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

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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 characterisation of resident microbiota in the productive environments, so that targeted sanitising 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* are 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 sanitising.

Metataxonomic survey highlighted a negligible impact of faecal 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 sanitising procedure.

IMPORTANCE

Our findings have emphasised 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.

INTRODUCTION

Poultry meat is one of the most consumed food 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 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 harboured 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,

71 feed type and additives (12). Apart from the animal conditions and diet, the microbial composition of
72 poultry gut and skin can be altered through the administration of probiotics, prebiotics and organic
73 acids (13).
74 Microorganisms colonising the processing environment during slaughtering cause cross-contamination,
75 with inevitable transmission of spoilage microorganisms and foodborne pathogens from the product to
76 the consumer. Therefore, an important step in food safety control is to elucidate the contamination
77 routes in food chain (14). In poultry slaughterhouses, pathogens such as *Campylobacter*, *Salmonella*,
78 and *Listeria monocytogenes* have been reported as commonly present (15, 16). Noteworthy, in the last
79 two years *Salmonella* has been the responsible pathogen for more than 94 % of alert notifications in
80 the European poultry market followed by *L. monocytogenes* (4%) and *Campylobacter* spp. (5). The
81 gastrointestinal tract of chickens has been identified as reservoir of several foodborne pathogens, with
82 *Campylobacter* spp. and *Salmonella* sp. as the most dominant ones. All have the potential to cause
83 gastroenteritis in humans with often severe impact on public health (17).
84 In this frame, the transmission routes of *Arcobacter* spp. in slaughterhouses cross-contamination, on
85 both environment and broiler's carcasses, are still poorly understood. *Arcobacter* is part of the
86 Campylobacterota phylum, which contains other two genera sources of human foodborne pathogens,
87 i.e. *Campylobacter* and *Helicobacter* (18, 19). *Arcobacter* (*A.*) *butzleri, A. cryaerophilus* and *A.*
88 *skirrowii* are the species most associated with human clinical cases of gastrointestinal disorders (20–
89 22). Particularly relevant is the association of *A. butzleri* with a food outbreak in the US linked to the
90 consumption of contaminated broasted chicken (20). Moreover, *A. butzleri* isolated from poultry and
91 slaughterhouse environments have shown biofilm production abilities (23), which can favour its
92 colonization aptitude (23–25). Several studies have been conducted to assess the importance of *A.*
93 *butzleri*, *A. cryaerophilus*, and *A. skirrowii* as contaminants of specific food products and
94 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 forty-nine poultry flocks were sampled during eight slaughtering processes over four months, whereas the microbiota accumulated on the equipment's surfaces of the abattoir was subsequently analysed after cleaning and sanitising 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, 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 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 pair-wise comparison analysis of similarities (ANOSIM) tests, as well 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, 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, BSN), the flock origin and processing run.

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

142 A total of 6681 unique ASVs were detected in the 151 samples analysed. After alignment to the Silva's
143 reference database, the majority (70 %) of ASVs were assigned to the genus taxonomic rank, while
144 only 7 % of the ASVs reached the species level assignment. Assignment to the species rank have been
145 made only for ASVs that aligned 100 % to the reference V3-V4 region of 16S rRNA gene, while for
146 higher taxonomic ranks the assignment was based on 99 % of similarity. Overall, in comparison to
147 BNS and SE a lower taxonomic resolution was achieved in BC, in which 21 % of the reads were only
148 assigned to order and family (**Supplementary Figure 1**).
149 The phyla Firmicutes, Proteobacteria, Bacteroidota, Actinobacteriota and Campylobacterota were
150 predominant and ubiquitously distributed, by representing up to 80 % of the relative abundance in all
151 samples (**Fig 3A**). While in the caeca microbiota predominated Firmicutes and Bacteroidota, the neck
152 skin was characterized by higher abundances of Firmicutes and Proteobacteria (**Fig 3B**). Proteobacteria
153 together with Actinobacteria represented the dominant phyla in the SE, and the relative presence of
154 Campylobacterota (formerly in the Proteobacteria phylum) was here significantly lower than in broilers
155 samples (BC, BNS).
156 At the family level, 21.1 % of the taxa were shared between SE and BNS, while 33 % of the taxa were
157 included in the core microbiota. The SE harboured 71 source-specific families, while only two families
158 were characteristic of BC, and none were exclusively present in BNS (**Fig 3C**). Accordingly, the most
159 abundant families belonged alternatively to the core microbiota and BNS_SE-specific subgroup (**Fig**
160 **3A**). Considering the core families in each sampling source, anaerobes like *Ruminococcaceae*,
161 *Rikenellaceae*, *Bacteroidaceae* and *Lachnospiraceae* accounted for more than 50 % of average
162 abundance in BC samples, whereas in BNS the same abundance percentage was represented by
163 *Enterobacteriaceae*, *Lactobacillaceae*, *Clostridiaceae* and *Aeromonadaceae*. The environmental
164 microbiota was dominated by *Moraxellaceae*, which were rarely detected in caecal samples, and by
165 other families exclusively present in the BNS-SE subgroup, namely *Micrococcaceae*, *Arcobacteraceae*,

166 *Weeksellaceae* and *Sphingomonadaceae*. While a stable community during the entire monitoring period
167 in BC was observed, a marked succession of dominant families occurred along production runs in
168 BNS. Indeed, apart from the constant presence of *Enterobacteriaceae* overtime, the *Aeromonadaceae*
169 were predominant in the first production run and were thus replaced by *Lactobacillaceae* in the middle
170 productions, while from the sixth run, the *Sphingomonadaceae* took over the dominance. A time course
171 succession of families was observed in SE as well, with *Arcobacteraceae* abundances that decreased
172 significantly from the first to the second sampling day, while in parallel *Lachnospiraceae* and
173 *Sphingomonadaceae* abundances were significantly higher (Wilcoxon's test; P [FDR] < 0.001) in the
174 second ones (**data not shown**).
175
176 **Microbiota snapshots at the highest taxonomic resolution**
177 At the highest taxonomic resolution, the three sampling sources showed distinct microbiota
178 composition and distribution at the genus or species level. In particular, the proportion of taxa
179 harboured in the BNS-SE subgroup was higher than in the core microbiota (**Supplementary Figure 2**).
180 To identify genera and species associated to caeca, neck skin and environment, the indicator species
181 analysis was performed based on point biserial correlation. Out of 568 taxa identified up to genus or
182 species rank level and present in more than two samples, 193 taxa showed significant associations
183 (*multipatt* statistic; R > 0.4; *P* < 0.001) with the three sampling sources or their pairwise combinations,
184 which were illustrated with a bipartite network (**Fig. 4**). The core taxon *Escherichia-Shigella* was the
185 most abundant in the entire dataset but appeared mainly associated to BNS and secondly to BC. Core
186 taxa included in the Bacteroidota phylum, like *Alistipes* and *Bacteroides*, were indicators of the caecal
187 microbiota, together with minor members of Firmicutes. *Rothia endophytica* was the more abundant
188 Actinobacteria and together with major Proteobacteria members like *Acinetobacter, Paracoccus* and
189 *Psychrobacter*, were the main taxa associated to SE, while *Clostridium isatidis*, *Lactobacillus*

190 *kitasatonis* and minor Proteobacteria members were the most significant indicators of BNS ecology.
191 Most of the taxa significantly associated with two sources were shared between BNS and SE (28 taxa),
192 while only four and three taxa were indicators of BC-BNS and BC-SE, respectively. Among the main
193 indicator taxa of BNS-SE, *Arcobacter* (*A.*) *butzleri* and *Sphingomonas* tended to be more associated
194 with the broiler's skin than to the environment, in contrast to *Acinetobacter*, *Moraxella* and *Rothia*
195 *endophytica* were markedly more abundant in the environment. Interestingly, *A. butzleri* was the only
196 Campylobacterota uniquely found and significantly associated to BNS and SE, while *Helicobacter*
197 *pullorum* and *Campylobacter jejuni* were indicators of BC although they were part of the core
198 microbiota.
199 Following the previous observations at the family rank, temporal successions of the dominant genera
200 and species occurred in BNS (along production runs) and SE (sampling days), but not in BC. *A.*
201 *butzleri* was constantly present on broiler's skin along production runs, but significantly more abundant
202 the first sampling day in SE. Considering the SE layout, the genera *Acinetobacter* and *Psychrobacter*
203 were significantly associated to the plucking sector and chilling line, respectively. Looking into BC and
204 BNS samples, no taxa were significantly associated to any extent with the different conditions of
205 flocks' rearing, such as the eventual need of antibiotic treatment or different types of diet (**data not**
206 **shown**).
207 Focusing on *Arcobacter* spp., *A. butzleri* was not detected in the scalder and defeathering tunnel of the
208 plucking sector, in contrast to *A. cryaerophilus* and *A. cibarius* (**Table 1**). Considering the
209 slaughterhouse layout, *A. butzleri* was the only Campylobacterota detected on the surfaces of the plastic
210 shackles in the final chilling line.
211
212 **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 relative 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 dataset and showed significantly higher values in the SE network (Pairwise Wilcoxon's test, *P* [FDR] < 0.001). By applying a cut-off value of three-fold the upper Inter-Quartile-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 networks structures (**Fig. 5 and Supplementary Table 4**).

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 (**Supplementary Table 5**). Analysing the composition of the modules in this network, many of the taxa included in the two largest modules, here coded as SE01 and SE02, co-occurred in BNS network modules as well (**Supplementary Figure 3**).

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 (**Supplementary Figure 3 C**).

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 been 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 and Supplementary Table 6**). Besides, *A. cibarius* (3 isolates) and *A. cryaerophilus* (4 isolates) were detected in the environment and caecum samples, but not on broilers neck skin. One isolate identified as *A. skirrowii*

261 and two *A. thereius* species were recovered from caeca and environment, respectively. Differentiation
262 among isolates of the same species was performed by considering the presence-absence profiles of
263 three virulence-associated genes, namely *irgA*, *hecA* and *hecB*, which are genetic elements
264 encompassed in variable regions of *Arcobacter* pangenome (25, 31). The species *A. cibarius*, *A.*
265 *cryaerophilus*, *A. skirrowii*, *A. thereius* and 50 % of the *A. butzleri* isolates did not possess these three
266 genes. Isolates of *A. butzleri* devoid of these genes and other four genotypes were detected in all three
267 sampling sources, whereas the genotypes *hecA-irgA*, *hecA-hecB* and *hecA-hecB-irgA* were not
268 recovered in the environment, which therefore harboured a lower number of the *A. butzleri* genotypes.
269 Overall, *Arcobacter* spp. have been isolated from broilers in all production runs, with 86 % and 88 %
270 positive samples in caeca and neck skins, respectively, while only one flock resulted *Arcobacter*-free in
271 both BC and BNS pooled samples. In the cleaned and sanitized SE only 31 % of the samples were
272 positive to the presence of *Arcobacter*. At least one positive sample was detected in each processing
273 phase considered in the plucking and slaughtering sectors, except for the automated evisceration (n = 5
274 samples). No *Arcobacter* was isolated from the shackles of the automated slaughtering line (n = 7) and
275 chilling line (n = 7). Surfaces of the scalding tank (sampling point 2A) and the rubber-fingers of the
276 defeathering tunnel (sampling point 3A) showed the highest number of positive samples and the
277 greatest biodiversity: i.e., the sites from which more different species and biotypes have been isolated.
278 Besides, species different from *A. butzleri* were only detected in the plucking sector and upon the
279 conveyor belt of manually eviscerated carcasses (**Fig 6**).
280 Comparing the distribution and numbers of isolated *Arcobacter* to the relative abundance of this genus,
281 no significant pairwise correlation was observed in the BC and SE samples (Spearman's correlation; P
282 > 0.05), while in BC samples it was not detected at all by the metataxonomic analysis. Moreover,
283 correlating samples 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 towards 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 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 (β-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 the three 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 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 negligeable 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 in 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 similarity with BNS. These two sampling sources shared several species included in *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 harboured in the poultry gut are unlikely to survive and colonise 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 washing step and air chilling tunnel.

Aside 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 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 to 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 α-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 to 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 minimal differences of temperature (~2-3 °C) between processing rooms have been sufficient to significantly modify the resident microbiota in favour of psychrotrophic taxa (32).

Besides the longitudinal variation along the process, the resident microbiota of SE changed, between the two days of sampling, in relation to the different flocks of broilers processed and the routine

356 turnover of sanitizers (32, 52). *Arcobacter* abundance varied significantly between the two days and
357 was thus mainly affected by these two variables, whereas it seemed to be minimally affected by
358 processing phases and environmental temperature during slaughtering. Indeed, it was homogeneously
359 distributed in all sectors and phases considered, with high abundances on the shackles of the second
360 line used for carcass chilling. *Arcobacter* is more resistant to cold temperature than other
361 Campylobacterota species (46, 53). This characteristic together with its aerotolerance can increase the
362 probability of final contamination and persistence on broiler carcasses at retail level (28). However, it
363 should be highlighted that microbiota of broiler's carcass undergoes more changes downstream of
364 slaughtering in relation to selective pressures of packaging type and storage (17, 54). Therefore, the
365 presence of spoilage or pathogenic bacteria detected in a poultry abattoir, such as
366 *Campylobacter* and *E. coli*, does not necessarily indicate a contamination of the product at the retail
367 level (55).
368 Previous observational studies based on 16S rRNA-amplicon sequencing did not detect *Arcobacter*
369 spp. in poultry slaughterhouses and processed carcasses (8, 28, 38, 56, 57). In other similar studies, this
370 emerging pathogen has been detected at lower abundance levels compared to our outcomes (< 3-5 %)
371 and only in water samples or on carcasses during defeathering steps (11, 58). Discrepancies of
372 sampling times, type of samples and slaughtering environments make always difficult direct
373 comparisons among different observational studies. However, the use of a metataxonomic approach
374 based on Amplicon Sequence Variants (ASVs) instead of Operational Taxonomic Units (OTUs) is
375 probably the technical reason behind the high *Arcobacter* recovering rate here observed, since ASVs
376 generally provide a more reliable assignment at the taxonomic levels of genus and species (59, 60).
377 This approach allowed to classify all Campylobacterota members at the highest taxonomic rank, which
378 was the species for *Arcobacter*: assigned with 100 % of similarity to reference sequences. On the other
379 hand, taxonomic assignment ended above the genus for other pathogens inhabiting the poultry

380 processing environments, such as *Shigella*, *Salmonella* and *Escherichia* (61). Accordingly, this
381 metataxonomic approach can be used as complementary analysis to the culture-based detection of
382 *Arcobacter* spp., although it usually provides a reliable overview of major taxa, but with a limited
383 capability in detecting the minor ones (32, 62).
384 The limited capability of detecting minor taxa raises the question whether the absence of *Arcobacter* in
385 the BC metataxonomic profiles was here determined by extremely low and thus undetectable
386 abundance levels for this pathogen. However, this seems unlikely since recent metagenomic studies did
387 not detect presence of *Arcobacter* in the microbiome of poultry gut contents (42, 63). Metagenomic has
388 been proved more powerful than metataxonomic in detecting low abundant taxa in chicken gut, when
389 enough reads per sample (> 500,000) are available (64). It has to be clarified that such sequencing
390 depth is not easily achievable in food matrices, where the nucleic acids extraction is often challenging
391 and the proportion of not-microbial DNA is high (9). Moreover, a minor taxa like *Aureimonas*
392 *altamirensis* have been detected in this study and in parallel isolated from the same samples (65),
393 underlining a satisfactory detection threshold for the metataxonomic analysis.
394 As far as the targeted detection of *Arcobacter* spp., isolates have collected from all the three sources,
395 including the BC, in contrast to the outcomes of relative abundances. Discrepancy between the
396 metataxonomic monitoring of a given pathogen and its presence detected through enrichment has been
397 already observed for *Salmonella* in chicken carcasses (50). It is not surprising in light of the two
398 different analytical targets: i.e., the total DNA of a population or few alive cells. In agreement with our
399 results, species of *Arcobacter* have been frequently isolated in poultry slaughterhouses during
400 processing or after cleaning-sanitising (23, 27, 66), and to a lesser ratio from broiler's skin (29, 67, 68).
401 Vice versa, this pathogen has not been isolated from intestines when the samples were collected
402 avoiding the contact with the environment (29, 68) or in live birds (69). The isolation from different
403 intestinal tracts has been reported when samples were collected during the slaughtering (69, 70). In our

404 case study it can be assumed that broilers' intestines were originally devoid of *Arcobacter* spp. and
405 have been contaminated immediately before the sampling, through the contact with the slaughterhouse
406 surfaces. Moreover, it can be speculated that limited numbers of cells have been transferred and thus
407 detected only by selective enrichment.
408 Despite the primary source of *Arcobacter* contamination in poultry process chain is still debated, the
409 intestines of birds is unlike to originally harbour this aerotolerant pathogen (71). Looking upstream the
410 slaughtering process, *Arcobacter* has not been found on live birds skin/feathers and rearing sheds, but
411 has been detected in effluent sludge and waters that may be in direct contact with chickens feet (66).
412 The presence of *Arcobacter* in the transport crates for live broilers might be linked to the feet-conveyed
413 contamination (29, 68). The high relative abundance of *Arcobacter* here observed on the shackles used
414 for live birds hanging seems to confirm the role of broilers feet as primary carrier of this pathogen into
415 abattoirs.
416 As far as the species isolated, *A. butzleri* has been detected at a much higher frequency than *A.*
417 *cryaerophilus* and others potential pathogenic *Arcobacter*. This is in agreement with previous studies
418 on the *Arcobacter* spp. prevalence in poultry slaughterhouses and carcasses (27, 66, 69). It is worth to
419 mentioning that *A. butzleri* and *A. cryaerophilus* tend to be detected at the same level in poultry
420 processing environments when direct counting is performed, while the enrichment method tends to
421 favour the development of *A. butzleri* over all other not-*butzleri* species (27, 29, 68). However, the
422 predominance of *A. butzleri* have been here confirmed by metataxonomic analysis by excluding the
423 risk of having overestimated its presence with the enrichment procedure (29).
424 During slaughtering *Arcobacter* spp. have been constantly isolated in almost all flocks and production
425 runs, while in SE the highest presence and biodiversity of the isolates were found in the plucking
426 sector, regardless of the sampling day. The internal surfaces of scalding and plucking tunnels are
427 difficult to clean and disinfect (72). In particular the plucker is a recognised collector and reservoir of

428 pathogens, such as *Campylobacter* and *Salmonella* (50). The resident microbiota of plucker and scalder
429 can determine cross-contamination within the processing runs (animal-to-animal or flock-to-flock) and
430 between different production runs (57). Moreover, *Arcobacter* spp. isolated from scalding water have
431 shown different genotypes than those recovered in the rest of the slaughterhouse (69), while Houf and
432 colleagues have reported a major environmental persistence of *A. cryaerophilus* in the slaughterhouse
433 (29). In this study *A. cryaerophilus* and other not-*butzleri* species were mostly detected in the plucking
434 sector by means of both enrichment and metataxonomic analyses. All together these observations lead
435 us to speculate the existence of *Arcobacter* species/strains persistent inside defeathering and scalding
436 tunnels, while others are more transiently connected to the processing runs. The confirmation of this
437 hypothesis is however beyond the scope of the present biogeographical study, and requires a
438 pangenomic approach that has been conducted in a parallel research (73). Indeed, the intra-species
439 isolates discrimination based on three putative virulence genes has been performed to grossly exclude
440 the multiple isolation of a strain from the same sample (25, 31), without the intention to fully
441 characterise the *Arcobacter* spp. ecology and virulence potential. Anyway, it is remarkable the
442 detection of putative virulence genes in 50% of the isolates (18, 25). This ratio highlights the potential
443 pathogenicity of *Arcobacter* and the importance of mitigating its presence in slaughterhouses.
444 Always with regard to the SE, *Arcobacter* was not isolated from the shackles of the first and second
445 line, unlike to what was observed in terms of relative abundances. This discrepancy highlights a much
446 more effective sanitising/inactivation of *Arcobacter* on the shackles in comparison to what observed for
447 other equipment's surfaces, such those inside plucking and defeathering tunnels. Furthermore, the high
448 *Arcobacter* abundance on shackle lines during slaughtering underline the role of these devices (together
449 with the carcasses themselves) in its spreading across the entire slaughtering environment. An
450 additional washing/spraying step with sanitizers with parallel re-hang of the carcasses between the
451 plucking and slaughter sectors could perhaps reduce the magnitude of *Arcobacter* contamination in this

process layout (74–76). Anyway, this mitigation would not be resolutive 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 (**Figure 1A and Supplementary Table 1)**. Broilers flocks were provided from 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 (**Supplementary Table 1)**. 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 slaughtering process at the moment of evisceration and the neck removal, respectively (**Figure 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 routinely cleaning and disinfection (**Supplementary Table 3)**, 40 and 270 days after the end of broilers sampling period, respectively (**Figure 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 **Figure 1B**, and specific environmental sampling points are listed in **Supplementary Table 2.**

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

Microbiological analysis and isolation of *Arcobacter* spp.

Isolation of *Arcobacter* spp. was performed, including selective enrichment, as described by Houf et al. (2001) (77), with slight modifications. All media and supplements were provided from 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 ten necks or intestines/faeces) and transferred in a sterile bag with 100 mL of enrichment broth, composed by: Arcobacter broth (CM0965; Oxoid, Basingstoke, UK) supplemented with 5% v/v 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 minutes with a Stomacher® 400 Circulator (LAB blender 400; PBI, Milan, Italy), and incubated for 48 hours 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 samples, as well the remaining half part of sponges, were resuspended in 100 mL of Ringer's solution and homogenized as previously described. Ten millilitres of homogenized suspension were centrifugated (7,000 g for 10 min), the pellet recovered, and stored at – 20 °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. Ten microliters

of enriched broth were plated in parallel on selective media with and without 10 % (v/v) of laked horse blood, and incubated for 96 hours 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 characterisation 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 µl of matrix α-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. (2015) (79), and primer pairs designed by Harmon and Wesley (1996) (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*, *hec*) with PCR protocol and conditions described by Douidah and colleagues (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 **Supplementary Table 7**.

534

535 **DNA extraction and amplicon-based sequencing**

536 Total DNA was extracted from SE and BNS samples using the Master Pure purification kit (Epicentre,
537 Madison, WI, USA) according to the manufacturer's instructions, whereas for BC samples the
538 NucleoSpin® kit (Macherey-Nagel, Düren, Germany) and related protocol for DNA purification has
539 been used. DNA quality and concentration was evaluated with a NanoDrop spectrophotometer and
540 Qubit fluorimeter (Thermo Scientific). Library of the V3-V4 region were constructed from the 16S
541 rRNA gene region using primers and conditions previously described (32). The PCR products were
542 purified using the Agencourt AMPure kit (Beckman Coulter, Milan, Italy), and the resulting products
543 were tagged with sequencing adapters using the Nextera XT library preparation kit (Illumina Inc, San
544 Diego, CA, USA), according to the manufacturer's instructions. Sequencing was performed using a
545 MiSeq Illumina instrument (Illumina) with V3 chemistry, which generated 2X250 bp paired-end reads.
546 MiSeq Control Software, V2.3.0.3, RTA, v1.18.42.0, and CASAVA, v1.8.2, were used for the base-
547 calling and Illumina barcode demultiplexing processes.

548

549 **Bioinformatic analysis**

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

557 Taxonomy was assigned with a 99 % of sequence similarity through Bayesian classifier method (84) by
558 matching ASVs to the 2021 release of Silva prokaryotic SSU reference database
559 (https://zenodo.org/record/4587955#.YObFvhMzZRE; version 138.1), with a species level assignment
560 performed at 100 % of sequence similarity with the *addSpecies* script. All assignments were double
561 checked by using BLASTn suite (https://blast.ncbi.nlm.nih.gov), and ASVs with uncertain
562 classification (to the Order rank or lower resolution) or matching (> 99 % similarity) with animal
563 genomes were removed from the frequency tables. Three samples with less than 1,000 reads were
564 excluded from the analysis: one from each sampling source (BNS, BC and SE). Finally, a total of
565 2,927,216 paired-end reads (average of 19,351 reads/sample) were used to construct ASVs frequency
566 table.
567 ASVs were aligned with *DECIPHER* package and an unrooted phylogenetic tree was constructed with
568 *phangorn* package (85, 86). Alpha diversity metrics and weighted UniFrac beta-diversity distance were
569 calculated with *phyloseq* and *picante* packages (87, 88): rarefaction limit was set to the lowest number
570 of sequences/sample.
571 Sequencing data were deposited at the Sequence Read Archive of the National Center for
572 Biotechnology Information under the bioproject accession number PRJNA1051949.
573
574 **Statistics**
575 Statistical analyses and data plotting were performed in R environment (version 4.1.1; http://www.r-
576 project.org), unless otherwise stated. Normality and homogeneity of the data were checked by means of
577 the Shapiro-Wilk W test and Levene's tests, respectively. Variation and differences between multiple
578 groups were assessed with one-way ANOVA (coupled with Tukey's post-hoc test) and Kruskal–
579 Wallis's test (coupled with pairwise Wilcoxon's test) for parametric and not parametric data,

580 respectively. Pairwise comparisons were alternatively performed with Wilcoxon and T-tests according
581 to data normality.
582 Principal-coordinate Analysis (PCoA) was used to visualize beta-diversity. Significant effects of
583 categorical variables (sampling sources, production runs, slaughterhouse sectors/lines) on the bacterial
584 community variations were evaluated with Permutational Multivariate Analysis of Variance
585 (PERMANOVA; *adonis* function based on 999 permutations and Brey-Curtis dissimilarity distances)
586 and Analysis of Similarities (*ANOSIM* function) based on the weighted UniFrac distance matrix. The
587 dispersion of bacterial communities was measured using the *betadisper* function.
588 To identify taxa that were specifically abundant in each type of sampling source, production run or
589 slaughterhouse sectors, indicator species analysis was conducted using the *multipatt* function and
590 verified with *strassoc-signassoc* functions in the package *indispecies* (89). Co-occurrence between taxa
591 were calculated with Sparse Correlations for Compositional data (*sparCC* algorithm) using default
592 parameters and 100 bootstraps in the R package *SpiecEasi* (90). Significance of the correlations were
593 calculated as the proportion of simulated bootstrapped and only significant positive correlation have
594 been considered ($R > 0.4$, P-values < 0.001). Significant taxa-sources associations and significant co-
595 occurrences among taxa were visualised with bipartite and co-occurrence networks, respectively.
596 Networks plotting and the analysis of network topology were performed with the Gephi suite (version
597 0.10.0; https://gephi.org).
598 Mantel's test was used to examine correlations between complex matrices, such as the bacterial
599 communities at the presence of *Arcobacter* detected though enrichment: function *mantel* in the *vegan*
600 package was conducted with Spearman's rank correlation and 999 permutations (Brey-Curtis
601 dissimilarity distance). Pairwise linear correlations were computed by the Pearson's moment
602 correlation.
603

604

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Cristian Botta: Conceptualization**,** Investigation, Data curation, Formal analysis, Software, Writing – original draft.

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860

861 **Tables**

862 **Table 1.**

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

863

Table 2.

		Sampling Source		
	Parameters:	**BC**	**BNS**	**SE**
General featues (counts)	Taxa analysed	148	163	285
	Nodes (taxa correlated)	47	81	214
	Edges (correlations)	50	128	908
	Triangles	7	81	2756
	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-occurrences 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%

Figure legends:

Fig. 1. Experimental design, spatiotemporal organisation of samples collection and process layout.

Graphical summary (**A**) of broiler flocks' origin with localisation (North-Italy), number of farmers, and type of samples collected from broilers during slaughter: i.e., caecum (BC) and neck skins (BNS). Detailed informations about flocks rearing conditions are provided in **Supplementary Table 1**. 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. At 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-

879 fingered pluckers on rotating disks. Once in the SS, the cloaca is cut (4A) by a vent cutter and most of
880 the carcasses processed (> 90 %) are transported by the FL to the neck cut (5A): collection point of
881 BNS during processing. Following: evisceration by spoon-shaped scoop (6A); giblet removal by rake-
882 like extractor and aspirator (7A); feet removal. Viscera and giblets are collected on a conveyor belt
883 (7B): collection point of BC during processing. Alternatively, from neck cut step onward the manual
884 evisceration is performed for broilers commercialised with head and feet, which are reunited to the
885 main line with a plastic conveyor belt (7C). At the end of the slaughter, carcasses are moved from FL
886 to plastic shackles (8A) of the second line [SL] and transported through further sectors for washing,
887 chilling (air chilling tunnel), and final portioning/packaging. Detailed information about SE sampling
888 points and cleaning-sanitizing procedures followed are described in **Supplementary Table 2** and
889 **Supplementary Table 3**, respectively.
890 Duration of the study (**C**) with the number of production runs (day slaughter processes) followed, total
891 samples collected from broilers (BC and BNS) and from SE.
892
893 **Fig. 2. Beta-diversity and Alpha-diversity of the bacteria communities.**
894 Principal Coordinates Analysis (PCoA) plot **(A)** displaying weighted UniFrac distances matrix (β-
895 diversity): sampling sources are shown by different colours as reported in the colour coding key.
896 Variance explained (R^2 value) by each sampling source (BC, BNS, SE) and pairwise biological
897 dissimilarity (R value) are quantified by Permutational Analysis of Variance (PERMANOVA) and
898 analysis of similarities (ANOSIM), respectively; since *P*-values result from a 999 permutations test,
899 they are only reported significant down to 0.001. Box plots illustrating beta-dispersion of the samples
900 from the centroid (**B**) and alpha-diversity metrics (**C**) in the three sampling sources: boxes represent the
901 interquartile range (IQR); central line indicates the median; whiskers indicate the furthest point within
902 (1.5 × IQR); black points beyond whiskers represent outliers; grey points display the samples.

903 Significant differences between sources are highlighted by *P*-value (Kruskal-Wallis and Pairwise
904 Wilcoxon's tests; FDR adjusted) or asterisks (*P*-value: *= <0.05; **= <0.01, ***= <0.001).
905
906 **Fig. 3. Overview of microbiota composition and distribution.**
907 Stacked bar plots **(A)** showing microbiota composition (relative abundance) in phylum and family taxa
908 ranks, with colour coding keys. Samples are grouped following the temporal sampling order in each
909 sampling source (BC, BNS, SE), and then according to the flock's slaughtering order and processing
910 phase order BC-BNS and SE, respectively. Abattoir sectors and transport lines are indicated: FL= first
911 line; PS= plucking sector; SS= slaughter sector; SL= second line. Taxa are sorted in the legend from
912 the most to the least abundant (> 1% average). Belonging to core microbiota or subgroup is reported
913 for each family. Box plots **(B)** displaying Log- transformed abundances of phyla. Different letters (a, b,
914 c, d) highlight significant differences (ANOVA coupled with Tukey's test; $P < 0.001$). Venn diagram
915 **(C)** showing the number of shared taxa at the family level among the three sampling sources; only taxa
916 present in more than 2 samples were considered.
917
918 **Fig. 4. Bipartite network revealing the taxa (genus or species level) associated to the three**
919 **sampling sources.**
920 Taxa (coloured nodes) are unidirectionally connected with arrows (edges) to the sampling sources (BC,
921 BNS, SE) if significant associations have been detected (Indicator Species Analysis: *multipatt*
922 statistics; R > 0.4 and P value < 0.001). Nodes are made proportional to taxa abundances (log
923 Transformed) and coloured in relation to the belonging Phylum (refer to colour coding key). Only the
924 taxa present in more than 2 samples were considered and most abundant taxa (> 0.5 % in average) are
925 reported in the legend with codifying number (from the most to the least abundant), together with their
926 belonging to core microbiota or subgroups (Supplementary Figure 2). Edges thicknesses and length are

927 respectively directly and indirectly proportional to the association strength (significance parameters in
928 *multipatt* statistic), while colour refers to the associated source. Network layout was constructed using
929 ForceAtlas2 algorithm: distance between node and associated source is proportional to the association
930 strength.
931
932 **Fig. 5. Co-occurrence networks of each sampling source.**
933 Taxa (nodes) are connected by lines (edges) in relation to significantly positive pairwise correlation
934 (*SparCC* algorithm with 100 bootstraps; *P-value* < 0.001, R > 0.4). Nodes are made proportional to
935 taxa occurrences and coloured in relation to the co-occurring modules (refer to colour coding keys);
936 hub taxa with the highest value of betweenness centrality (> 3× of upper IQR) are reported. Edges
937 thicknesses are made proportional to *SparCC* correlation values and network layout was constructed
938 using ForceAtlas2 algorithm. Detailed information on modules composition is reported in
939 **Supplementary Table 4**.
940
941 **Fig. 6. Pseudo-heatmap summarising the frequency of *Arcobacter spp*. isolation and abundances**
942 **in the three sampling sources**.
943 Species and biotypes of the isolates are reported on the X axis. Samples (Y axis) are ordered by
944 production runs in BC and BNS, while in SE the order follows the succession of sectors/lines and
945 slaughtering phases (sampling points). Sectors/lines: (FL) first shackles line for live birds and
946 carcasses; (PL) plucking sector; (SS) slaughter sector; (SL) second shackles. Sampling points: (1A)
947 shackles automated line; (2A) scalding tunnel; (3A) defeathering tunnel; (4A) vent cutter; (5A) neck
948 cutter; (6A) spoon-shaped scoop eviscerator; (7A) rake-like extractor and aspirator for giblets; (7B)
949 conveyor belt for viscera and giblets; (7C) conveyor belt for manually eviscerated carcases; (8A)
950 shackles of chilling line. For the number of isolates and abundances refer to colours coding key (ND=

not detected).

Tables legends:

Table 1. Distribution of *Arcobacter*, *Campylobacter* and *Helicobacter* genera in broilers and environmental samples. For the SE sector codes (*) refer to **Figure 1** and Supplementary table 2: first line [FL] of stainless stell shackles; plucking sector [PS]; slaughter sector [SS]; second line [SL] of plastic shackles.

Table 2. Summary of *SparCC*-based co-occurrence networks features and topology. 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; Path length= number of edges along the shortest path for all possible pairs of nodes

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 sanitise
- 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 relaying 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