance levels for this pathogen. However, this seems unlikely since recent metagenomic studies did not detect the presence of *Arcobacter* in the microbiome of poultry gut contents (42, 63). Metagenomic has been proved more powerful than metataxonomic in detecting low abundant taxa in chicken gut, when enough reads per sample (>500,000) are available (64). It has to be clarified that such sequencing depth is not easily achievable in food matrices, where the nucleic acids extraction is often challenging and the proportion of non-microbial DNA is high (9). Moreover, a minor taxa like *Aureimonas altamirensis* have been detected in this study and in parallel isolated from the same samples (65), underlining a satisfactory detection threshold for the metataxonomic analysis.

As far as the targeted detection of *Arcobacter* spp., isolates have been collected from all three sources, including the BC, in contrast to the outcomes of relative abundances. Discrepancy between the metataxonomic monitoring of a given pathogen and its presence detected through enrichment has been already observed for *Salmonella* in chicken carcasses (50). It is not surprising in light of the two different analytical targets: i.e., the total DNA of a population or few alive cells. In agreement with our results, species of *Arcobacter* have been frequently isolated in poultry slaughterhouses during processing or after cleaning-sanitizing (23, 27, 66), and to a lesser ratio from broiler's

skin (29, 67, 68). Vice versa, this pathogen has not been isolated from intestines when the samples were collected avoiding the contact with the environment (29, 68) or in live birds (69). The isolation from different intestinal tracts has been reported when samples were collected during the slaughtering (69, 70). In our case study it can be assumed that broilers' intestines were originally devoid of *Arcobacter* spp. and have been contaminated immediately before the sampling, through the contact with the slaughterhouse surfaces. Moreover, it can be speculated that limited numbers of cells have been transferred and thus detected only by selective enrichment.

Despite the primary source of *Arcobacter* contamination in the poultry process chain is still debated, the intestines of birds are unlike to originally harbor this aerotolerant pathogen (71). Looking upstream the slaughtering process, *Arcobacter* has not been found on live birds' skin/feathers and rearing sheds, but has been detected in effluent sludge and waters that may be in direct contact with chickens' feet (66). The presence of *Arcobacter* in the transport crates for live broilers might be linked to the feet-conveyed contamination (29, 68). The high relative abundance of *Arcobacter* here observed on the shackles used for live birds hanging seems to confirm the role of broilers' feet as primary carrier of this pathogen into abattoirs.

As far as the species isolated, *A. butzleri* has been detected at a much higher frequency than *A. cryaerophilus* and other potential pathogenic *Arcobacter*. This is in agreement with previous studies on the *Arcobacter* spp. prevalence in poultry slaughterhouses and carcasses (27, 66, 69). It is worth mentioning that *A. butzleri* and *A. cryaerophilus* tend to be detected at the same level in poultry processing environments when direct counting is performed, while the enrichment method tends to favor the development of *A. butzleri* over all other non-*butzleri* species (27, 29, 68). However, the predominance of *A. butzleri* has been here confirmed by metataxonomic analysis by excluding the risk of having overestimated its presence with the enrichment procedure (29).

During slaughtering, *Arcobacter* spp. have been constantly isolated in almost all flocks and production runs; while in SE, the highest presence and biodiversity of the isolates were found in the plucking sector, regardless of the sampling day. The internal surfaces of scalding and plucking tunnels are difficult to clean and disinfect (72). In particular, the plucker is a recognized collector and reservoir of pathogens, such as *Campylobacter* and *Salmonella* (50). The resident microbiota of plucker and scalding can determine cross-contamination within the processing runs (animal-to-animal or flock-to-flock) and between different production runs (57). Moreover, *Arcobacter* spp. isolated from scalding water have shown different genotypes than those recovered in the rest of the slaughterhouse (69), while Houf et al. have reported a major environmental persistence of *A. cryaerophilus* in the slaughterhouse (29). In this study, *A. cryaerophilus* and other non-*butzleri* species were mostly detected in the plucking sector by means of both enrichment and metataxonomic analyses. Altogether these observations lead us to speculate the existence of *Arcobacter* species/strains persistent inside defeathering and scalding tunnels, while others are more transiently connected to the processing runs. The confirmation of this hypothesis is however beyond the scope of the present biogeographical study, and requires a pangenomic approach that has been conducted in a parallel research (73). Indeed, the intra-species isolates discrimination based on three putative virulence genes has been performed to grossly exclude the multiple isolations of a strain from the same sample (25, 31), without the intention to fully characterize the *Arcobacter* spp. ecology and virulence potential. Anyway, it is remarkable the detection of putative virulence genes in 50% of the isolates (18, 25). This ratio highlights the potential pathogenicity of *Arcobacter* and the importance of mitigating its presence in slaughterhouses.

Always with regard to the SE, *Arcobacter* was not isolated from the shackles of the first and second lines, unlike what was observed in terms of relative abundances. This discrepancy highlights a much more effective sanitizing/inactivation of *Arcobacter* on the shackles in comparison to what was observed for other equipment's surfaces, such as those inside plucking and defeathering tunnels. Furthermore, the high *Arcobacter*

abundance on shackle lines during slaughtering underlines the role of these devices (together with the carcasses themselves) in its spreading across the entire slaughtering environment. An additional washing/spraying step with sanitizers with parallel re-hang of the carcasses between the plucking and slaughter sectors could perhaps reduce the magnitude of *Arcobacter* contamination in this process layout (74–76). Anyway, this mitigation would not be resolute in relation to the high presence of this pathogen on the whole carcass and in the resident microbiota of equipment's surfaces of the abattoir.

To conclude, the combination of untargeted metataxonomic monitoring and *Arcobacter*-targeted enrichment applied here *in situ* allowed to improve the knowledge on the pathways followed by this emerging pathogen in the contamination of poultry slaughterhouses. The environmental contamination has been largely conveyed by broilers skin, which represented also the main source of *Arcobacter*. The high prevalence of *Arcobacter* in the abattoir and its viable persistence after sanitizing in specific environmental niches highlighted the importance of monitoring and mitigating its presence, which could soon be regarded as indicator of food safety and quality in poultry slaughtering.

## MATERIALS AND METHODS

### Broilers and environmental sampling

The study was conducted in a local poultry abattoir (average of 90,000 birds per day) operating in Piedmont (North-West of Italy). From January to May 2021, eight production runs were followed, and a total of 49 broiler flocks (breed Ross 308; 50 days age and 3.3 kg of weight in average; 10,000 broilers per flock in average) were sampled, each of them corresponding to a group of chickens reared in sheds with the same procedures by a unique farmer until the moment of delivering to the abattoir (Fig. 1A; Table S1). Broilers flocks were provided by farmers located in the North-West and Nord-Centre of Italy and differed in relation to the type of feeding and eventual need of curative antibiotic treatment (Table S1). According to the sampling procedures recommended to verify microbiological quality in broilers (35, 36), samples of broiler caecum (BC) and broiler neck skin (BNS) were collected during the slaughtering process at the moment of evisceration and the neck removal, respectively (Fig. 1B). Ten neck skins and ten caeca were randomly sampled during the slaughtering process of each flock, and separately pooled in two sterile bags; no link between BC and BNS at the level of the individual carcass level was kept.

Samples from the slaughterhouse equipment and environment (SE) were collected on two sampling days after routine cleaning and disinfection (Table S3), 40 and 270 days after the end of broilers sampling period, respectively (Fig. 1C). Sampling was performed on areas in contact with the carcasses (processing line) or viscera/giblet (by-products/waste line), using sterile sponges (VWR International, Leuven, Belgium) previously hydrated with 10 mL of buffered peptone water (BPW; Sigma, St. Louis, MO, USA). The same types of area were considered on the two sampling days. The plant used the semi-automated slaughtering process displayed in Fig. 1B, and specific environmental sampling points are listed in Table S2.

A total of 154 samples were collected from the three sampling sources (49 of BC; 49 of BNS; and 56 of SE) and kept at 4°C until the microbiological analysis, performed within 2 h after sampling.

### Microbiological analysis and isolation of *Arcobacter* spp.

Isolation of *Arcobacter* spp. was performed, including selective enrichment, as described by Houf et al. (77), with slight modifications. All media and supplements were provided by Merck & Co. (Readington Township, NJ, USA), unless stated otherwise.

Briefly, each pooled BSN or BC sample was aseptically cut with a scalpel, and 25 g was randomly collected (~2–3 g pieces from each of the 10 necks or intestines/feces) and transferred in a sterile bag with 100 mL of enrichment broth, composed by:

Arcobacter broth (CM0965; Oxoid, Basingstoke, UK) supplemented with 5% vol/vol of laked horse blood, 16 mg/L cefoperazone (C4292), 10 mg/L amphotericin B (A2411), 100 mg/L 5-fluorouracil (F6627), 32 mg/L novobiocin sodium salt (74675), and 64 mg/L trimethoprim (T7883). Hydrated sponges (SE) were aseptically cut in half lengthwise and one half placed in a sterile bag with 100 mL of the Arcobacter enrichment broth. All samples were homogenized for 2 min with a Stomacher 400 Circulator (LAB blender 400; PBI, Milan, Italy), and incubated for 48 h at 28°C in microaerobic conditions (AnaeroBox; Thermo Scientific, Waltham, MA, USA).

In parallel to the enrichment analysis, 25 g from each pooled BNS or BC sample, as well as the remaining half part of sponges, was resuspended in 100 mL of Ringer's solution and homogenized as previously described. About 10 mL of homogenized suspension were centrifugated ( $7,000 \times g$  for 10 min), the pellet recovered, and stored at  $-20^{\circ}\text{C}$  for further DNA extraction and metataxonomic analysis.

Isolation of *Arcobacter* spp. was carried out using as selective media the Arcobacter broth supplemented with agar (15 g/L) (77), with the same antibiotics used as for enrichment. About 10 mL of enriched broth was plated in parallel on selective media with and without 10% (vol/vol) of laked horse blood, and incubated for 96 h at 28°C in microaerobic conditions. After incubation, at least eight colonies (with a maximum of 12) with characteristic morphology were stored for further analysis.

### Identification and characterization of *Arcobacter* spp. isolates

Identification of the isolates was performed by Matrix Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS; Bruker, Billerica, MA, USA). Pure culture colonies were placed on a Micro Scout Plate spot (Bruker, Billerica, MA, USA) to which was later added 1  $\mu\text{L}$  of matrix  $\alpha$ -Cyano-4-hydroxycinnamic acid (CHCA; bioMérieux, C8982). After the crystallization of the matrix on the samples, the plate was read at MALDI-TOF MS comparing the spectra obtained with those present in the instrument database. All isolates with a threshold value below 1.7 were not considered *Arcobacter* spp., and were discarded (78).

Total genomic DNA of all isolates identified as *Arcobacter* spp. through MALDI-TOF analysis was extracted as previously described (25). The assignment of the isolates to *Arcobacter* genus was verified following the PCR protocol described by Valverde Bogantes et al. (79), and primer pairs designed by Harmon and Wesley (80). Species assignment was confirmed with a multiplex species-specific PCR assay for the simultaneous identification of *Arcobacter* (*A.*) *butzleri*, *A. thereius*, *A. cibarius*, *A. skirrowii*, and *A. cryaerophilus* (22).

Characterization of the isolates was performed by amplifying three virulence-related genes (*irgA*, *hecA*, and *hec*) with PCR protocol and conditions described by Doudah et al. (81). These genes are highly variable among *Arcobacter* spp. genomes and therefore their presence/absence have been used here as biomarkers to differentiate within isolates of the species (25, 31). All reagents for PCR assays and primer pairs were provided by Sigma-Aldrich (St. Louis, MO, USA). List of primers and thermal cycle parameters used are reported in Table S7.

### DNA extraction and amplicon-based sequencing

Total DNA was extracted from SE and BNS samples using the Master Pure purification kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions; whereas for BC samples, the NucleoSpin kit (Macherey-Nagel, Düren, Germany) and related protocol for DNA purification have been used. DNA quality and concentration were evaluated with a NanoDrop spectrophotometer and Qubit fluorimeter (Thermo Scientific). Library of the V3-V4 region was constructed from the 16S rRNA gene region using primers and conditions previously described (32). The PCR products were purified using the Agencourt AMPure kit (Beckman Coulter, Milan, Italy), and the resulting products were tagged with sequencing adapters using the Nextera XT library preparation kit (Illumina Inc, San Diego, CA, USA), according to the manufacturer's instructions. Sequencing



was performed using a MiSeq Illumina instrument (Illumina) with V3 chemistry, which generated 2 × 250 bp paired-end reads. MiSeq Control Software, V2.3.0.3, RTA, v1.18.42.0, and CASAVA, v1.8.2, were used for the base-calling and Illumina barcode demultiplexing processes.

## Bioinformatic analysis

A total of 5,469,573 raw-reads were produced by the 16S amplicon-based sequencing of the 154 samples. To obtain ASVs, the raw-reads were analyzed with *DADA2* package (82) in R environment (version 4.1.1; <http://www.r-project.org>). The pipeline previously described was followed for raw-reads filtering [*truncLen* = *c*(250,250); *trimLeft* = *c*(36, 36); *maxEE* = *c*(5, 5); *minLen* = *c*(50, 50); *truncQ* = 6], paired-end merging (minOverlap = 20) and *de-novo* chimera removal (83). All parameters not reported for filtering/merging steps are intended as default *DADA2* settings.

Taxonomy was assigned with a 99% of sequence similarity through Bayesian classifier method (84) by matching ASVs to the 2021 release of Silva prokaryotic SSU reference database (<https://zenodo.org/record/4587955#.YObFvhMzZRE>; version 138.1), with a species-level assignment performed at 100% of sequence similarity with the *addSpecies* script. All assignments were double-checked by using BLASTn suite (<https://blast.ncbi.nlm.nih.gov>), and ASVs with uncertain classification (to the Order rank or lower resolution) or matching (>99% similarity) with animal genomes were removed from the frequency tables. Three samples with less than 1,000 reads were excluded from the analysis: one from each sampling source (BNS, BC, and SE). Finally, a total of 2,927,216 paired-end reads (average of 19,351 reads/samples) were used to construct ASVs frequency table.

ASVs were aligned with *DECIPHER* package and an unrooted phylogenetic tree was constructed with *phangorn* package (85, 86). Alpha diversity metrics and weighted UniFrac beta-diversity distance were calculated with *phyloseq* and *picante* packages (87, 88): rarefaction limit was set to the lowest number of sequences/samples.

## Statistics

Statistical analyses and data plotting were performed in R environment (version 4.1.1; <http://www.r-project.org>), unless otherwise stated. Normality and homogeneity of the data were checked by means of the Shapiro-Wilk *W* test and Levene's tests, respectively. Variation and differences between multiple groups were assessed with one-way ANOVA (coupled with Tukey's post hoc test) and Kruskal-Wallis's test (coupled with pairwise Wilcoxon's test) for parametric and nonparametric data, respectively. Pairwise comparisons were alternatively performed with Wilcoxon and *t* tests according to data normality.

PCoA was used to visualize beta-diversity. Significant effects of categorical variables (sampling sources, production runs, and slaughterhouse sectors/lines) on the bacterial community variations were evaluated with Permutational Multivariate Analysis of Variance (PERMANOVA; *adonis* function based on 999 permutations and Bray-Curtis dissimilarity distances) and Analysis of Similarities (*ANOSIM* function) based on the weighted UniFrac distance matrix. The dispersion of bacterial communities was measured using the *betadisper* function.

To identify taxa that were specifically abundant in each type of sampling source, production run or slaughterhouse sectors, indicator species analysis was conducted using the *multipatt* function and verified with *strassoc-signassoc* functions in the package *indispecies* (89). Co-occurrence between taxa was calculated with Sparse Correlations for Compositional data (*sparCC* algorithm) using default parameters and 100 bootstraps in the R package *SpiecEasi* (90). Significance of the correlations was calculated as the proportion of simulated bootstrapped and only significant positive correlations were considered ( $R > 0.4$ ,  $P$  values < 0.001). Significant taxa-sources associations and significant co-occurrences among taxa were visualized with bipartite and co-occurrence

networks, respectively. Network plotting and the analysis of network topology were performed with the Gephi suite (version 0.10.0; <https://gephi.org>).

Mantel's test was used to examine correlations between complex matrices, such as the bacterial communities at the presence of *Arcobacter* detected through enrichment: function *mantel* in the *vegan* package was conducted with Spearman's rank correlation and 999 permutations (Brey-Curtis dissimilarity distance). Pairwise linear correlations were computed by the Pearson's moment correlation.

## Highlights

*Arcobacter* spp. is part of the resident microbiota of poultry slaughterhouses and is particularly persistent in certain environmental niches that are difficult to clean and sanitize. The introduction of this emerging pathogen in the processing environments is related to the broiler's skin microbiota, of which it represents a characteristic species. Metataxonomic analysis relying on 16S-rRNA gene sequencing represents a valid technique to track the presence of *Arcobacter* spp. An analytical approach combining metataxonomic and culture-dependent detection of this pathogen could be soon implemented to define food safety and quality in poultry slaughtering.

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## AUTHOR AFFILIATIONS

<sup>1</sup>Department of Agricultural, Forest and Food Sciences, University of Torino, Torino, Italy

<sup>2</sup>Department of Veterinary Sciences, University of Torino, Torino, Italy

<sup>3</sup>Local Health Company, ASL CN2, Cuneo, Italy

<sup>4</sup>Department of Veterinary and Biosciences, Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium

## AUTHOR ORCIDs

Cristian Botta  <http://orcid.org/0000-0003-1102-3294>

Davide Buzzanca  <http://orcid.org/0000-0003-4734-7151>

Francesco Chiesa  <http://orcid.org/0000-0001-6781-682X>

Ilario Ferrocino  <http://orcid.org/0000-0002-1657-0054>

Kalliopi Rantsiou  <http://orcid.org/0000-0002-5649-608X>

Kurt Houf  <http://orcid.org/0000-0002-0909-7756>

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## AUTHOR CONTRIBUTIONS

Cristian Botta, Conceptualization, Data curation, Formal analysis, Investigation, Software, Writing – original draft | Davide Buzzanca, Data curation, Investigation, Writing – review and editing | Elisabetta Chiarini, Data curation, Investigation, Writing – review and editing | Francesco Chiesa, Conceptualization, Investigation, Writing – review and editing | Selene Rubiola, Investigation, Writing – review and editing | Ilario Ferrocino, Investigation, Writing – review and editing | Edoardo Fontanella, Investigation, Writing – review and editing | Kalliopi Rantsiou, Writing – review and editing | Kurt Houf, Supervision, Writing – review and editing | Valentina Alessandria, Conceptualization, Funding acquisition, Supervision, Writing – review and editing

## DATA AVAILABILITY

Sequencing data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information under the BioProject accession number [PRJNA1051949](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1051949).

## ADDITIONAL FILES

The following material is available [online](#).

### Supplemental Material

**Figure S1 (AEM00296-24-S0001.pdf).** Taxonomic resolution achieved in the three sampling sources: relative distribution of the reads in relation to their assigned taxonomic rank, following the formula: (no. of reads assigned to a given taxa rank) / (total number of reads in a sample) x 100.

**Figure S2 (AEM00296-24-S0002.pdf).** (A) Stacked bar plots showing microbiota composition at the genus/species rank level, and (B) Venn diagram displaying the number of shared genus-species among the three sampling sources (only taxa present in more than 2 samples were considered).

**Figure S3 (AEM00296-24-S0003.pdf).** Co-occurrence networks of SE source with the projection of taxa belonging modules in broiler cecum (A), broiler neck skin (B), and co-occurrence network (C).

**Supplemental tables (AEM00296-24-S0004.xlsx).** Tables S1 to S7.

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