



UNIVERSITÀ DEGLI STUDI DI TORINO

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

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

This is the author's manuscript	
Original Citation:	
Availability:	
This version is available http://hdl.handle.net/2318/1974193 since 2024-04-22T22:48:51Z	
Published version:	
DOI:10.1128/aem.00296-24	
Terms of use:	
Open Access	
Anyone can freely access the full text of works made available as "Open Access". Works made available	le
under a Creative Commons license can be used according to the terms and conditions of said license. of all other works requires consent of the right holder (author or publisher) if not exempted from convi	Use

(Article begins on next page)

protection by the applicable law.

1	Microbial contamination pathways in a poultry abattoir provided clues on the distribution and
2	persistence of <i>Arcobacter</i> spp.
3	
4	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
5	Ferrocino <sup>1</sup> , Edoardo Fontanella <sup>3</sup> , Kalliopi Rantsiou <sup>1</sup> , Kurt Houf <sup>4</sup> , Valentina Alessandria <sup>1</sup>
6	
7	<sup>1</sup> Department of Agricultural, Forest and Food Sciences, University of Torino, Italy.
8	<sup>2</sup> Department of Veterinary Sciences, University of Torino, Italy
9	<sup>3</sup> Local health company, ASL CN2, Cuneo, Italy
10	<sup>4</sup> Department of Veterinary and Biosciences, Faculty of Veterinary Medicine, Ghent University,
11	Belgium
12	
13	Key words: poultry slaughtering process, emerging foodborne pathogen, detection, microbiota,
14	Arcobacter, metataxonomic
15	
16	*Corresponding author: davide.buzzanca@unito.it
17	
18	

#### 19 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.

38

#### **39 IMPORTANCE**

40 Our findings have emphasised the persistence of *Arcobacter* spp. in a modern poultry abattoir and its 41 establishment as part of the resident microbiota in specific environmental niches. Although the 42 responses provided here are not conclusive for the identification of the primary source of 43 contamination, this biogeographic assessment underscores the importance of monitoring *Arcobacter* 44 spp. from the early stages of the production chain with the integrative support of metataxonomic 45 analysis. Through such combined detection approaches, the presence of this pathogen could be soon 46 regarded as hallmark indicator of food safety and quality in poultry slaughtering.

#### 47 INTRODUCTION

Poultry meat is one of the most consumed food worldwide, and its market in the European Union (EU) 48 has grown constantly in the last decade, reaching in 2019 an estimated threshold of 13.3 million tonnes 49 50 produced (1). Italy is the fifth largest producer of poultry meat in the EU and it has shown a steadfast 51 export growth over the past few years, notwithstanding the adverse impacts of the global pandemic. At 52 the national level, most poultry farms are concentrated in the northern regions, and broilers are 53 slaughtered in few large-scale abattoirs (2–4). Proportionally to the dimension of this food trade, cases 54 of recall related to poultry meat and poultry products are frequent and often associated with foodborne 55 outbreaks in different countries (5, 6).

56 Contamination along the poultry processing chain is a common event that extends from farm to fork. 57 Each step of this process plays a role in shaping the ultimate microbiota profile of poultry meat, 58 encompassing both spoilage and pathogenic microorganisms (7). It has been documented that the 59 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 60 61 contamination of live birds prior to slaughter that changes composition and abundance over the various 62 production stages, determining the final meat spoilage (8, 9). In particular, the microorganisms found 63 on chicken skin represent the initial contributors to the carcass surface microbiota. Most of the 64 microbial populations harboured on feathers, feet and carcass surface are eventually concentrated on 65 the neck skin through the percolation of water that occurs in different processing stages, including 66 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).

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 gastroenteritis in humans with often severe impact on public health (17). 83

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 Campylobacterota phylum, which contains other two genera sources of human foodborne pathogens, 86 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 95 ecology within the environmental contamination dynamics of the whole microbiome in space and time.
96 Since microbial species actively coexists, mutualistically or competitively, within the communities of
97 ecological niches, it is of pivotal importance a holistic evaluation of the transmission routes of
98 *Arcobacter* spp. in the frame of the entire bacterial population of a poultry slaughterhouse.

99 The present study aims to assess the extent to which skin and caeca of incoming broilers contribute to 100 the composition of the resident microbiota in a modern slaughterhouse, as well as to elucidate how the 101 environmental persistence and distribution of Arcobacter spp. are affected by these cross-102 contamination patterns. Therefore, neck skins and caeca of forty-nine poultry flocks were sampled 103 during eight slaughtering processes over four months, whereas the microbiota accumulated on the 104 equipment's surfaces of the abattoir was subsequently analysed after cleaning and sanitising in two 105 distinct sampling campaigns (Fig 1). The DNA amplicon-based sequencing of the 16S rRNA gene was 106 applied to characterize bacterial communities and to detect the presence of *Arcobacter* species, which 107 was benchmarked in parallel with selective isolation.

108

#### 109 **RESULTS**

110

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

113 Transfer of microbiota from the broilers neck skin (BNS) and caecum (BC) through the processing 114 steps on surfaces of a slaughtering environment (SE), and the establishment of a resident in-house 115 microbiota, have been assessed in an abattoir localized in the north-western Italy (**Fig 1**). Bacterial 116 communities of all three sampling sources (BNS, BC, SE) were examined through metataxonomic 117 analysis based on Amplicon Sequence Variants (ASVs), and BC and BNS microbiota refer to samples 118 that represent a pool for each slaughtered flock.

The phylogenetic variation of the samples was visualized with a PCoA plot based on weighted UniFrac 119 120 beta-diversity distance (Fig 2A). Bacterial communities of BC, BNS and SE were graphically 121 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 122 [FDR] < 0.001). Bacterial communities of BC were clearly different from BNS and SE, as significantly 123 indicated by both PERMANOVA and pair-wise comparison analysis of similarities (ANOSIM) tests, 124 125 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 126 127 similarity. In addition, the dispersion of bacterial communities in each source was examined by 128 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 129 130 limited phylogenetic and compositional variation among samples, while more dispersed bacterial 131 communities were observed in BNS and in SE.

132 The comparison of alpha-diversity metrics between the three sources showed significantly (P[FDR] <133 0.001) higher values for the number of observed taxa, richness (Chao1), evenness estimators (Shannon, 134 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 135 136 [FDR] < 0.05) higher in the samples collected from the first compared to the second shackles line, and 137 has progressively decreased along processing phases (data not shown). No other significant variations 138 in alpha-diversity metrics were observed in each source as function of the sampling area and surface 139 material (SE) or among broilers' samples (BC, BSN), the flock origin and processing run.

140

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

A total of 6681 unique ASVs were detected in the 151 samples analysed. 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 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 (**Supplementary Figure 1**).

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, 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 composition and distribution at the genus or species level. In particular, the proportion of taxa 178 179 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 the first sampling day in SE. Considering the SE layout, the genera Acinetobacter and Psychrobacter 202 203 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).

Focusing on *Arcobacter* spp., *A. butzleri* was not detected in the scalder and defeathering tunnel of the plucking sector, in contrast to *A. cryaerophilus* and *A. cibarius* (**Table 1**). Considering the slaughterhouse layout, *A. butzleri* was the only Campylobacterota detected on the surfaces of the plastic 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.

219 Overall, the slaughterhouse environment showed the highest proportion of taxa significantly correlated 220 and thus included in the graph. Indeed, SE network showed a greatest number of edges and triangles, as 221 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 222 223 pairwise co-occurrences, the taxa in SE were not more densely connected and did not show a relative 224 higher tendency to group in modules than what was observed in BC and BNS. Focusing on the co-225 occurrences type, the intra-family and mainly the intra-genus pairwise correlations were more frequent 226 in BC and BNS networks than in SE.

227 To examine whether certain taxa exhibited keystone roles in the microbial ecosystem of each sampling 228 source, the betweenness centrality, which measures the number of shortest paths going through a node 229 and it is directly correlated to the core location of a given node in a network, was considered. Overall, 230 this topological parameter of the node (taxon) was not correlated (Pearson's correlation; P > 0.05) to its 231 abundance or occurrence in the dataset and showed significantly higher values in the SE network 232 (Pairwise Wilcoxon's test, P [FDR] < 0.001). By applying a cut-off value of three-fold the upper Inter-233 Quartile-Range (IQR), a set of 15 potential keystone taxa in the SE network was identified, which 234 comprised also Arcobacter butzleri, and only 4 taxa in both BC and BNS networks. The keystone taxa 235 varied in the three sampling sources, with the exception of Ruminococcus torques which represented a 236 central taxon in both SE and BC networks structures (Fig. 5 and Supplementary Table 4).

237 Next, networks were partitioned in group of highly interconnected (co-occurring) nodes, defined as 238 modules (30), which were more numerous and dimensionally larger in the SE network. Modules 239 segregation within the SE network was significantly dependent (Kruskal-Wallis and Pairwise 240 Wilcoxon's tests; P[FDR] < 0.001) to the distribution of the taxa between the two sampling days and 241 along the lines-sectors of the slaughterhouse, i.e., automated line, plucking, evisceration, and chilling 242 line (Supplementary Table 5). Analysing the composition of the modules in this network, many of the 243 taxa included in the two largest modules, here coded as SE01 and SE02, co-occurred in BNS network 244 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**).

251

### 252 Isolation of *Arcobacter* from production runs and specific niches in the slaughterhouse 253 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 three virulence-associated genes, namely *irgA*, *hecA* and *hecB*, which are genetic elements 263 264 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 phase considered in the plucking and slaughtering sectors, except for the automated evisceration (n = 5273 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 defeathering tunnel (sampling point 3A) showed the highest number of positive samples and the 276 277 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**).

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 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).

286

#### 287 DISCUSSION

288 The investigation of microbiota distribution, diversity and dynamic within an ecosystem is the ultimate 289 goal of any biogeographical study. When applied to food processing environments it becomes pivotal 290 to reconstruct contamination routes of productive processes, which are in turn highly influenced by 291 factors like the premise layout, sanitizing interventions, productive flow chart and temperature (14, 32– 292 34). DNA-based metataxonomic analysis and parallel targeted isolation of Arcobacter spp. have been 293 performed in this study towards that scope. Common microbiological analysis consists of neck skins 294 (representative of the entire carcass microbiota) and caeca sampling during poultry slaughtering (35, 295 36), where their metataxonomic profiles can provide a reliable picture of the incoming contaminant 296 microbiota. On the other hand, metataxonomic profiles of environmental samples collected after 297 cleaning-sanitizing provide insights on the resident populations and the potential pathogens prevalence 298 during processing (32, 37).

299 Confirming previous metataxonomic studies, neck skins (BNS) and caecal samples (BC) represented 300 two distinct microbiotas (38, 39). In comparison to BNS microbiota, BC was characterized by higher 301 biodiversity and presence of anaerobes included in Bacteroidota phylum (12). Moreover, BC 302 microbiota showed a lower level of intra-communities' phylogenetic variability ( $\beta$ -dispersion) in 303 comparison with BNS and SE, as well as a stable composition in time among the production runs. On 304 the other hand, BNS microbiota showed a temporal succession of taxa during the three months, in 305 relation to the production runs and despite the different flock origins.

306 Microbiota composition of both BNS and BC was not influenced by rearing conditions like the diet 307 type and antibiotic treatments. It has been reported that diet can influence the intestinal and skin 308 microbiota composition of broilers, which however are more dependent to the stocking density and the 309 housing conditions, such as the sharing transport crates (40, 41). Indeed, caecal microbiomes of 310 poultries reared in conventional and antibiotic free farms showed distinct taxonomic and functional 311 profiles, although this separation was completely lost in the carcasses' microbiome collected 312 downstream the slaughtering process (42). Another metagenomic investigation highlighted a major 313 impact of packaging and processing environments on chicken breast microbiome in comparison to the 314 antibiotic usage (9). Therefore, in this study the negligeable effect of different diets and antibiotics 315 observed on broilers microbiota can be explained by closeness and contact among animals or carcasses 316 during processing, as well as flock-to-flock contamination conveyed in the same processing day by 317 devices and surfaces. Taking in consideration the new limiting European regulation on the use of 318 veterinary medical products and medicated feed (43, 44), the identification of processing phases in 319 which the microbiota/microbiome analyses can be useful to define a previous use of antibiotics will be 320 fundamental in the near future.

321 The composition and structure of SE microbiota have shown similarity with BNS. These two sampling 322 sources shared several species included in Arcobacteraceae, Weeksellaceae and Sphingomonadaceae 323 families, which were not detected in BC samples. This aspect indicates that skin, feet and feathers (here 324 represented by the BNS samples) are the major sources of contamination that determine the resident 325 microbiota in a modern automated abattoir. It suggests that Good Manufacturing Practices (GMPs) 326 were properly followed in the monitored abattoir, but it is also determined by intrinsic characteristics of 327 BC microbiota. Indeed, the none aerophilic and thermophilic taxa harboured in the poultry gut are 328 unlikely to survive and colonise the processing environment (38, 45). Noteworthy, A. butzleri 329 represented a predominant and abundant taxon in BNS and SE, but was not detected in BC. In contrast, 330 more thermophilic members of the Campylobacterota phylum, such H. pullorum and C. jejuni, were 331 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 conveyslaughtered carcasses through washing step and air chilling tunnel.

Aside the direct association between taxa and sources, co-transferring phenomena have been observed 334 335 for certain groups of abundant taxa through the network analysis of co-occurrences. In particular, co-336 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 337 338 interaction in a given habitat (47), the resulting network analysis can help to decipher spatial 339 segregation and contamination routes in food processing environments (32, 48, 49). In this frame, 340 transferring of A. butzleri between BNS and SE did not occur together with other abundant taxa 341 associated to BNS, perhaps highlighting a primary origin different from the animal's skin and/or a 342 reciprocal exchange between the two sources (BNS-SE).

343 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 344 reduction of biodiversity along the poultry slaughtering process is not surprising since several steps can 345 346 progressively act on carcasses' microbiota composition through mechanical removal, washing and high 347 temperatures. These phenomena have been already observed in rinsates of broiler carcasses collected 348 after plucking and chilling (50), and are often associated to a parallel reduction of the viable bacterial 349 counts (51). Taking into account that both shackles' lines undergone the same cleaning-sanitizing 350 intervention, this aspect seems to indirectly highlight a temperature-related selective pressure as well. 351 Noteworthy, in cattle slaughterhouses minimal differences of temperature (~2-3 °C) between 352 processing rooms have been sufficient to significantly modify the resident microbiota in favour of 353 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 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).

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).

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 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 between different production runs (57). Moreover, Arcobacter spp. isolated from scalding water have 430 431 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 the multiple isolation of a strain from the same sample (25, 31), without the intention to fully 440 441 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 452 process layout (74–76). Anyway, this mitigation would not be resolutive in relation to the high 453 presence of this pathogen on the whole carcass and in the resident microbiota of equipment's surfaces 454 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.

#### 462 MATERIALS AND METHODS

463

#### 464 **Broilers and environmental sampling**

465 The study was conducted in a local poultry abattoir (average of 90,000 birds per day) operating in 466 Piedmont (North-West of Italy). From January to May 2021, eight production runs were followed, and 467 a total of 49 broiler flocks (breed Ross 308®; 50 days age and 3.3 kg of weight in average; 10,000 468 broilers per flock in average) were sampled, each of them corresponding to a group of chickens reared 469 in sheds with the same procedures by a unique farmer until the moment of delivering to the abattoir 470 (Figure 1A and Supplementary Table 1). Broilers flocks were provided from farmers located in the 471 North-West and Nord-Centre of Italy and differed in relation to the type of feeding and eventual need 472 of curative antibiotic treatment (Supplementary Table 1). According to the sampling procedures 473 recommended to verify microbiological quality in broilers (35, 36), samples of broiler caecum (BC) 474 and broiler neck skin (BNS) were collected during slaughtering process at the moment of evisceration 475 and the neck removal, respectively (Figure 1B). Ten neck skins and ten caeca were randomly sampled 476 during the slaughtering process of each flock, and separately pooled in two sterile bags; no link 477 between BC and BNS at the level of the individual carcass level was kept.

478 Samples from the slaughterhouse equipment and environment (SE) were collected on two sampling 479 days after routinely cleaning and disinfection (Supplementary Table 3), 40 and 270 days after the end 480 of broilers sampling period, respectively (Figure 1C). Sampling was performed on areas in contact 481 with the carcasses (processing line) or viscera/giblet (by-products/waste line), using sterile sponges 482 (VWR International, Leuven, Belgium) previously hydrated with 10 ml of buffered peptone water 483 (BPW; Sigma, St. Louis, MO, USA). The same types of area were considered on the two sampling 484 days. The plant used the semi-automated slaughtering process displayed in Figure 1B, and specific 485 environmental sampling points are listed in Supplementary Table 2.

486 A total of 154 samples were collected from the three sampling sources (49 of BC; 49 of BNS; 56 of

487 SE) and kept at 4 °C until the microbiological analysis, performed within two hours after sampling.

488

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

490 Isolation of *Arcobacter* spp. was performed, including selective enrichment, as described by Houf et al.

491 (2001) (77), with slight modifications. All media and supplements were provided from Merck & Co.

492 (Readington Township, NJ, USA), unless stated otherwise.

493 Briefly, each pooled BSN or BC sample was aseptically cut with a scalpel, and 25 g was randomly 494 collected (~2-3 g pieces from each of the ten necks or intestines/faeces) and transferred in a sterile bag 495 with 100 mL of enrichment broth, composed by: Arcobacter broth (CM0965; Oxoid, Basingstoke, UK) 496 supplemented with 5% v/v of laked horse blood, 16 mg/L cefoperazone (C4292), 10 mg/L 497 amphotericin B (A2411), 100 mg/L 5-fluorouracil (F6627), 32 mg/L novobiocin sodium salt (74675) 498 and 64 mg/L trimethoprim (T7883). Hydrated sponges (SE) were aseptically cut in half lengthwise and 499 one half placed in a sterile bag with 100 mL of the Arcobacter enrichment broth. All samples were 500 homogenized for 2 minutes with a Stomacher® 400 Circulator (LAB blender 400; PBI, Milan, Italy), 501 and incubated for 48 hours at 28 °C in microaerobic conditions (AnaeroBox ®; Thermo Scientific, 502 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.

508 Isolation of *Arcobacter* spp. was carried out using as selective media the Arcobacter broth 509 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

512 colonies (with a maximum of 12) with characteristic morphology were stored for further analysis.

513

#### 514 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  $\mu$ 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. (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).

528 Characterization of the isolates was performed by amplifying three virulence-related genes (*irgA*, *hecA*, 529 *hec*) with PCR protocol and conditions described by Douidah and colleagues (81). These genes are 530 highly variable among *Arcobacter* spp. genomes and therefore their presence/absence have been used 531 here as biomarkers to differentiate within isolates of the species (25, 31). All reagents for PCR assays 532 and primer pairs were provided by Sigma-Aldrich (St. Louis, MO, USA). List of primers and thermal 533 cycle parameters used are reported in **Supplementary Table 7**.

#### 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**

A total of 5,469,573 raw-reads were produced by the 16S amplicon-based sequencing of the 154 samples. To obtain Amplicon Sequence Variants (ASVs) the raw-reads were analysed 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* setting. 557 Taxonomy was assigned with a 99 % of sequence similarity through Bayesian classifier method (84) by 558 release of Silva prokaryotic matching ASVs to the 2021 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 classification (to the Order rank or lower resolution) or matching (> 99 % similarity) with animal 562 563 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 564 565 2,927,216 paired-end reads (average of 19,351 reads/sample) were used to construct ASVs frequency 566 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/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, respectively. Pairwise comparisons were alternatively performed with Wilcoxon and T-tests accordingto data normality.

Principal-coordinate Analysis (PCoA) was used to visualize beta-diversity. Significant effects of categorical variables (sampling sources, production runs, slaughterhouse sectors/lines) on the bacterial community variations were evaluated with Permutational Multivariate Analysis of Variance (PERMANOVA; *adonis* function based on 999 permutations and Brey-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.

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.

#### 605 ANKNOWLEDGEMENTS

- 606 This study was funded by grant ALEV CRT 20 01-Fondazione CRT 2019: Diffusion of Arcobacter
- spp. in Piedmont poultry meats and study of its pathogenic potential founded by *Cassa di Risparmio di Torino* (CRT).
- 609 The authors thank Davide Bongiovanni for his invaluable help during the samples collection and610 analysis.
- 611
- 612 **CRediT authorship contribution statement**
- 613 Cristian Botta: Conceptualization, Investigation, Data curation, Formal analysis, Software, Writing –
- 614 original draft.
- 615 Davide Buzzanca: Investigation, Data curation, Writing Review & Editing
- 616 Elisabetta Chiarini: Investigation, Data curation, Writing Review & Editing
- 617 Francesco Chiesa: Conceptualization, Investigation, Writing Review & Editing
- 618 Selene Rubiola: Investigation, Writing Review & Editing
- 619 Ilario Ferrocino: Investigation, Writing Review & Editing
- 620 Edoardo Fontanella: Investigation, Writing Review & Editing
- 621 Kalliopi Rantsiou: Writing Review & Editing
- 622 Kurt Houf: Supervision, Writing Review & Editing
- 623 Valentina Alessandria: Conceptualization, Supervision, Funding acquisition, Writing Review &
- 624 Editing
- 625

#### 627 **REFERENCES**

- 628 1. Eurostat Statistics Explained. 2021. Agricultural production livestock and meat.
- 629 2. AVEC. 2021. Annual Report 2020 (AVEC).
- 630 3. UNA-Italia. 2020. Annual Report 2020.
- 631 4. UNA-Italia. 2021. Annual Report 2021.
- 632 5. EC. 2022. RASFF Window: searchable online database of rapid alert system for food and feed
  633 notifications classified as alert.
- 634 6. CDC. 2022. National Outbreak Reporting System (NORS). Centers for Disease Control and
  635 Prevention.
- Marmion M, Ferone MT, Whyte P, Scannell AGM. 2021. The changing microbiome of poultry
  meat; from farm to fridge. Food Microbiol 99:103823.
- 8. Wang H, Qin X, Li X, Wang X, Gao H, Zhang C. 2020. Changes in the microbial communities
  of air- and water-chilled yellow-feathered broilers during storage at 2 °C. Food Microbiol
  87:103390.
- 641 9. Li S, Mann DA, Zhang S, Qi Y, Meinersmann RJ, Deng X. 2020. Microbiome-informed food
  642 safety and quality: longitudinal consistency and cross-sectional distinctiveness of retail chicken
  643 breast microbiomes. mSystems 5:1–21.
- Rouger A, Tresse O, Zagorec M. 2017. Bacterial contaminants of poultry meat: sources, species,
  and dynamics. Microorganisms 5:50.
- Wages JA, Feye KM, Park SH, Kim SA, Ricke SC. 2019. Comparison of 16S rDNA next
  sequencing of microbiome communities from post-scalder and post-picker stages in three
  different commercial poultry plants processing three classes of broilers. Front Microbiol 10:1–
  11.
- 650 12. Xiao Y, Xiang Y, Zhou W, Chen J, Li K, Yang H. 2017. Microbial community mapping in

- 651 intestinal tract of broiler chicken. Poult Sci 96:1387–1393.
- 13. Yadav S, Jha R. 2019. Strategies to modulate the intestinal microbiota and their effects on
  nutrient utilization, performance, and health of poultry. J Anim Sci Biotechnol 10:1–11.
- Lim ES, Kim JJ, Sul WJ, Kim J-S, Kim B, Kim H, Koo OK. 2021. Metagenomic analysis of
  microbial composition revealed cross-contamination pathway of bacteria at a foodservice
  facility. Front Microbiol 12:1–12.
- Song X, Wang H, Xu X. 2021. Investigation of microbial contamination in a chicken
  slaughterhouse environment. J Food Sci 86:3598–3610.
- 659 16. Gonçalves-Tenório A, Silva B, Rodrigues V, Cadavez V, Gonzales-Barron U. 2018. Prevalence
  660 of pathogens in poultry meat: a meta-analysis of european published surveys. Foods 7:69.
- Rouger A, Moriceau N, Prévost H, Remenant B, Zagorec M. 2018. Diversity of bacterial
  communities in French chicken cuts stored under modified atmosphere packaging. Food
  Microbiol 70:7–16.
- Buzzanca D, Kerkhof P, Alessandria V, Rantsiou K, Houf K. 2023. Arcobacteraceae
  comparative genome analysis demonstrates genome heterogeneity and reduction in species
  isolated from animals and associated with human illness. Heliyon 9:e17652.
- 667 19. Oren A, Garrity GM. 2021. Valid publication of the names of forty-two phyla of prokaryotes. Int
  668 J Syst Evol Microbiol 71.
- Lappi V, Archer JR, Cebelinski E, Leano F, Besser JM, Klos RF, Medus C, Smith KE,
  Fitzgerald C, Davis JP. 2013. An outbreak of foodborne illness among attendees of a wedding
  reception in wisconsin likely caused by *Arcobacter butzleri*. Foodborne Pathog Dis 10:250–255.
- 672 21. Ramees TP, Dhama K, Karthik K, Rathore RS, Kumar A, Saminathan M, Tiwari R, Malik YS,
- 673 Singh RK. 2017. Arcobacter: an emerging food-borne zoonotic pathogen, its public health
- 674 concerns and advances in diagnosis and control a comprehensive review. Vet Q 37:136–161.

- Douidah L, De Zutter L, Vandamme P, Houf K. 2010. Identification of five human and mammal
  associated *Arcobacter* species by a novel multiplex-PCR assay. J Microbiol Methods 80:281–
  286.
- Ferreira S, Fraqueza MJ, Queiroz JA, Domingues FC, Oleastro M. 2013. Genetic diversity,
  antibiotic resistance and biofilm-forming ability of *Arcobacter butzleri* isolated from poultry and
  environment from a Portuguese slaughterhouse. Int J Food Microbiol 162:82–88.
- 681 24. Šilha D, Sirotková S, Švarcová K, Hofmeisterová L, Koryčanová K, Šilhová L. 2021. Biofilm
  682 formation ability of *Arcobacter*-like and *Campylobacter* Strains under different conditions and
  683 on food processing materials. Microorganisms 9:2017.
- Buzzanca D, Botta C, Ferrocino I, Alessandria V, Houf K, Rantsiou K. 2021. Functional
  pangenome analysis reveals high virulence plasticity of *Aliarcobacter butzleri* and affinity to
  human mucus. Genomics 113:2065–2076.
- Chieffi D, Fanelli F, Fusco V. 2020. *Arcobacter butzleri*: up-to-date taxonomy, ecology, and
  pathogenicity of an emerging pathogen. Compr Rev Food Sci Food Saf 19:2071–2109.
- 689 27. Houf K, De Zutter L, Verbeke B, Van Hoof J, Vandamme P. 2003. Molecular characterization
  690 of *Arcobacter* isolates collected in a poultry slaughterhouse. J Food Prot 66:364–369.
- 691 28. Yu Z, Joossens M, Houf K. 2020. Analyses of the bacterial contamination on belgian broiler
  692 carcasses at retail level. Front Microbiol 11.
- Houf K, De Zutter L, Van Hoof J, Vandamme P. 2002. Occurrence and distribution of
   *Arcobacter* species in poultry processing. J Food Prot 65:1233–1239.
- Blondel VD, Guillaume JL, Lambiotte R, Lefebvre E. 2008. Fast unfolding of communities in
  large networks. J Stat Mech Theory Exp 10:1–12.
- 697 31. Isidro J, Ferreira S, Pinto M, Domingues F, Oleastro M, Gomes JP, Borges V. 2020. Virulence
  698 and antibiotic resistance plasticity of *Arcobacter butzleri*: Insights on the genomic diversity of an

699 emerging human pathogen. Infect Genet Evol 80:1567–1348.

- Botta C, Ferrocino I, Pessione A, Cocolin L, Rantsiou K. 2020. Spatiotemporal distribution of
   the environmental microbiota in food processing plants as impacted by cleaning and sanitizing
   procedures: the case of slaughterhouses and gaseous ozone. Appl Environ Microbiol 86:1–15.
- 33. Bokulich NA, Ohta M, Richardson PM, Mills DA. 2013. Monitoring seasonal changes in
  winery-resident microbiota. PLoS One 8:e66437.
- 34. Bokulich NA, Bergsveinson J, Ziola B, Mills DA. 2015. Mapping microbial ecosystems and
  spoilage-gene flow in breweries highlights patterns of contamination and resistance. Elife
  4:e04634.
- 35. EFSA. 2011. Scientific opinion on *Campylobacter* in broiler meat production: control options
   and performance objectives and/or targets at different stages of the food chain. EFSA J 9.
- Ministero della Salute. 2017. Linee guida per l'uso prudente degli antimicrobici negli
  allevamenti zootecnici per la prevenzione dell'antimicrobico-resistenza e proposte alternative
  41.
- 713 37. Møretrø T, Langsrud S. 2017. Residential bacteria on surfaces in the food industry and their
  714 implications for food safety and quality. Compr Rev Food Sci Food Saf 16:1022–1041.
- Rothrock MJ, Locatelli A, Feye KM, Caudill AJ, Guard J, Hiett K, Ricke SC. 2019. A
  microbiomic analysis of a pasture-raised broiler flock elucidates foodborne pathogen ecology
  along the farm-to-fork continuum. Front Vet Sci 6:1–14.
- Oakley BB, Morales CA, Line J, Berrang ME, Meinersmann RJ, Tillman GE, Wise MG,
  Siragusa GR, Hiett KL, Seal BS. 2013. The poultry-associated microbiome: network analysis
  and farm-to-fork characterizations. PLoS One 8.
- 40. Cholewińska P, Michalak M, Wojnarowski K, Skowera S, Smoliński J, Czyż K. 2021. Levels of
   Firmicutes, Actinobacteria phyla and *Lactobacillaceae* family on the skin surface of broiler

chickens (Ross 308) depending on the nutritional supplement and the housing conditions. Agric
11.

Kers JG, Velkers FC, Fischer EAJ, Hermes GDA, Lamot DM, Stegeman JA, Smidt H. 2019.
Take care of the environment: housing conditions affect the interplay of nutritional interventions
and intestinal microbiota in broiler chickens. Anim Microbiome 1:1–14.

- 42. De Cesare A, Oliveri C, Lucchi A, Savini F, Manfreda G, Sala C. 2022. Pilot study on poultry
  meat from antibiotic free and conventional farms: can metagenomics detect any difference?
  Foods 11.
- 43. EC. 2019. Regulation (EU) 2019/6 of the European parliament and of the council of 11
  december 2018 on veterinary medicinal products and repealing directive 2001/82/EC (Text with
  EEA relevance). J Eur Union 43–167.
- 44. EC. 2019. Regulation (EU) 2019/4 of the European Parliament and of the Council of 11
  December 2018 on the manufacture, placing on the market and use of medicated feed, amending
  Regulation (EC) No 183/2005. J Eur Union 1–23.
- Cegar S, Kuruca L, Vidovic B, Antic D, Hauge SJ, Alvseike O, Blagojevic B. 2022. Risk
  categorisation of poultry abattoirs on the basis of the current process hygiene criteria and
  indicator microorganisms. Food Control 132:108530.
- Kaakoush NO, Sodhi N, Chenu JW, Cox JM, Riordan SM, Mitchell HM. 2014. The interplay
  between *Campylobacter* and *Helicobacter* species and other gastrointestinal microbiota of
  commercial broiler chickens. Gut Pathog 6:18.
- Freilich MA, Wieters E, Broitman BR, Marquet PA, Navarrete SA. 2018. Species co-occurrence
  networks: can they reveal trophic and non-trophic interactions in ecological communities?
  Ecology 99:690–699.
- 48. Botta C, Franciosa I, Coisson JD, Ferrocino I, Colasanto A, Arlorio M, Cocolin L, Rantsiou K.

- 747 2023. Beef carcass microbiota after slaughtering and primary cooling: a metataxonomic
  748 assessment to infer contamination drivers. Food Res Int 174:113466.
- Chaillou S, Chaulot-Talmon A, Caekebeke H, Cardinal M, Christieans S, Denis C, Hélène
  Desmonts M, Dousset X, Feurer C, Hamon E, Joffraud J-J, La Carbona S, Leroi F, Leroy S,
  Lorre S, Macé S, Pilet M-F, Prévost H, Rivollier M, Roux D, Talon R, Zagorec M,
  Champomier-Vergès M-C. 2015. Origin and ecological selection of core and food-specific
  bacterial communities associated with meat and seafood spoilage. ISME J 9:1105–1118.
- Kim SA, Hong Park S, In Lee S, Owens CM, Ricke SC. 2017. Assessment of chicken carcass
   microbiome responses during processing in the presence of commercial antimicrobials using a
   next generation sequencing approach. Sci Rep 7:1–14.
- 51. Huezo R, Northcutt JK, Smith DP, Fletcher DL, Ingram KD. 2007. Effect of dry air or
  immersion chilling on recovery of bacteria from broiler carcasses. J Food Prot 70:1829–1834.

52. Cremonesi P, Morandi S, Ceccarani C, Battelli G, Castiglioni B, Cologna N, Goss A, Severgnini

- M, Mazzucchi M, Partel E, Tamburini A, Zanini L, Brasca M. 2020. Raw milk microbiota
   modifications as affected by chlorine usage for cleaning procedures: The Trentingrana PDO
   Case. Front Microbiol 11:1–14.
- 53. Kjeldgaard J, Jørgensen K, Ingmer H. 2009. Growth and survival at chiller temperatures of
   *Arcobacter butzleri*. Int J Food Microbiol 131:256–259.
- Meng J, Huang X, Song L, Hou B, Qiao M, Zhang P, Zhao Q, Zhang B, Liu F. 2019. Effect of
  storage temperature on bacterial diversity in chicken skin. J Appl Microbiol 126:854–863.
- Duffy LL, Blackall PJ, Cobbold RN, Fegan N. 2014. Quantitative effects of in-line operations on
   *Campylobacter* and *Escherichia coli* through two Australian broiler processing plants. Int J Food
   Microbiol 188:128–134.
- 770 56. Wang H, Qin X, Mi S, Li X, Wang X, Yan W, Zhang C. 2019. Contamination of yellow-

feathered broiler carcasses: microbial diversity and succession during processing. Food
Microbiol 83:18–26.

- 773 57. Rothrock MJ, Locatelli A, Glenn TC, Thomas JC, Caudill AC, Kiepper BH, Hiett KL. 2016.
  774 Assessing the microbiomes of scalder and chiller tank waters throughout a typical commercial
  775 poultry processing day. Poult Sci 95:2372–2382.
- 58. Kunert-Filho HC, Furian TQ, Sesterhenn R, Chitolina GZ, Willsmann DE, Borges KA, Salle
  CTP, Moraes HL de S, do Nascimento VP. 2022. Bacterial community identification in poultry
  carcasses using high-throughput next generation sequencing. Int J Food Microbiol 364:109533.
- 779 59. Callahan BJ, McMurdie PJ, Holmes SP. 2017. Exact sequence variants should replace
  780 operational taxonomic units in marker-gene data analysis. ISME J 11:2639–2643.
- 60. Botta C, Coisson JD, Ferrocino I, Colasanto A, Pessione A, Cocolin L, Arlorio M, Rantsiou K.
- 782 2021. Impact of electrolyzed water on the microbial spoilage profile of Piedmontese Steak
  783 Tartare. Microbiol Spectr 9.
- Devanga Ragupathi NK, Muthuirulandi Sethuvel DP, Inbanathan FY, Veeraraghavan B. 2018.
  Accurate differentiation of *Escherichia coli* and *Shigella* serogroups: challenges and strategies.
  New Microbes New Infect 21:58–62.
- Wang H, He A, Yang X. 2018. Dynamics of microflora on conveyor belts in a beef fabrication
  facility during sanitation. Food Control 85:42–47.
- 63. De Cesare A, Sala C, Castellani G, Astolfi A, Indio V, Giardini A, Manfreda G. 2020. Effect of
  Lactobacillus acidophilus D2/CSL (CECT 4529) supplementation in drinking water on chicken
  crop and caeca microbiome. PLoS One 15:1–18.
- Durazzi F, Sala C, Castellani G, Manfreda G, Remondini D, Cesare A De. 2021. Comparison
  between 16S rRNA and shotgun sequencing data for the taxonomic characterization of the gut
  microbiota. Sci Rep 1–10.

- Buzzanca D, Chiarini E, Mania I, Chiesa F, Alessandria V. 2023. *Aureimonas altamirensis*: first
  isolation from a chicken slaughterhouse in Italy followed by genotype and phenotype
  evaluations. Microbiol Res (Pavia) 14:1319–1330.
- 66. Gude A, Hillman TJ, Helps CR, Allen VM, Corry JEL. 2005. Ecology of *Arcobacter* species in
  chicken rearing and processing. Lett Appl Microbiol 41:82–87.
- Atanassova V, Kessen V, Reich F, Klein G. 2008. Incidence of *Arcobacter* spp. in poultry:
  quantitative and qualitative analysis and PCR differentiation. J Food Prot 71:2533–2536.
- 802 68. Van Driessche E, Houf K. 2007. Discrepancy between the occurrence of *Arcobacter* in chickens
  803 and broiler carcass contamination. Poult Sci 86:744–751.
- 804 69. Ho HTK, Lipman LJA, Gaastra W. 2008. The introduction of *Arcobacter* spp. in poultry
  805 slaughterhouses. Int J Food Microbiol 125:223–229.
- Schönknecht A, Alter T, Gölz G. 2020. Detection of *Arcobacter* species in different intestinal
   compartments of broiler chicken during slaughter and processing. Microbiologyopen 9:1–6.
- Shange N, Gouws P, Hoffman LC. 2019. *Campylobacter* and *Arcobacter* species in foodproducing animals: prevalence at primary production and during slaughter. World J Microbiol
  Biotechnol 35:146.
- 811 72. Barker D, Lankhaar J, Stals P. 2004. Primary processing of poultrypoultry meat processing and
  812 quality: a volume in woodhead publishing series in food science, technology and nutrition.
- 813 73. Chiarini E, Buzzanca D, Chiesa F, Botta C, Ferrocino I, Fontanella E, Rantsiou K, Houf K,
- 814 Alessandria V. 2023. Occurrence and antibiotic resistance of *Arcobacter* spp. isolated from
- poultry slaughterhouses in Northern Italy, p. 208. *In* Simtrea (ed.), 7TH Internation Conference
  on Microbial Diversity. Simtrea, Parma.
- 817 74. Loretz M, Stephan R, Zweifel C. 2010. Antimicrobial activity of decontamination treatments for
  818 poultry carcasses: A literature survey. Food Control 21:791–804.

- Bucher O, Rajić A, Waddell LA, Greig J, McEwen SA. 2012. Do any spray or dip treatments,
  applied on broiler chicken carcasses or carcass parts, reduce *Salmonella* spp. prevalence and/or
  concentration during primary processing? a systematic review–meta-analysis. Food Control
  27:351–361.
- Ku T, Marmion M, Ferone M, Wall P, Scannell AGM. 2019. Processing and retail strategies to
  minimize *Campylobacter* contamination in retail chicken. J Food Process Preserv 43:1–17.
- 825 77. Houf K, Devriese LA, De Zutter L, Van Hoof J, Vandamme P. 2001. Development of a new
  826 protocol for the isolation and quantification of *Arcobacter* species from poultry products. Int J
  827 Food Microbiol 71:189–196.
- Ferreira L, Sánchez-Juanes F, García-Fraile P, Rivas R, Mateos PF, Martínez-Molina E,
  González-Buitrago JM, Velázquez E. 2011. MALDI-TOF mass spectrometry is a fast and
  reliable platform for identification and ecological studies of species from family rhizobiaceae.
  PLoS One 6.
- Valverde Bogantes E, Fallas-Padilla KL, Rodríguez-Rodríguez CE, Fernández Jaramillo H,
  Arias Echandi ML. 2015. Zoonotic species of the genus *Arcobacter* in poultry from different
  regions of Costa Rica. J Food Prot 78:808–811.
- 835 80. Harmon KM, Wesley I V. 1996. Identification of *Arcobacter* isolates by PCR. Lett Appl
  836 Microbiol 23:241–244.
- 837 81. Douidah L, de Zutter L, Bare J, De Vos P, Vandamme P, Vandenberg O, Van den Abeele A-M,
- Houf K. 2012. Occurrence of putative virulence genes in *Arcobacter* species isolated from
  humans and animals. J Clin Microbiol 50:735–741.
- 840 82. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2:
  841 High-resolution sample inference from Illumina amplicon data. Nat Methods 13:581–583.
- 842 83. Botta C, Franciosa I, Alessandria V, Cardenia V, Cocolin L, Ferrocino I. 2022. Metataxonomic

- signature of beef burger perishability depends on the meat origin prior grinding. Food Res Int
  156:1–13.
- 845 84. Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naïve Bayesian classifier for rapid assignment
  846 of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267.
- 847 85. Schliep KP. 2011. phangorn: Phylogenetic analysis in R. Bioinformatics 27:592–593.
- 848 86. Wright ES. 2016. Using DECIPHER v2.0 to analyze big biological sequence data in R. R J
  849 8:352–359.
- 850 87. McMurdie PJ, Holmes S. 2013. Phyloseq: an R Package for Reproducible Interactive Analysis
  851 and Graphics of Microbiome Census Data. PLoS One 8.
- 852 88. Kembel SW, Cowan PD, Helmus MR, Cornwell WK, Morlon H, Ackerly DD, Blomberg SP,
  853 Webb CO. 2010. Picante: R tools for integrating phylogenies and ecology. Bioinformatics
  854 26:1463–1464.
- 855 89. De Cáceres M, Legendre P. 2009. Associations between species and groups of sites: Indices and
  856 statistical inference. Ecology 90:3566–3574.
- 857 90. Friedman J, Alm EJ. 2012. Inferring correlation networks from genomic survey data. PLoS
  858 Comput Biol 8:1–11.

### 861 Tables

### **Table 1.**

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

#### 864 **Table 2.**

		S	ampling Sour	ce
	Parameters:	BC	BNS	SE
	Taxa analysed	148	163	285
Conoral foatuor	Nodes (taxa correlated)	47	81	214
(counts)	Edges (correlations)	50	128	908
(counts)	Triangles	7	81	2756
	Diameter	8	7	12
	Degree	2.128	3.16	8.486
Network	Density	0.046	0.040	0.040
topology	Modularity	0.709	0.772	0.664
(average value)	Clustering Coefficient	0.357	0.474	0.513
	Path length	3.418	2.657	4.793
Co-occurrences	Intra-Phylum	88.0%	66.4%	59.4%
type (% on the	Intra-Family	34.0%	25.0%	12.0%
total)	Intra-Genus	4.0%	7.8%	1.5%

865

#### 866 **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 rubber879 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 to plastic shackles (8A) of the second line [SL] and transported through further sectors for washing, 886 887 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.

Buration 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.

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 (βdiversity): sampling sources are shown by different colours as reported in the colour coding key. 895 Variance explained ( $R^2$  value) by each sampling source (BC, BNS, SE) and pairwise biological 896 897 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 interguartile range (IQR); central line indicates the median; whiskers indicate the furthest point within 902  $(1.5 \times IQR)$ ; 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

# Fig. 4. Bipartite network revealing the taxa (genus or species level) associated to the three sampling sources.

Taxa (coloured nodes) are unidirectionally connected with arrows (edges) to the sampling sources (BC, BNS, 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 coloured in relation to the belonging Phylum (refer to colour coding key). Only the taxa present in more than 2 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 (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.

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 coloured in relation to the co-occurring modules (refer to colour 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 modules composition is reported in **Supplementary Table 4**.

940

# Fig. 6. Pseudo-heatmap summarising the frequency of *Arcobacter spp.* isolation and abundances 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= 951 not detected).

#### 952

953 Tables legends:

#### 954 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.

958

# 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

962 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

964 length= number of edges along the shortest path for all possible pairs of nodes

965

967	Highlights
967	Highlights

969	•	Arcobacter spp. is part of the resident microbiota of poultry slaughterhouses and is particularly
970		persistent in certain environmental niches that are difficult to clean and sanitise
971	•	The introduction of this emerging pathogen in the processing environments is related to the
972		broiler's skin microbiota, of which it represents a characteristic species
973	•	Metataxonomic analysis relaying on 16S-rRNA gene sequencing represents a valid technique to
974		track the presence of Arcobacter spp.
975	•	An analytical approach combining metataxonomic and culture-dependent detection of this
976		pathogen could be soon implemented to define food safety and quality in poultry slaughtering
977		
978		
979		
980		