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Exploring multi-antibiotic resistance in *Arcobacter butzleri* isolates from a poultry processing plant in northern Italy: An in-depth inquiry

Elisabetta Chiarini [a,1], Davide Buzzanca [a,*,1], Francesco Chiesa [b], Cristian Botta [a], Kalliopi Rantsiou [a], Kurt Houf [c], Valentina Alessandria [a]

[a] Department of Agricultural, Forest and Food Sciences, University of Turin, Grugliasco, TO, Italy
[b] Department of Veterinary Sciences, University of Turin, Grugliasco, TO, Italy
[c] Department of Veterinary and Biosciences, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

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ABSTRACT

Arcobacter butzleri is a foodborne pathogen that has been identified in various animal-derived foods, notably in poultry meat. The prevalence of this bacterium in poultry underscores the need for comprehensive investigations into its dissemination within poultry meat processing plants. Such assessments are critical due to the potential cross-contamination that may occur, impacting the safety of the final food product. This study endeavours to evaluate the genomic similarity of 56 *A. butzleri* isolates obtained from chicken carcasses (specifically neck skins and caecum, post-slaughter) and equipment surfaces following cleaning and sanitizing procedures. All samples originate from a poultry slaughterhouse in Northern Italy. The genomic analysis includes single nucleotide polymorphism, average nucleotide identity, and core genome multi-locus sequence typing analysis, aiming to discern potential cross-contamination between carcasses and equipment. The study evaluates antibiotic resistance, biofilm production, and host cell colonization of *A. butzleri* strains through *in vitro* assays. Genomic dereplication reveals the presence of 31 distinct strains. Results elucidate that a portion of these strains is evident in multiple sources, indicative of cross-contamination even post-cleaning and sanitization. The *in vitro* tests underline multi-antibiotic resistance of *A. butzleri* with genes associated with antibiotic resistance, among them *mexAB-oprM*, showing a correlation to the observed resistance. This study establishes the colonization capability of *A. butzleri* strains on a human gut mucus-secreting cell model, suggesting a potential virulence factor. Detection of various putative virulence genes further supports the hypothesis of strains virulence potential. This study holds importance of *A. butzleri* as it unveils the antibiotic resistance and pathogenic capabilities inherent in this species, identifying associated genetic traits. Through comprehensive genomic analysis, it was conclusively validated instances of cross-contamination between various sources and equipment surfaces, underscoring the pervasive dissemination of this bacterium within a food processing plant and its persistence. This research serves as an exploration into the intricate dynamics of *A. butzleri* within poultry processing environments. It accentuates the imperative for stringent hygiene protocols and ongoing surveillance to curtail the risk of cross-contamination, thereby ensuring the safety of poultry products for discerning consumers.

1. Introduction

Foodborne pathogens are of great concern for human health and contaminated foods can be the result of cross-contamination along the food production, requiring an in-depth risk assessment of potential contamination sources (Gonçalves-Tenório, Silva, Rodrigues, Cadavez, & Gonzales-Barron, 2018).

The family Arcobacteraceae includes Gram-negative species that have been isolated from a variety of matrices like food, animals, and human clinical specimens (Ramees et al., 2017). Among them, the species *Arcobacter butzleri* has been considered as a foodborne zoonotic pathogen associated with gastrointestinal disorders (Ramees et al., 2017), with diarrhoea as main symptom (Lappi et al., 2013; Ramees et al., 2017). Poultry meat represents an important reservoir of *Arcobacter* spp. (Houf, De Zutter, Van Hoof, & Vandamme, 2002), and chicken meat contaminated by *A. butzleri* has been linked to foodborne outbreaks (Lappi et al., 2013). The slaughter process is regarded as a

* Corresponding author.
E-mail address: davide.buzzanca@unito.it (D. Buzzanca).
[1] These two authors contributed equally to this work.

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Abbreviations

(ANI) Average nucleotide identity
(AR) antibiotic resistance
(BFI) biofilm formation index
(BC) broiler caecum
(BNS) broiler neck skins
(CDS) coding DNA sequences
(CFU) colony forming unit
(cgMLST) core genome multi-locus sequences typing
(COGs) clusters of orthologous genes
(CRISPR) clustered regularly interspaced short palindromic repeats
(DMEM) Dulbecco's modified Eagle Medium European Collection of Authenticated Cell Cultures
(ECACC) European Collection of Authenticated Cell Cultures
(EUCAST) European Committee on Antimicrobial Susceptibility Testing
(FBS) Foetal Bovine Serum
(GTDB-Tk) genome taxonomy database Toolkit
(MIC) minimum inhibitory concentration
(MDR) multi drug resistance
(OD) optical density
(PBS) phosphate-buffered saline
(PVGs) putative virulence genes
(SE) slaughterhouse environment
(SNPs) single nucleotide polymorphisms
(WGS) Whole Genome Sequencing

major risk for cross-contamination due to carcass manipulation and persistent bacterial contamination in the slaughter house environment (Rani, Mhlongo, & Hugo, 2023). The presence of *Arcobacter* spp. on equipment surfaces is favoured by the ability to adhere to different materials, and their capacity to form biofilm under different conditions (Girbau et al., 2017). Furthermore, *A. butzleri* showed resistance to biocides (Rasmussen, Kjeldgaard, Christensen, & Ingmer, 2013) and detergents (Šilha, Šilhová, Vytřasová, Brožková, & Pejchalová, 2016) suggesting its potential to persist after standard cleaning and disinfection procedures. The pathogenicity of *A. butzleri* in humans is well recognised. It has been demonstrated that *A. butzleri* can invade and colonize intestinal host cells *in vitro*, overcoming the mucus barrier (Buzzanca, Alessandria, et al., 2023; Buzzanca et al., 2021). Furthermore, the antibiotic resistance of several strains has been demonstrated (Fanelli et al., 2019; Isidro et al., 2020). Whole genome studies on *A. butzleri* showed an open pangenome that includes putative virulence genes involved in several virulence pathways (Buzzanca et al., 2021; Isidro et al., 2020). A genomic study aimed at assessing Arcobacteraceae taxonomy and genomic similarity have highlighted a clear distinction between species related to animals, including *A. butzleri* and those associated with the environmental habitats (Buzzanca, Kerkhof, Alessandria, Rantsiou, & Houf, 2023).

Considering these aspects, the objectives of the present study are to encompass a genomic and pangenome assessment of *A. butzleri* isolates obtained from an Italian chicken slaughterhouse, to assess the route of *A. butzleri* contamination and its potential risks as a meat contaminant.

The study focuses on the genomic analysis and physiological characterisation of the pathogenicity of *A. butzleri* isolated from broiler neck skins and caeca after slaughter and slaughterhouse surfaces after the cleaning procedure. Analyses performed have concentrated on establishing a connection between existing phenotypical results and genomic characteristics.

2. Materials and methods

2.1. Bacterial isolates and antibiotic resistance assay

A. butzleri isolates (n = 106) were collected during a sampling campaign conducted in a poultry slaughterhouse (average of 90,000 birds per day) from January 2021 to March 2022 (Supplementary table 1) (Chiarini et al., 2023), the flocks comes from extensive indoor breeding. The isolates were obtained from *Gallus domesticus* Ross 308, more specifically the isolation was performed from ten broiler neck skins (BNS) and ten caeca (BC) from a total of 49 flocks in North Italy during slaughter (Supplementary table 1). Samples from surfaces were collected from the slaughterhouse environment (SE) after cleaning and disinfecting procedures (Supplementary table 1). *A. butzleri* was isolated from BC, BNS and SE following the procedure designed by Houf and colleagues (Chiarini et al., 2023; Houf, Devriese, De Zutter, Van Hoof, & Vandamme, 2001b). *A. butzleri* was identified at species level using MALDI-TOF MS and multiplex species-specific PCR assay designed by Douidah and colleagues for *A. butzleri*, *A. cryaerophilus*, *A. cibarius*, *A. skirrowii*, *A. thereius* (Chiarini et al., 2023; Douidah, De Zutter, Vandamme, & Houf, 2010).

A. butzleri isolates were subjected to antibiotic resistance assay. The thresholds for the Minimum Inhibitory Concentration (MICs) breakpoints of seven antibiotics (μg/ml) followed to consider an isolate resistant or susceptible were obtained from Isidro and colleagues (Isidro et al., 2020) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST Breakpoint tables v12.0; http://www.eucast.org/). The antibiotics evaluated were all provided by Sigma-Aldrich (Milan, Italy) and are listed below: amoxicillin (A8523-5G), amoxicillin-clavulanic acid (SMB00607-1G), ampicillin (A9518-5G), azithromycin (PHR1088-16), clarithromycin (PHR1038-500 MG), erythromycin (E5389-1G) and gentamicin (G1264-5 GR). Values for *Campylobacter coli* (gentamicin, erythromycin, ampicillin, azithromycin, amoxicillin-clavulanic acid) and *Enterobacteraceae* (clarithromycin, amoxicillin) were used. The following values were the concentrations related to resistance breakpoint 1X to which bacterial growth corresponded to resistance: gentamicin (GEN) 2 μg/ml, erythromycin (ERY) 8 μg/ml, ampicillin (AMP) 16 μg/ml, clarithromycin (CLA) 2 μg/ml, azithromycin (AZI) 2 μg/ml, amoxicillin (AMO) 8 μg/ml, amoxicillin-clavulanic acid (AMC) 83 μg/ml. The antibiotic concentrations (resistance breakpoints) tested were reference MIC 1X and its double (2X) and quadruple (4X) concentrations. The antibiotic resistance test was performed in three independent biological replicates following the procedure described by Isidro and colleagues with some modification (Isidro et al., 2020). Briefly, the antibiotics were diluted in Müeller-Hinton agar medium (Oxoid, CM0337B), supplemented with 5% (V/V) defibrinated horse blood (Microbiol, Italy; 17.0159). Two microliters of bacterial suspension brought to 0.5 McFarland (Remel, Thermo Fisher Scientific) were inoculated on the plates. The plates were then incubated for 48 h at 30 °C, under microaerophilic conditions (Thermo Fisher, CN0035A).

2.2. Whole genome sequencing, genome identification, functional annotation and pangenome evaluation

A selection of the strains was carried out on the base of the antibiotic resistance results as shown in Fig. 1. Fifty-six isolates of *A. butzleri* (Table 1) were selected from each isolation source (BC, BNS and SE) among the most resistant and most susceptible isolates to antibiotics and were sequenced by Illumina Whole Genome Sequencing (WGS). Illumina WGS was conducted by the company Novogene (Cambridge, United Kingdom). In brief, the gDNA was randomly fragmented into short fragments, end-repaired, A-tailed and ligated with Illumina

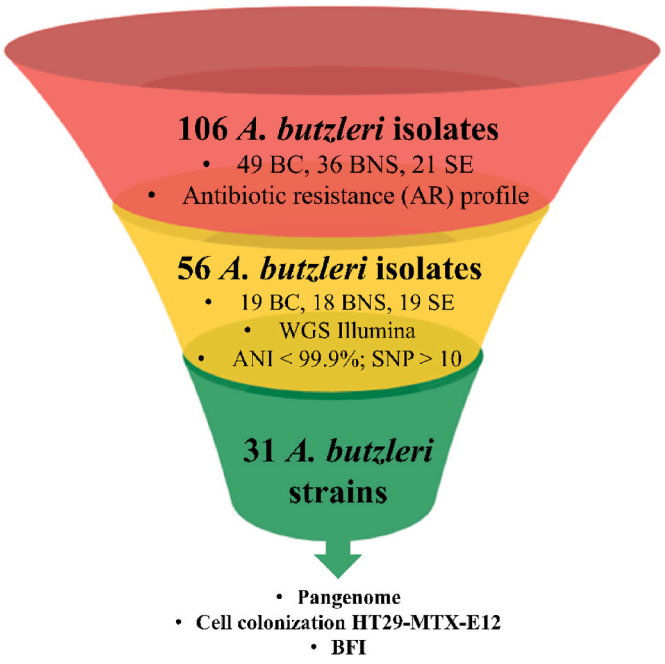

Fig. 1. Schematic representation of the selection of the isolates. A total of 106 isolates were tested for antimicrobial resistance. After evaluation of antimicrobial resistance profile, 56 from each isolation source were selected for Illumina sequencing and genomic analysis. The results of ANI <99.9% and SNP value > 10, allowed the identification of 31 *A. butzleri* strains on which subsequent analyses were performed: pangenome and tests for cell colonization and biofilm formation index.

adapter (NEBNext® library prep Kit). The fragments were PCR amplified, size selected, and purified. The libraries were analysed with Qubit and real-time PCR. The fragment size distribution was performed by bioanalyzer analysis (Agilent 2100 Bioanalyzer). The libraries were pooled and sequenced using Illumina Novaseq6000 (paired-end 150). The following bioinformatic tools were used with default option unless otherwise indicated. The raw sequence reads were checked with fastQC v0.11.9 and trimmed by sequence quality using fastP software v1.0.4 (Chen, Zhou, Chen, & Gu, 2018). The cleaned reads were assembled with Shovill software v1.1.0 using Spades assembler software v3.15.4 (min contigs length 200 bp) (Bankevich et al., 2012). The assemblies quality was evaluated with Quast v5.0.2 (Gurevich, Saveliev, Vyahhi, & Tesler, 2013).

After the completeness and contamination evaluation of the genomes with CheckM v1.1.2 (Parks, Imelfort, Skennerton, Hugenholtz, & Tyson, 2015), the genome taxonomy database Toolkit (GTDB-Tk) was used to perform species identification (Chaumeil, Mussig, Hugenholtz, & Parks, 2019). The genomes were compared in pairs using OrthoANIu v1.2 (Yoon, Ha, Lim, Kwon, & Chun, 2017). Pangenome evaluation and visualization were performed with the software Anvi'o v7.1 (mcl 10) (Eren et al., 2015). Functional gene annotation was performed using Prokka v1.14.5 (database HAMAP, CMs: Bacteria, Kingdoms: Bacteria) (Seemann, 2014), Dfast v1.2.18 (Tanizawa, Fujisawa, & Nakamura, 2017), and egg-NOG mapper 2.1.9 (query and subject coverage 60%) (Huerta-Cepas et al., 2019). Pangenome partitions were evaluated with Panaroo v1.2.8 (Tonkin-Hill et al., 2020), PPanGGolin v1.1.136 (Gautreau et al., 2020) and Roary v3.13.0 (Sitto & Battistuzzi, 2020). The orthogroups were analysed with OrthoFinder v2.5.4 (Emms & Kelly, 2019). CSIphylogeny v1.4 (https://cge.food.dtu.dk/services/CSIPhylogeny/) was used for single nucleotide polymorphisms (SNPs) analysis (Kaas, Leekitcharoenphon, Aarestrup, & Lund, 2014). Correlations between particular orthogroups and genes with strain characteristics were evaluated with Scoary v1.6.16 (Brynildsrud, Bohlin, Scheffer, & Eldholm, 2016). MegaX v10.1.7 (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) was used to align and produce dendrograms from specific sequences (e.g., 16 s RNA). BcgTree v1.1.0 was applied to obtain a dendrogram based on 107 single-copy amino-acid sequences (Ankenbrand & Keller, 2016). Nucmer v 4. x (Marçais et al., 2018) was used to perform alignment between genes annotated by Prokka and putative virulence genes from a set of *A. butzleri* strains analysed by Buzzanca and colleagues (Buzzanca et al., 2021). The core genome multi-locus sequences typing (cgMLST) analysis was performed using chewBBACA v3.0.0 (Silva et al., 2018). CRISPRCasFinder (CRISPR-Cas++ 1.1.2) was used to detect clustered regularly interspaced short palindromic repeats (CRISPR)-Cas sequences (Couvin et al., 2018).

2.3. Evaluation of biofilm formation index

A. butzleri strains were analysed to assess the ability to form biofilm following the procedure recently used in literature with some modifications (Martinez-Malaxetxebarria et al., 2022; Salazar-Sánchez et al., 2022). All strains were grown overnight in 3 ml of *Arcobacter* broth at 30 °C, and then diluted to an optical density (OD) of 0.25 at 630 nm (Bioteck; Synergy HT). Two hundred microliters of bacterial suspension were placed into each well of a 96-weel sterile polystyrene microplate (Enrico Bruno; 05,327,393) and incubated for 48 h at 30 °C (normal atmosphere), each strain was loaded in technical duplicates. After two days, the OD at 630 nm was measured (G value). The microplate was washed two times with 200 μl of sterile distilled water and dried at 42 °C for 30 min. After drying, the wells were stained with 200 μl of crystal violet 0.5% (Sigma-Aldrich; C6158) and incubated for 15 min at room temperature. The crystal violet was removed, and the wells were washed three times with 200 μl of sterile distilled water and dried at 42 °C for 30 min.

Two hundred microliters of 98% ethanol (Supelco; 1,009,831,011) were added to each well. Ethanol was then transferred to a new sterile 96-well microplate to perform the spectrophotometer OD measurement at 595 nm (AB). Another microplate containing only *Arcobacter* broth was also prepared on which the same procedure was performed to subsequently calculate the background noise (CW).

The biofilm formation index (BFI) was used to express the biofilm formation ability of each *A. butzleri* strain. The BFI was determined by applying the formula: BFI=(AB-CW)/G, in which AB is the optical density of the stained attached microorganisms, CW is the optical density of the stained control wells containing microorganisms-free medium only and G is the optical density of the bacteria grown in suspended culture. Semiquantitative biofilm production is then divided into four categories as previously described by (Teh, Flint, & French, 2010): strong ≥1.10, moderate 0.70 to 1.09, weak 0.35 to 0.69 and none <0.35. The BFI evaluation was performed in three independent biological replicates.

2.4. Colonization ability on human cells lines

Based on the WGS results, the cell colonization ability of 31 *A. butzleri* strains was assessed following the protocol previously described by Buzzanca et al. (2021, 2023) with some modifications (Buzzanca, Alessandria, et al., 2023; Buzzanca et al., 2021). A human colon carcinoma cell lines HT29-MTX-E12 (12,040,401; European Collection of Authenticated Cell Cultures, ECACC) was cultured in DMEM (Dulbecco's modified Eagle Medium; 6429; Sigma-Aldrich, St. Louis, MO, USA) with 10% v/v FBS (Foetal Bovine Serum; F7524; Sigma-Aldrich) and EmbryoMax penicillin-streptomycin (TMS-AB2-C; Sigma-Aldrich). The cells were grown in flasks (Corning, New York, NY, USA) at 37 °C in a humidified atmosphere (5% CO_2), and sub-passaged every 48 h (Galaxy 170 S; Eppendorf, Hamburg, Germany). The cells were seeded at a density of 35,000 cells/cm^2 on 1.93 cm^2 Falcon ® 24-well Clear Flat Bottom TC-treated Polystyrene Multiwell Cell Culture Plate (Falcon, 353047). The cells were cultivated in a complete culture medium (0.5 ml/well) under the same conditions as described above for

Table 1

Information about 106 *A. butzleri* isolates from BC, BNS and SE. The table shows code, flock code, source of isolation and antibiotic resistance profile of the *A. butzleri* isolates object of study. The genome group column indicates isolates that represent the same strain (ANI >99.9% and SNPs <10), "s" indicates unique strain while "-" isolates not subjected to genome sequencing. The strains selected for biofilm formation index and host cells colonization are indicated by asterisks (*). The source of isolation is indicated through the codes BC (broilers caecum), BNS (broilers neck skin) and SE (environmental surfaces). The antibiotics profile is related to the antibiotics amoxicillin + clavulanic acid (AMC), amoxicillin (AMO), ampicillin (AMP), azithromycin (AZI), clarithromycin (CLA), erythromycin (ERY) and gentamicin (GEN) at resistance breakpoints 1X (1), 2X (2) and 4X (4). The flock code includes the sampling day, in the case of isolates from SE the letters a and b indicate different sampling days.

Isolate	Genome group	Flock/sample_code	Source	AMC	AMO	AMP	AZI	CLA	ERY	GEN
BZg213	A	3_DAY8	BC	1	4	4	4	4	1	1
BZg214*	A	3_DAY8	BC	1	4	4	4	4	1	1
BZe306*	B	7b	SE	2	4	4	4	4	2	1
BZg113	B	6_DAY4	BC	2	4	4	4	4	2	1
BZg114	B	6_DAY4	BC	2	4	4	4	4	2	1
BZg115	B	6_DAY4	BC	2	4	4	4	4	2	1
BZe348	C	5b	SE	2	4	4	4	4	1	0
BZe363*	C	5b	SE	2	4	4	4	4	1	0
BZs142	C	5_DAY5	BNS	2	4	4	4	4	1	0
BZe307	D	7b	SE	2	4	4	4	4	4	1
BZe314*	D	4b	SE	2	4	4	4	4	4	1
BZe315	D	4b	SE	2	4	4	4	4	4	1
BZe316	D	4b	SE	2	4	4	4	4	4	1
BZe287	D	3b	SE	2	4	4	4	4	4	1
BZe300	D	2b	SE	2	4	4	4	4	4	1
BZs99*	E	4_DAY4	BNS	4	4	4	4	4	4	1
BZs89	E	1_DAY4	BNS	4	4	4	4	4	4	1
BZe327*	F	6b	SE	4	4	4	4	4	4	4
BZs166	F	3_DAY6	BNS	4	4	4	4	4	4	4
BZs250*	G	6_DAY9	BNS	2	0	4	1	4	4	4
BZs174	G	6_DAY6	BNS	2	0	4	1	4	4	4
BZe291	H	5a	SE	0	4	4	1	2	1	1
BZs7*	H	3_DAY2	BNS	0	4	4	1	2	1	1
BZg193	I	6_DAY7	BC	4	4	4	4	4	2	4
BZg277	I	2_DAY9	BC	4	4	4	4	4	2	4
BZg278*	I	2_DAY9	BC	4	4	4	4	4	2	4
BZg131	J	5_DAY5	BC	0	2	0	1	1	0	1
BZg132	J	5_DAY5	BC	0	2	0	1	1	0	1
BZg134*	J	5_DAY5	BC	0	2	0	1	1	0	1
BZg117	J	4_DAY4	BC	0	2	0	1	1	0	1
BZg64	J	3_DAY2	BC	0	2	0	1	1	0	1
BZe322*	K	3b	SE	2	4	4	4	4	2	0
BZe400	K	2b	SE	2	4	4	4	4	2	0
BZg177*	L	1_DAY6	BC	4	4	4	0	2	0	0
BZg14	L	1_DAY2	BC	4	4	4	0	2	0	0
BZs252*	M	5_DAY9	BNS	4	4	4	4	2	2	2
BZs155	M	2_DAY5	BNS	4	4	4	4	2	2	2
BZe401	M	12b	SE	4	4	4	4	2	2	2
BZs242*	s	8_DAY9	BNS	0	2	1	1	1	0	0
BZg269*	s	7_DAY9	BC	0	0	4	1	1	0	1
BZg262*	s	6_DAY9	BC	0	1	4	0	0	0	1
BZs173*	s	5_DAY6	BNS	4	2	4	4	4	2	4
BZs143*	s	5_DAY5	BNS	1	1	0	4	4	4	4
BZg74*	s	5_DAY3	BC	0	4	2	0	1	0	0
BZe344*	s	4b	SE	4	4	4	0	0	0	0
BZs170*	s	4_DAY6	BNS	1	4	4	0	2	0	2
BZg135*	s	4_DAY5	BC	0	4	4	1	0	0	0
BZs104*	s	3_DAY4	BNS	0	4	2	0	0	0	0
BZs83*	s	3_DAY3	BNS	0	4	4	1	1	0	0
BZs21*	s	3_DAY2	BNS	0	4	4	2	2	0	0
BZe301*	s	2b	SE	2	4	4	4	4	4	1
BZe283*	s	19a	SE	2	2	1	4	4	2	0
BZe296*	s	17a	SE	2	4	4	4	4	4	0
BZe310*	s	11b	SE	2	4	4	4	4	2	0
BZs206*	s	1_DAY7	BNS	1	4	2	1	1	0	0
BZs158*	s	1_DAY5	BNS	4	4	4	0	2	4	4
BZg258	–	8_DAY9	BC	1	0	4	0	0	0	0
BZg260	–	8_DAY9	BC	0	0	4	2	2	0	1
BZs235	–	7_DAY9	BNS	0	4	4	1	0	0	0
BZs236	–	7_DAY9	BNS	0	4	4	2	1	0	1
BZs240	–	3_DAY9	BNS	1	4	4	4	4	2	0
BZs44	–	7_DAY2	BNS	0	4	4	0	0	0	0
BZs248	–	6_DAY9	BNS	0	0	4	2	0	0	1
BZg268	–	5_DAY9	BC	2	4	1	4	1	0	1
BZg267	–	5_DAY9	BC	1	2	4	2	2	2	2
BZg265	–	5_DAY9	BC	2	2	2	4	4	1	1
BZs253	–	5_DAY9	BNS	1	4	4	4	2	0	1
BZg181	–	5_DAY6	BC	2	1	4	0	0	4	4

(*continued on next page*)

Table 1 (*continued*)

Isolate	Genome group	Flock/sample_code	Source	AMC	AMO	AMP	AZI	CLA	ERY	GEN
BZs172	–	5_DAY6	BNS	4	1	1	0	0	0	4
BZg110	–	5_DAY4	BC	0	0	1	4	4	2	1
BZg111	–	5_DAY4	BC	4	4	4	4	4	1	1
BZs96	–	5_DAY4	BNS	2	4	4	1	1	0	1
BZg39	–	5_DAY2	BC	4	4	4	0	1	0	1
BZg276	–	4_DAY9	BC	1	0	1	1	2	0	1
BZg279	–	4_DAY9	BC	0	4	1	4	4	2	1
BZs280	–	4_DAY9	BNS	0	1	2	2	4	0	1
BZs254	–	4_DAY9	BNS	4	0	4	4	1	4	0
BZg209	–	4_DAY8	BC	1	4	4	1	2	0	1
BZg185	–	4_DAY7	BC	0	0	0	0	4	0	1
BZg188	–	4_DAY7	BC	2	4	4	0	4	0	1
BZg182	–	4_DAY6	BC	1	4	4	4	4	4	0
BZg136	–	4_DAY5	BC	0	4	1	0	0	0	0
BZg257	–	3_DAY9	BC	0	2	4	2	2	0	1
BZs196	–	3_DAY7	BNS	0	1	2	0	1	1	1
BZs199	–	3_DAY7	BNS	2	4	4	4	4	2	0
BZg107	–	3_DAY4	BC	0	2	4	4	2	0	1
BZg109	–	3_DAY4	BC	2	4	4	2	2	0	0
BZe341	–	20a	SE	4	4	4	4	4	4	0
BZg275	–	2_DAY9	BC	0	2	0	1	1	0	1
BZg216	–	2_DAY8	BC	1	0	0	0	1	0	4
BZg123	–	2_DAY5	BC	0	4	4	4	1	0	0
BZs119	–	2_DAY4	BNS	0	4	4	4	4	0	0
BZg36	–	2_DAY2	BC	0	0	1	0	1	0	1
BZg34	–	2_DAY2	BC	2	4	4	4	2	0	4
BZe500	–	1b	SE	4	4	4	4	4	4	0
BZs243	–	1_DAY9	BNS	0	0	4	0	0	0	1
BZg204	–	1_DAY7	BC	2	4	4	4	2	1	1
BZg203	–	1_DAY7	BC	2	4	4	4	4	2	0
BZg175	–	1_DAY6	BC	1	4	4	1	1	0	1
BZg176	–	1_DAY6	BC	4	4	4	4	4	2	0
BZs162	–	1_DAY6	BNS	2	4	4	0	2	0	1
BZg130	–	1_DAY5	BC	2	4	0	4	1	2	0
BZs91	–	1_DAY4	BNS	0	0	4	1	2	0	1
BZg78	–	1_DAY3	BC	1	4	4	4	2	2	0
BZs28	–	1_DAY2	BNS	0	4	4	2	2	2	0
BZs9	–	1_DAY2	BNS	0	4	4	4	2	1	0

14 days after cell layer confluence, with regular medium refreshments (Buzzanca et al., 2021). Three days before the bacterial inoculum, the monolayer was washed twice with phosphate-buffered saline (PBS 1X, Sigma-Aldrich). The culture medium was then replaced with the same medium without antibiotics. *A. butzleri* was inoculated at an average bacterial load of 7.87 Log_{10} (st. err 0.34).

The colonization test was performed by replacing 0.25 ml of DMEM with 0.25 ml bacterial in DMEM. The models were incubated for 90 min at 37 °C (normal atmosphere) and washed twice with 1 ml of PBS. Bacterial colonization (bacteria present in the cell model after washes) was assessed by applying 0.5 ml of Triton X-100 0.25% (v/v, in PBS). After 30 min of incubation at 37 °C, dilutions in Ringer's solution were made and plated on *Arcobacter* agar (w/o laked horse blood and isolation supplements (Houf, Devriese, De Zutter, Van Hoof, & Vandamme, 2001a) to determine the bacterial load. Plates were incubated for 48 h at 30 °C in microaerophilic conditions.

The bacterial colonization was expressed as ΔLog CFU, following the formula: Log CFU ml^{-1}TC (bacteria count after washing steps) - Log CFU ml-1T0 (bacteria initial inoculum). The colonization evaluation was performed in three independent biological replicates.

2.5. Statistical analysis

The data homogeneity was evaluated by Shapiro-Wilk's W and Modified Levene's tests (Brown-Forsythe test), while Kruskal-Wallis (K-W) and ANOVA tests were used to assess differences between multiple groups. Wilcoxon Rank sum test (WRS) and two-sample *t*-test were used to assess differences between two groups for data nonparametric (K-W, WRS) and parametric (Anova, *t*-test) data respectively. Dunn's and Tukey's tests were performed as post-hoc analyses for nonparametric and parametric data respectively. The accessory genes were analysed using Pagoo v0.3.17 (Ferrés & Iraola, 2021). Statistical analyses were performed using RStudio 2022.07.2 (R version 4.2.1).

3. Results & discussions

3.1. Antibiotic resistance assay

One-hundred six isolates of *A. butzleri* (Fig. 1, Table 1), were analysed to evaluate the antibiotic resistance (AR). Results of the antimicrobial evaluation showed that all isolates were resistant to at least two antibiotics (Table 1), with specifically 35.5% of the isolates found to be resistant to all antibiotics tested.

The least effective antibiotic was ampicillin with 90.6% of the isolates resulted resistant. The 88.7% of the isolates were resistant to clarithromycin while 86.9% to amoxicillin. Among the isolates, 80.3% showed to be resistant to azithromycin and 66.3% exhibited resistance to amoxicillin and clavulanic acid. The lowest percentages of antibiotic resistance were observed for gentamicin and erythromycin, with resistance rates of 64.4% and 55.1%, respectively.

Results reported show that the isolates not only exhibit a resistant phenotype but are also able to grow at the highest concentration tested (reference breakpoint 4X). Resistance to ampicillin was observed in 81 isolates, 73 for amoxicillin, 55 for azithromycin, 47 for clarithromycin, 22 for amoxicillin clavulanic acid, 21 for erythromycin and 14 isolates showed resistance to gentamycin.

The high resistance to ampicillin has been reported in previous studies (Fanelli et al., 2020; Gungor et al., 2023; Isidro et al., 2020; Shirzad Aski, Tabatabaei, Khoshbakht, & Raeisi, 2016; Van den Abeele, Vogelaers, Vanlaere, & Houf, 2016) and it is in line with what Ferreira

and colleagues previously reported (Ferreira, Fraqueza, Queiroz, Domingues, & Oleastro, 2013). Specifically, Van den Abeele and colleagues reported that 91% of the tested *A. butzleri* strains were resistant to ampicillin (Van den Abeele et al., 2016).

Out of 106 isolates analysed, 70 were found to be resistant to amoxicillin in combination with clavulanic acid, although the resistance rate to this combination was lower than that observed for other antibiotics within the penicillin class. The resistance of *Arcobacter* spp. to penicillin class seems to be associated with the presence of the β-lactamase enzyme in the genome of *A. butzleri* (Ferreira, Luís, Oleastro, Pereira, & Domingues, 2019). Moreover, a possible cause of β-lactam resistance could be related to the combined effect given by the presence and activity of β-lactamase genes, the binding affinity of target proteins (such as penicillin-binding protein) and the permeability of the bacterial cell membrane, particularly for Gram-negative bacteria (Fanelli et al., 2020). The susceptibility to amoxicillin in combination with clavulanic acid is related to β-lactamase inhibition of clavulanic acid (Ferreira et al., 2019).

The antibiotics analysed which belong to the class of macrolides, are clarithromycin, azithromycin, and erythromycin. The results showed the resistance of *A. butzleri* to clarithromycin and azithromycin and the susceptibility to erythromycin.

As also previously observed in a study conducted on *Arcobacter* spp. isolates from broiler carcasses, in the present study, most of the phenotypes displayed resistance to azithromycin but were susceptible to erythromycin (Son, Englen, Berrang, Fedorka-Cray, & Harrison, 2007). According to Ferreira et al. (2019), azithromycin is the macrolide antibiotic for which the resistance rate appears to be highest(Ferreira et al., 2019). More than half of the isolates tested were resistant to gentamicin, an antibiotic belonging to the aminoglycoside class, in contrast to studies conducted that have shown *Arcobacter* spp. to be susceptible to this antibiotic (Fanelli et al., 2019; Vicente-Martins, Oleastro, Domingues, & Ferreira, 2018). Considering *Arcobacter* spp. isolated from several sources Van den Abeele and colleagues showed that 99% of the strains were susceptible to gentamicin (Van den Abeele et al., 2016) and similarly, Müller and colleagues observed cases of intermediate resistance to gentamicin (Müller, Abdel-Glil, Hotzel, Hänel, & Tomaso, 2020). In this study the multiple antibiotic resistance of *A. butzleri* was evidenced.

3.2. Selection process of isolates

In this study, a total of 106 isolates of *A. butzleri* were examined for antibiotic resistance. This set included 85 isolates obtained from broiler carcasses (49 BC and 36 BNS) and 21 isolates from various surfaces within the slaughterhouse (SE) (Fig. 1) (Supplementary table 1). Following evaluation of the resistance profiles (Table 1), 56 isolates (19 BC, 18 BNS and 19 SE) were selected for Whole Genome Sequencing. This selection was conducted to obtain a representative group (n = 56) that encompassed both the most susceptible and the most resistant isolates. Isolates originating from the same sample were included to identify the potential presence of multiple strains (ANI <99.9% and SNPs >10). The genomic analyses (Average Nucleotide Identity, ANI <99.9% and SNP >10) (Fig. 2, Supplementary figures 1-2) indicated a differentiation of a total of 31 distinct strains of *A. butzleri* destinated to phenotypical tests (Fig. 1).

3.3. Whole genome sequencing and pangenome evaluation

The evaluation of antibiotic resistance allowed the selection of 56 isolates designed for whole genome sequencing (WGS) (Fig. 1). Isolates were selected to form groups comprising *A. butzleri* characterized by different AR profiles and originating from the three isolation sources (BC, BNS and SE) (Fig. 1, Table 1). The WGS (Table 1 and Supplementary table 2) performed on 56 isolates (19 from slaughterhouse environment after cleaning procedures "SE", 19 from caecum "BC" and 18 from chicken neck skins "BNS") confirms the identification of the isolates as *A. butzleri*.

The analysis of average nucleotide identity (ANI) and single nucleotide polymorphisms (SNPs) indicates that some of the isolates represents the same strain isolated from multiple sources (ANI >99.9% and <10 SNPs) (Olm et al., 2017; Stimson et al., 2019), showing 31 different strains from 13 strains groups (A-M) and 18 unique strains (Fig. 2, Table 1 and Supplementary figures 1, 2).

One hundred and sixty-seven assembled *A. butzleri* genomes were downloaded from GenBank NCBI (https://www.ncbi.nlm.nih.gov/genbank/; 09-01-2023) to perform a cgMLST analysis (Supplementary Table 3). The aim was to assess the potential similarity of genomes in the database with the subject of the study. The cgMLST analysis doesn't show a similarity between isolation sources (Supplementary figure 3). Fifteen BC isolates exhibited ANI and SNPs values within the designated threshold, thereby qualifying them as distinct entities closely related to at least one other isolate originating from the same source of isolation (Table 1, Fig. 2 and Supplementary figures 1, 2). The same observation was possible for ten and six isolates from SE and BNS respectively (Table 1, Figs. 1 and 2 and Supplementary figures 1, 2). Moreover, some strains were found to be present in multiple isolation sources. The strains BZs7, BZs252, BZe327 and BZe363, were found on BNS and SE (9 isolates), while BZe306 was isolated from BC and SE (4 isolates). Six isolates from SE representing the same strain, were found on plucking area/station (scalder tank and plucker) and on evisceration equipment surfaces. The genomic analysis performed demonstrate cross-contamination routes between carcasses, surfaces and even between different environmental surfaces. Results showed the presence of nine persistent strains on different sampling days. Persistent strains from chicken carcasses (BC and BNS) and SE were found to be present after four months (Table 1). The persistence of strains may be attributed to the ability of the bacterial strains to persist or to strains in subsequent flocks acting as carriers of *A. butzleri.*

The analysis of isolates from the same flock shows multiple strains at the same time (SNPs >10; ANI <99.9%) in SE belonging to the groups s/D/K (2b), D/s (4b) and D/K (2b and 3b) (Table 1 and Supplementary Table 1). Similarly, strains of groups G/s (6_DAY9), C/s/J (5_DAY5), E/J (4_DAY4) and H/J/s (3_DAY2) were found to be in chicken carcasses (BC, BNS) of the same flock (Table 1 and Supplementary Table 1). The isolation of multiple strains from the same sources suggests co-contamination caused by distinct *A. butzleri* strains. The isolates selection allowed the phenotypical evaluation of bacteria characterized by different genotypes avoiding interference in pangenome partitions analysis due to multiple isolates corresponding to the same strain.

The pangenome and phenotypical analysis were focused on 31 strains after de-replication (ANI <99.9% and >10 SNPs) (Figs. 2 and 3; Supplementary figures 1, 2). The Scoary analysis demonstrated a correlation between genes and isolation sources (multiple isolation sources were considered; Scoary *naive-p < 0.03*). The strains isolated from environmental samples (9/12) showed a positive correlation to *regX,* a gene linked to hypoxic stress (Mahatha et al., 2022) and *mcpU* linked to chemotaxis (Corral-Lugo et al., 2018). The strains isolated from SE demonstrated a positive correlation with *oprM* and *mexA* (present in 10 out of 12 strains). These genes were identified as positively correlated with all strains isolated from the plucking sector, along with *mdtL* (González-Plaza et al., 2018). The gene *mexA* has been associated with antibiotic resistance in *A. cryaerophilus* (On et al., 2019), while *oprM* has been associated to antibiotic resistance in Gram-negative bacteria (Pesingi et al., 2019). The correlation of genes linked to environmental stress and antibiotic resistance suggests a genome adaptation of strains present on environmental surfaces after the cleaning procedure. The positive correlation between environmental strains and antibiotic resistance genes can be linked to selective pressure due use of sanitizers on environmental surfaces (van Dijk et al., 2022).

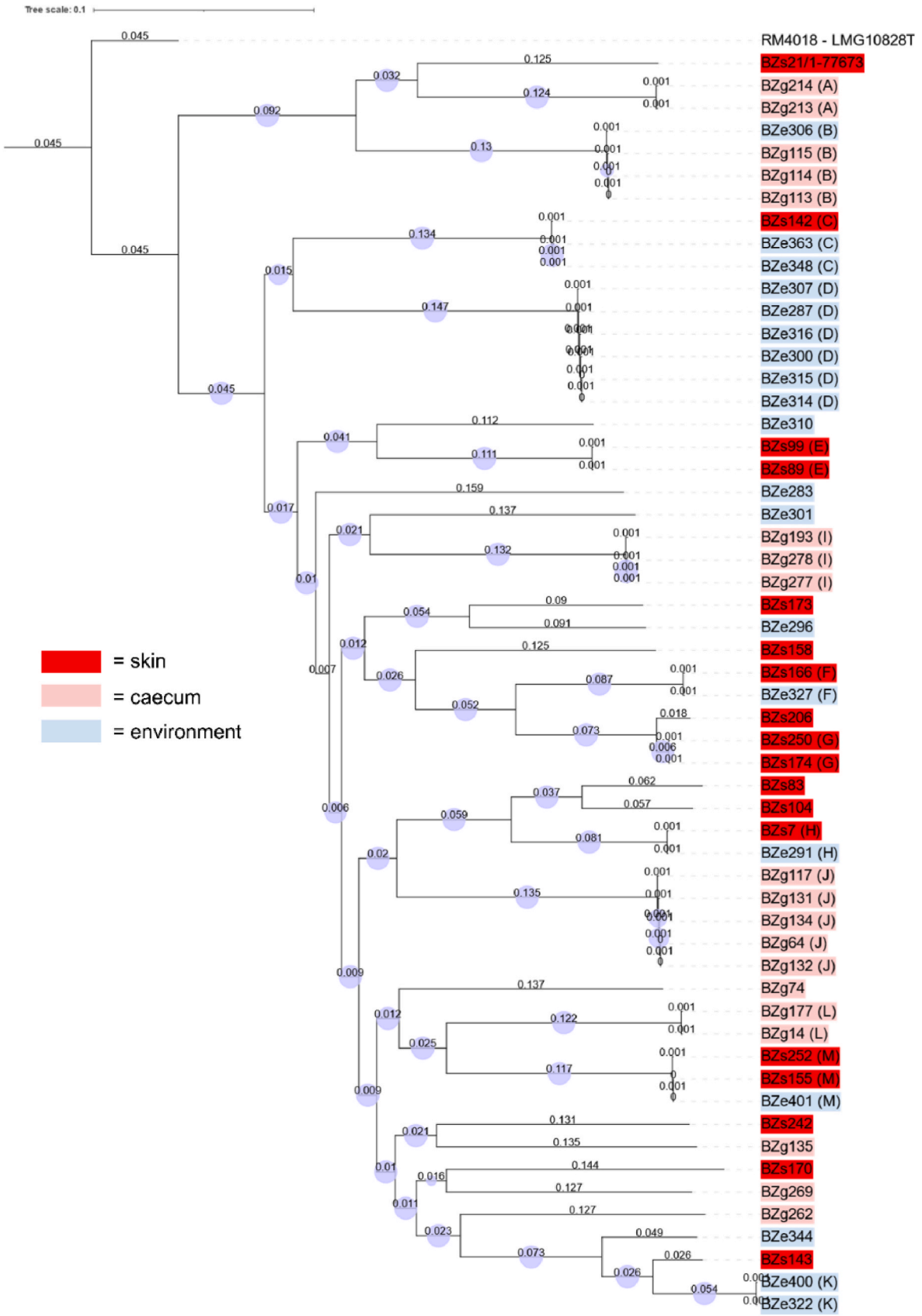

Fig. 2. Dendrogram produced on SNPs about 56 *A. butzleri* isolates. The dendrogram shows distances between *A. butzleri* isolates. The sources of isolation are indicated by blue box for slaughterhouse environment (SE), pink box for chicken cloacae (BC) and red box for chicken neck skins (BNS). The numbers on tree branches indicate distance values, while the pink circles are bootstrap values (from 0.5 to 1). The letters A-M indicates isolates groups that includes multiple isolates (Table 1; ANI >99.9% and SNPs <10). The genome of LMG 10828^{T} (RM4018) was included as a reference.

Fig. 3. Pangenome visualization of *A. butzleri* strains. The figure includes information about *A. butzleri* pangenome. The strains order follows the gene clusters presence absence (D. Euclidean, L. Ward). "Environment" box shows strains isolated from slaughterhouse environment (SE); multiple isolation sources are shown in Fig. 1 and Table 1 (supplementary table 1). GC content, number of genes per kbp, genome size, singleton gene cluster (SCGs) and number of gene cluster are shown under the dendrogram. The number of genome where a gene was found to be present is indicated by "number of contributing genomes". The blue box indicates that a strain was isolated from SE while dark red and red indicate strains isolated from BNS and BC respectively. The genome of LMG 10828^T (RM4018) was included in the analysis.

3.4. Gene annotation and pangenome evaluation

The genome functional annotations showed an average of 2170 genes (st.dev. 78), among them 52% (st.dev. 1.38) with known function (Supplementary table 2).

The *A. butzleri* clusters of orthologous genes (COGs) included 166 orthologues related to amino acid transport and metabolism (Supplementary figure 4 and Supplementary table 4), confirming the importance of amino acid metabolism for *A. butzleri* (Buzzanca et al., 2021). However, the functional evaluation of 16.8 % COGs was not possible. The importance of amino acid transport and metabolism was confirmed by gene enrichment analysis directly on amino acid sequences annotated with Prokka, showing an average of 151 genes (st. dev. 5) related to amino acid metabolism. Despite this, the "unknown function" group remains numerically relevant, comprising 312 genes (st. dev. 10). The class composition is relatively constant between isolation sources. Associated CRISPR-Cas sequences were found to be present in BZs21, BZg74, BZs99, BZg134, BZe301 and BZe327.

The pangenome evaluation performed using several tools indicates a high number of accessory genes. An average of 1674 core genes was detected by several tools (Supplementary table 5). The wide accessory genome demonstrates again the open pangenome of *A. butzleri*, a phenomenon related to horizontal gene transfer (Buzzanca et al., 2021). However, the analysis of accessory genes shows the absence of a strong correlation between isolation sources and genomic traits (Figs. 3, 4A-B). The absence of correlation between isolation sources and accessory genes is related to the isolation of multiple isolates from different sources resulted the same strain (Table 1, Fig. 4A and B, Supplementary figures 1, 2). The ability of *A. butzleri* to survive and colonize different matrices (BC, SE, BNS) is confirmed suggesting the presence of genomic traits that favours its survive in the environment. These genome characteristics are present in the core genome, alternatively, different

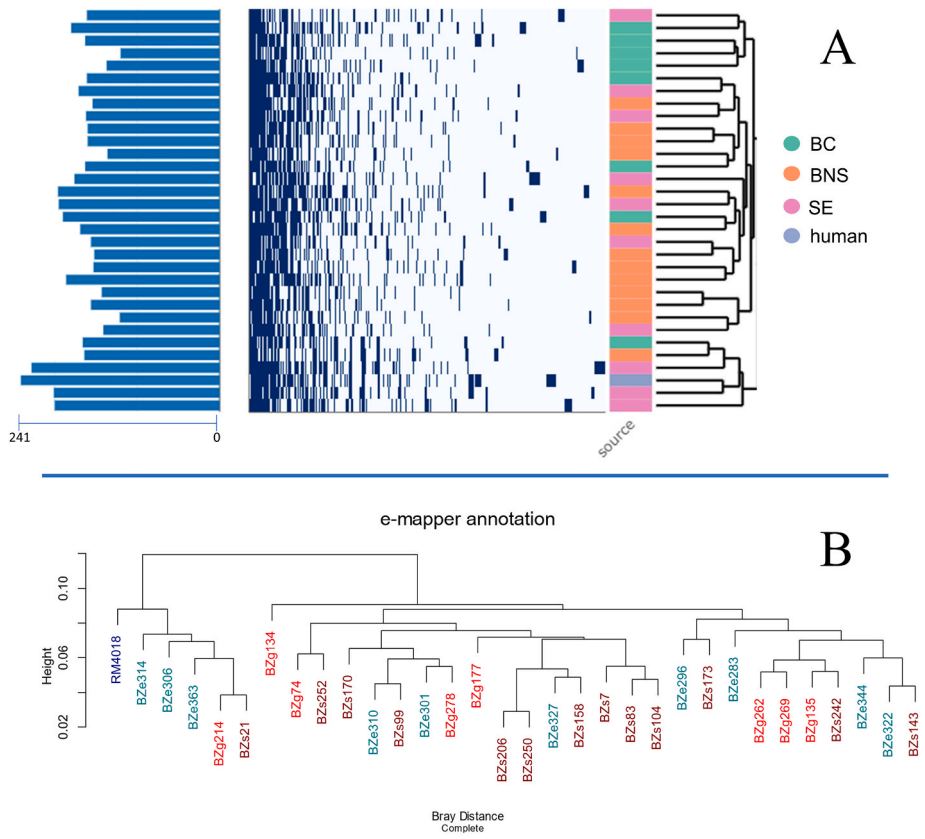

Fig. 4. Presence/absence matrix (A) and distance tree performed on accessory genes annotation of e-mapper (B). Panel A shows the presence/absence binary matrix produced from e-mapper annotation. The histogram shows the number of genes (total accessory genes clusters 831). The Bray distance tree (panel B) is related to Pagoo (R package) analysis on gene functions annotated by e-mapper. The dendrograms are produced on Bray distance matrix calculated on accessory gene functions. Different isolation sources are indicated by strain name colours: BNS = dark red, BC = red, SE = blue. The strain RM4018 (LMG 10828^{T}; dark blue; human) has been included as an outgroup.

accessory genes with the same functions can compensate for needs related to bacteria subjected to different environmental conditions. However, the presence of many hypothetical proteins doesn't allow a punctual evaluation of these genomic traits.

3.5. Antibiotic resistance genomic traits

The antibiotic resistances (AR) (Fig. 5) were correlated to genes and orthologues of the 31 *A. butzleri* strains (Scoary *naive-p* < *0.05*). The AR analysis of the 31 *A. butzleri* strains (de-replication by average nucleotide identity and SNPs analysis), demonstrates higher antibiotic resistance in the case of strain BZe327 isolated from slaughterhouse equipment samples. This strain exhibited resistance to all antibiotics tested, to breakpoint 4X, despite it being identified as the most effective antibiotic in the study (Fig. 5). The presence of multidrug resistance (MDR) highlights the public health concern posed by of *A. butzleri* which is considered an emerging foodborne pathogen worldwide (Ferreira et al., 2019; Gungor et al., 2023; Son et al., 2007). Even more so, considering the possible transmission of antibiotic resistance genes between bacteria from animals to humans. Moreover, the MDR phenotype makes treatment for infections caused by *Arcobacter* spp. potentially highly problematic (Ferreira, Queiroz, Oleastro, & Domingues, 2016).

Nine strains were found to be resistant to all antibiotics at the lower concentration tested. An orthologue of *hlyD* (efflux transporter, ABC transporter) (Lee et al., 2012) was associated with antibiotic resistance at resistance breakpoint 1X. The gene *hlyD* was associated with virulence and drug susceptibility in uropathogenic *Escherichia coli* (UPEC) (Harwalkar, Gupta, Rao, & Srinivasa, 2014). The presence of four *hlyD* orthologues in antibiotic resistant *A. butzleri* strains suggests different functions linked to this secretion membrane fusion protein variants.

Two efflux pump orthologues already linked to Gram-negative AR were found to be correlated to antibiotic resistance. RND efflux pump (Venter, Mowla, Ohene-Agyei, & Ma, 2015) and hydrophobe/amphiphile efflux-1 (Huang et al., 2022) were found to be correlated to AR, as they were present in 8 of the 9 AR strains. As well as orthologues, the Scoary analysis on genes reveals correlations to AR at seven-on-seven antibiotics in *A. butzleri*. The genes *mexA* and *mexB* are present in all strains, while *oprM* was absent in twelve strains (BZg74, BZg134, BZg135, BZg214, BZs21, BZs143, BZs158, BZs173, BZs206, BZs242, BZs250 and Bzs252). The *mexAB-oprM* operon has been implicated in antibiotic resistance (AR) to β-lactam, chloramphenicol, lincomycin, macrolides, novobiocin, tetracyclines, and quinolones in *Pseudomonas aeruginosa* (Pesingi et al., 2019). Similarly, *mexA* and *mexB* have been associated with AR in *A. cryaerophilus* (On et al., 2019). The presence of the *mexAB-oprM* operon in *A. butzleri* aligns with its observed antibiotic resistance. Furthermore, the presence of a β-lactamase gene in all strains is consistent with the known antibiotic resistance profile of *A. butzleri* (Ferreira et al., 2019). Also, acetyltransferase domains were associated with AR (Baumgartner et al., 2021).

The presence of *cydB*, associated with AR in *E. coli* (Shukla et al., 2017) and was observed in 6 of 9 *A. butzleri* resistant strains at seven-on-seven antibiotics. The gene *epsM* was found only in 3 AR strains, this protein is linked to biofilm production that in turn is related to AR (Høiby, Bjarnsholt, Givskov, Molin, & Ciofu, 2010). The correlation of a gene encoding an acetyltransferase (GNAT) domain protein was observed (6/9 strains). Part of the orthologues showed a correlation to AR to at least 6 antibiotics at resistance breakpoint 1X. Two orthologues of *tetR* were found to be associated respectively with 12 and 14 antibiotic resistant strains. TetR is a repressor associated with antibiotic resistance (Aleksandrov, Schuldt, Hinrichs, & Simonson, 2009). This

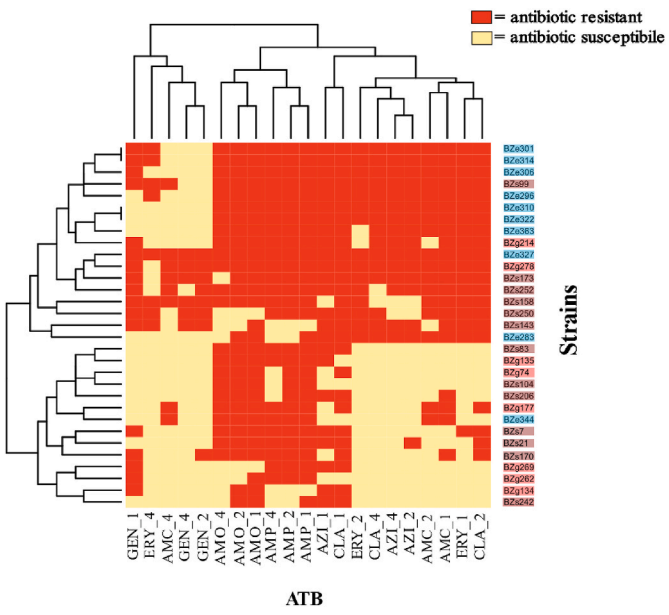

Fig. 5. Heatmap about antibiotic resistance test on the 31 *A. butzleri* strains. The heatmap shows the antibiotic resistance (red) or susceptibility (yellow) of 31 *A. butzleri* strains isolated from BNS (brown), BC (red), SE (blue). The antibiotics shown are indicated by abbreviations as follows: amoxicillin + clavulanic acid (AMC), amoxicillin (AMO), ampicillin (AMP), azithromycin (AZI), clarithromycin (CLA), erythromycin (ERY) and gentamicin (GEN) at resistance breakpoints 1X (1), 2X (2) and 4X (4). The heatmap was produced on 1(resistant)/0 (susceptible) binary matrix (heatmap {stats} R).

association was observed also for a drug resistance transporter MFS encoding gene (Li & Nikaido, 2009) and the gene *bcrC* (Bernard, El Ghachi, Mengin-Lecreulx, Chippaux, & Denizot, 2005), associated with AR. Moreover, a bleomycin resistance protein encoding gene (Dortet et al., 2017) was associated with antibiotic resistant strains being present in 12 of the 17 strains resistant to at least six antibiotics. The high number of AR gene agrees with the multidrug resistance of *A. butzleri* strains. The isolation procedure involves the use of antibiotics and antimicrobials as a selective supplement, this may have led to a selection of resistant strains (Houf & Stephan, 2007). Although the genes listed above have been associated with resistance to specific antibiotics in other bacteria, these genes in *A. butzleri* can allow AR to different antibiotics and with different mechanisms that remain to be investigated.

The analysis of genes associated with antibiotic-specific resistance genes didn't show correlation between specific gene clusters and AR. The orthologues analysis reveals a positive association of *hlyD*, and *tetR* to amoxicillin–clavulanic acid, while hydrophobe/amphiphile efflux-1 family RND trasporter, *tetR* and *bcrC* orthologues to erythromycin resistance (resistance breakpoint 1X). These genes/orthologues have been linked to AR in other bacteria as stated above. The AR genes have been correlated to the presence of specific genomic traits suggesting a chromosomic AR in *A. butzleri.* Moreover, part of these genes was correlated to SE strains suggesting multiple resistance to antibiotics, detergents/disinfectants, and environmental conditions to which these strains have been exposed. However, the isolation procedure involves the use of antibiotics and antimicrobials as selective supplement, which might have resulted in the selection of antibiotic resistant strains (Houf & Stephan, 2007).

3.6. Biofilm formation and cell colonization ability of A. butzleri

The finding of *A. butzleri* on slaughterhouse surfaces, after disinfection practices of the slaughterhouse, made the study of biofilm formation of interest. The Biofilm Formation Index (BFI), was calculated following the proposed BFI values for *C. jejuni* taken as a reference (Teh et al., 2010). The results show that 29 out of the 31 *A. butzleri* strains examined do not exhibit biofilm production (BFI <0.35) (Supplementary table 6). Only strains BZg278 and BZe306, display strong biofilm production (BFI ≥1.10) (*p-value* < *0.05*). The weak biofilm production found in this study has already been described for *A. butzleri* (Ferreira et al., 2013; Girbau et al., 2017; Gungor et al., 2023). Although biofilm production of *A. butzleri* was found to be weak, it could explain the high presence of *Arcobacter* spp. found on slaughterhouse surfaces (Ferreira et al., 2013). The ability to adhere to surfaces could clarify the high prevalence and possible route of cross-contamination in meat processing surfaces. Salazar-Sánchez and colleagues studied genes *flaA, flaB, fliS*, *luxS*, *pta* and *spoT* in *A. butzleri* and their role in biofilm production. They observed a higher biofilm production on polystyrene for *A. butzleri* mutants for genes *pta* and *spoT* (Salazar-Sánchez et al., 2022). These two genes were found to be present in the 31 strains tested on polystyrene in this study. Moreover, different materials can be related to different adhesion and biofilm production, Martinez-Malaxetxebarria and colleagues demonstrate that *Arcobacter* spp. adhesion was higher on borosilicate glass when compared to stainless steel and polystyrene (Martinez-Malaxetxebarria et al., 2022). The BFI results, and literature information about *A. butzleri* suggest possible biofilm production of the 31 *A. butzleri* under specific conditions related to surfaces materials.

In this study, the ability of the 31 selected *A. butzleri* strains to colonize the cell layer of the HT29-MTX-E12 cell models has been evaluated. The host cell invasion was not studied since in previous work, high bacterial invasion capacity did not emerge (Buzzanca et al., 2021). Co-incubation of the bacterial suspension with the mucus-secreting human colon adenocarcinoma cells took place for 90 min, the results are expressed as Δ Log CFU (Fig. 6A; Supplementary table 7). All strains showed a moderate ability to colonize cells; no significant difference emerges between strains considering the isolation source (BNS, BC, SE). Of the thirty-one strains of *A. butzleri* assayed, eleven strains show a high colonization phenotype (Δ Log CFU >0; Fig. 6A; Supplementary table 7), and the highest value of colonization was recorded for the strain BZg214 (Anova, *p-value* < *0.05*); of these four strains from SE and six from BNS. The lowest colonization phenotypes were found in the following strains: BZs7, BZg134, BZg177, BZe344, and in the reference strain LMG 10828^{T} (Fig. 6A). No significant correlation emerged between the ability to colonize cells and biofilm production by the strains.

The high ability of *A. butzleri* to colonize mucus-secreting cells agrees with previous work about *A. butzleri* and the pathogenic bacterium *C. jejuni* and highlights how strains can be considered highly pathogenic (Alemka, Corcionivoschi, & Bourke, 2012; Buzzanca, Alessandria, et al., 2023; Karadas et al., 2016). In both cases, an interesting element is the presence of the flagella, which may not only affect motility but also the ability to colonize (Ramees et al., 2017). The ability of intestinal pathogens to colonize cellular mucus could be considered an adaptive feature that allows them to explicate their pathogenic function and infect cells bypassing the barrier formed by mucus (Sicard, Bihan, Vogeleer, Jacques, & Harel, 2017).

The *in vitro* colonization of *A. butzleri* was evaluated jointly with the detection of putative virulence genes (PVGs). The WGS analysis showed 120 genes considered putative virulence related in *A. butzleri* (Fig. 6B–Supplementary table 8) (Buzzanca et al., 2021). The alignment between the coding DNA sequences (CDSs) object of study and the putative virulence genes previously annotated for *A. butzleri* strains allowed the detection of these PVGs (Buzzanca et al., 2021). Moreover, genes related to *tonB* (virulence) and *actP* (acetate metabolism) were found to be present in all strains. These genes were found to be overexpressed during *A. butzleri in vitro* colonization test (Buzzanca, Alessandria, et al., 2023). The sequence variability of the nine putative virulence genes (*cadF, ciaB, cj1349, hecA, hecB, irgA, mviN, pldA,* and *tlyA*) currently evaluated in the study of *Arcobacter* spp. was confirmed (Buzzanca et al., 2021; Douidah et al., 2012).

The heatmap (**B**) represents the presence of virulence genes in the analysed strains. At the right, the colour legend, where the numbers 2

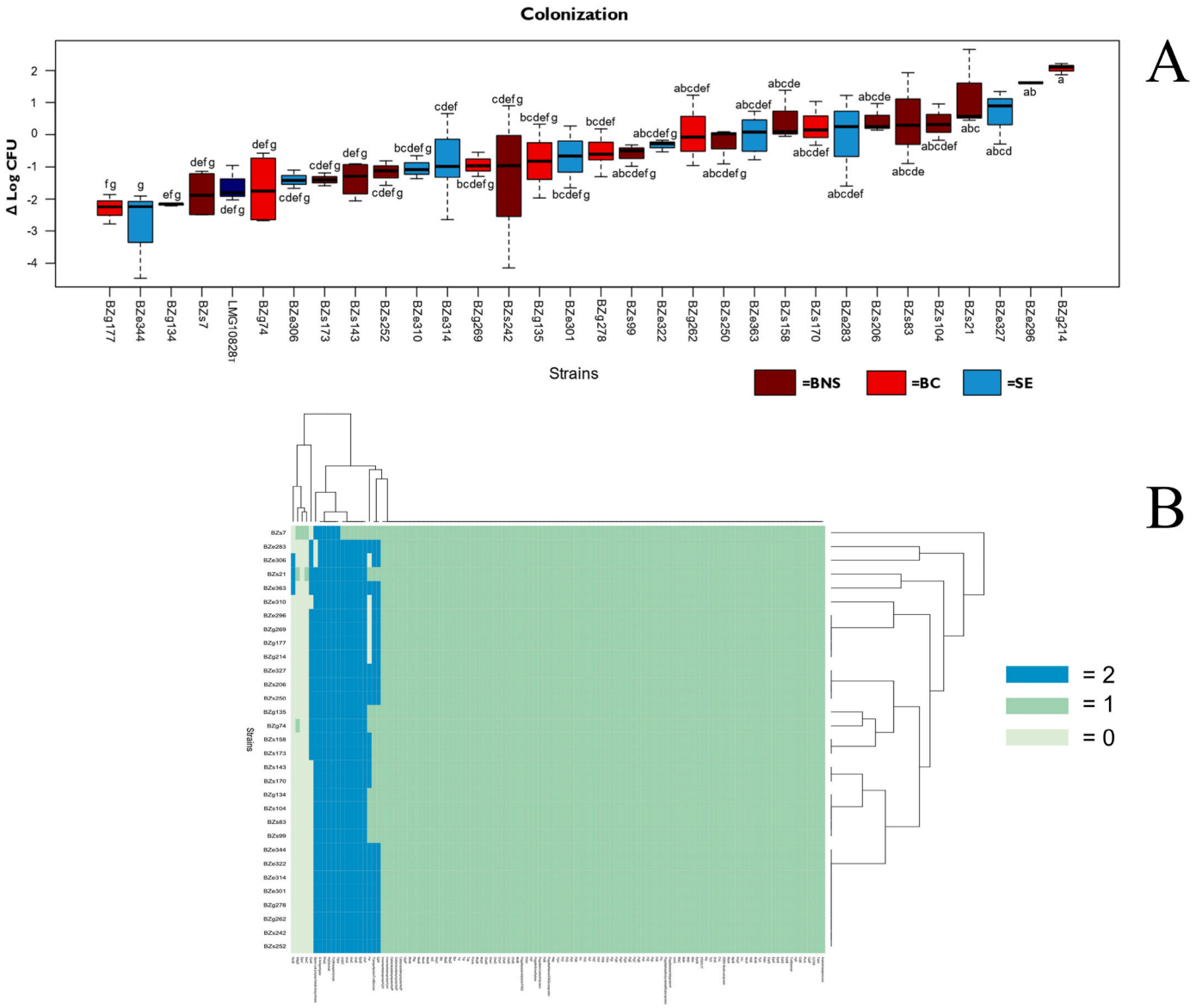

Fig. 6. The panel **A** shows the colonization of the 31 strains of *A. butzleri* on the mucus secreting cell line HT29-MTX. The legend in the figure represents the sources of isolations. Values > 0 indicate bacterial growth after the 90 min of colonization. The box plot refers to the median value, while the letters refer to the mean (*Anova, p-value* < *0.05*).

(from Dfast annotation) and 1 (from alignment with sequences annotated by Buzzanca and colleagues) indicate the presence of virulence genes in the strain, while 0 indicates the absence of the gene.

4. Conclusions

The isolation of *A. butzleri* in the slaughterhouse underlines the occurrence of this bacterium in poultry meat processing plants. This aspect suggests meat contamination due to cross-contamination during the slaughtering procedures, a risk already observed and suggested for other pathogens (Gonçalves-Tenório et al., 2018; Houf et al., 2001b, 2002). The whole genome analysis (ANI and SNPs analysis) demonstrated the simultaneous presence of strains in several sources (BC, BNS and SE). The multiple isolations confirmed cross-contamination routes in the slaughterhouse. However, the absence of *A. butzleri* in chicken guts has been reported (Van Driessche & Houf, 2007) indicating that BC contamination came from environmental surfaces and the external of chicken carcasses (e.g. plumage and skin). The isolation of *A. butzleri* from SE demonstrated its ability to adhere to environmental surfaces enhancing its spread and persistence. Even if some genes were correlated to specific isolation sources, this correlation was not strong probably due to multiple isolation sources demonstrating again cross-contamination events.

The AR results showed a high level of AR on part of *A. butzleri*, suggesting the importance of further investigation of this pathogen considering the potential exposure to humans. Genes related to AR are probably involved in detergents/sanitizers and environmental conditions resistance, suggesting multiple functions of AR genes. Moreover, the multiple antibiotic resistance jointly to bacterial survival after cleaning and disinfection procedures suggest broad-spectrum resistance mechanisms to multiple classes of molecules. The *in vitro* host cell colonization of *A. butzleri* strains was observed on mucus secreting cell models demonstrating their *in vitro* pathogenic potential (Buzzanca, Alessandria, et al., 2023; Buzzanca et al., 2021).

The isolation of antibiotic resistant and *in vitro* potentially pathogenic strains suggests the necessity of new studies focused on *A. butzleri* along the poultry meat production chain. Future studies should be focused on strains isolation from farms to consumers via

slaughterhouses to trace cross-contamination. The use of WGS techniques will be decisive for discriminating between isolates and confirming the possible presence of strains on multiple sources up to the final consumer.

Founding

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CRediT authorship contribution statement

Elisabetta Chiarini: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Davide Buzzanca:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Francesco Chiesa:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Cristian Botta:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Kalliopi Rantsiou:** Writing – review & editing, Supervision, Conceptualization. **Kurt Houf:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Valentina Alessandria:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization, Funding acquisition, Methodology.

Declaration of competing interest

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

Data availability

The raw sequences reads of A. butzleri Illumina WGS can be accessed on the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/, accessed on December 19, 2023) under the bioproject PRJNA986324.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2024.110500.

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