



Genomic Characterization of *Arcobacter butzleri* Isolated From Shellfish: Novel Insight Into Antibiotic Resistance and Virulence Determinants

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Arcobacter (*A.*) *butzleri* is an emerging pathogenic microorganism, whose taxonomy has been recently suggested to be emended to the *Aliarcobacter* (*Al.*) *butzleri* comb. nov. Despite extensive taxonomic analysis, only few fragmented studies have investigated the occurrence and the prevalence of virulence and antibiotic resistance determinants of this species in strains isolated from shellfish. Herein we report for the first time the whole genome sequencing and genomic characterization of two *A. butzleri* strains isolated from shellfish, with particular reference to the antibiotic, heavy metals and virulence determinants. This study supported the taxonomic assignment of these strains to the *Al. butzleri* species, and allowed us to identify antibiotic and metal resistance along with virulence determinants, also additional to those previously reported for the only two *A. butzleri* strains from different environments genomically characterized. Moreover, both strains showed resistance to β -lactams, vancomycin, tetracycline and erythromycin and susceptibility to aminoglycosides and ciprofloxacin. Beside enlarging the availability of genomic data to perform comparative studies aimed at correlating phenotypic differences associated with ecological niche and geographic distribution with the genetic diversity of *A. butzleri* spp., this study reports the endowment of antibiotic and heavy metal resistance and virulence determinants of these shellfish-isolated strains. This leads to hypothesize a relatively high virulence of *A. butzleri* isolated from shellfish and prompt the need for a wider genomic analysis and for *in vitro* and *in vivo* studies of more strains isolated from this and other ecological niches, to unravel the mechanism of pathogenicity of this species, and the potential risk associated to their consumption.

Keywords: *Aliarcobacter butzleri*, *Arcobacter butzleri*, antibiotic and heavy metal resistance, virulence genes, genomics, food safety, emerging foodborne pathogen, shellfish

INTRODUCTION

The current validated taxonomy places the *Arcobacter* genus within the *Campylobacteraceae* family (belonging to the class *Epsilonproteobacteria* of the phylum *Proteobacteria*) together with *Campylobacter* and *Sulfurospirillum* genera. Recently, based on a wide comparative genomic analysis, Waite et al. (2017) proposed the re-classification of the class *Epsilonproteobacteria* with the *Arcobacter* genus ascribed to the *Arcobacteraceae* fam. nov. (type genus: *Arcobacter*, order: *Campylobacteriales*, class: *Campylobacteriia*, class. nov., phylum: *Campylobacteraeota* phyl. nov.) (Waite et al., 2017, 2018).

Arcobacter spp. have a wide diversity of hosts and habitats, with water being one of the main routes of transmission (Collado and Figueras, 2011). *Arcobacter* spp. have been indeed detected in environmental water sources, including rivers, lakes, sewage and plankton (Kim et al., 2010; Levican et al., 2013; Park et al., 2016), marine, domestic (Merga et al., 2014), and drinking water (Jalava et al., 2014; Talay et al., 2016), water distribution pipes (Phillips, 2001), groundwater (Fong et al., 2007), and recreational water (Lee et al., 2012). Four species (namely, *A. butzleri*, *A. cryaerophilus*, *A. theaeus*, and *A. skirrowii*) have also been isolated in humans and animals (Banting and Figueras Salvat, 2017) and are able to cause human bacteraemia, endocarditis, peritonitis, gastroenteritis, and diarrhea (Jiang et al., 2010; Figueras et al., 2014; Arguello et al., 2015; Ferreira et al., 2016).

To date this genus includes 26 species (Ramees et al., 2017; Pérez-Cataluña et al., 2018a), which inhabit various ecological niches (Collado and Figueras, 2011; Ferreira et al., 2016). Recently, Pérez-Cataluña et al. (2018a) used a polyphasic approach to revisit the taxonomy of the genus. By setting specific cut-off values for each method (identity of 16S rRNA, genomic indexes such as ANI, AAI, and DDH, multilocus sequence analysis, etc.), they delimited genomic and phylogenetic groups and defined six different genera including *Aliarcobacter* gen. nov. This genus comprises seven species including also *Aliarcobacter* (*Al.*) *butzleri* comb. nov., whose type strain was confirmed to be LMG 10828^T (ATCC 49616^T; RM4018; Miller et al., 2007), and the species description as the one given by Vandamme et al. (1992) and Pérez-Cataluña et al. (2018a,b).

However, it must be considered that both the species description of *A. butzleri* and *Al. butzleri* comb. nov., were obtained by analyzing an exiguous number of *A. butzleri* strains (12 by Vandamme et al., 1992 and only two by Pérez-Cataluña et al., 2018a, respectively). Thus, it might be expected that the likely increasing availability of *A. butzleri* genomic sequences could lead this species to a further redefinition.

Recent outcomes and progress related to the pathogenicity of *A. butzleri* have led to the allocation of this species in the list of microbes considered a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002). *A. butzleri* is considered an emerging food-borne enteropathogen and, in recent years, has been associated with enteritis, severe diarrhea, bacteraemia, and septicemia in humans and enteritis, mastitis, abortion,

and stillbirth in cows, sheep, and pigs (Engberg et al., 2000; Lau et al., 2002; Collado and Figueras, 2011; Collado et al., 2014; Ferreira et al., 2014a,b, 2016; Figueras et al., 2014; Van den Abeele et al., 2014; Heimesaat et al., 2015; Gözl et al., 2016; Webb et al., 2016; Franz et al., 2018; Fusco et al., 2018; Flynn et al., 2019). Enteritis due to the ingestion of food contaminated with *Arcobacter* spp. can be self-limiting. Nevertheless, the severity and protraction of the symptoms might require an antibiotic treatment, which might be affected by the (multiple) antibiotic resistance of the strain, thus complicating the treat of the relevant infections. *A. butzleri* is the most prevalent species of this genus in meat products (chicken, pork, beef, lamb), milk, cheese, and shellfish (Figueras et al., 2011a,b; Patyal et al., 2011; Shah et al., 2012; Hausdorf et al., 2013; Lee and Choi, 2013; Rahimi, 2014; Ramees et al., 2014; Lehmann et al., 2015; Ferreira et al., 2016). The presence and persistence in these niches, also endowed by the ability to form biofilms (Ferreira et al., 2013; Girbau et al., 2017), favor its spread and transmission to shellfish and farm animal, and increase the risk associated with food consumption. Although few fragmented studies have been carried out to assess the occurrence of this species in shellfish, *A. butzleri* has been found as the most common species in bivalve molluscs (mussels, clams, oysters, etc.) (Nieva-Echevarria et al., 2013; Levican et al., 2014; Mottola et al., 2016; Leoni et al., 2017). This is most likely due to capture by the filter feeding process of bivalves and to a fecal contamination of the relevant environment, so that *Escherichia coli* has been proposed by Leoni et al. (2017) as an index organism for *A. butzleri* contamination in bivalve molluscs, leading to suggest that these shellfishes could be a reservoir of *A. butzleri* (Leoni et al., 2017). For this reason, consumption of contaminated shellfish, especially if raw or undercooked, which is still a widespread practise (Schauer Weissfeld, 2014), may be source of *A. butzleri* infections in humans. The ability to survive in different environments, the endowment of antibiotic resistance genes and virulence potential found by genomic approaches (Miller et al., 2007; Pérez-Cataluña et al., 2018a), as well as the genetic plasticity conferred by the presence of mobile elements, which allow the transfer of genes (Doudah et al., 2014), are important determinants for the evolution and the fitness of this and the other species of this genus.

As far as we know, extensive genome-based characterization of the species has been carried out only on two *A. butzleri* strains, namely, RM4018 (Culture collection n. LMG 10828^T), isolated from human feces, and ED-1 isolated from microbial fuel cells (Miller et al., 2007; Pérez-Cataluña et al., 2018a). Considering that contaminated shellfish may be source of *A. butzleri* infection and given that the prevalence and expression of putative virulence and antibiotic resistance genes within this species may vary with the source of the strain (Doudah et al., 2012; Ferreira et al., 2014b; Girbau et al., 2015; Zacharow et al., 2015a), herein we report the antibiotic susceptibility and genomic-based characterization of two *A. butzleri* strains isolated from shellfish, with particular reference to the genetic determinants of the above mentioned traits of pathogenicity.

MATERIALS AND METHODS

Strains and Culture Condition

Arcobacter butzleri (Ab) strains 6V and 55 were isolated on 2016 from clams (*Tapes philippinarum*) (Mottola et al., 2016) and mussels (*Mytilus galloprovincialis*) obtained from local fish market in the Apulia region (Italy). These strains were previously identified and typed by MLST (Mottola et al., 2016; Mottola, 2017). Allelic profiles and sequences are available on the *Arcobacter* MLST database¹ under the ID numbers 717 (Ab 6V) and 839 (Ab 55).

Pure cultures were isolated and maintained in the microbial collection of the Institute of Sciences of Food Production, CNR, Bari². Bacterial strains were maintained at -80°C as pure stock cultures in Brain Heart Infusion (BHI) broth (Oxoid S.p.A., Rodano, Milan, Italy) supplemented with glycerol (30% vol/vol). Cultures were streaked on Agar blood plates (Oxoid, Milan, Italy) and grown at 37°C for 48 h. Working cultures were obtained growing a single colony in 20 mL of BHI broth with 0.6% yeast extract (BHI-YE), at 37°C for 48 h.

Genome Sequencing and Assembly

DNA isolation was performed by using the Wizard® Genomic DNA Purification Kit (Promega), as previously described by Ercolini et al. (2005). The integrity, purity, and quantity of DNA were assessed as previously described by Fusco et al. (2011), by agarose gel electrophoresis, by NanoDrop-2000 (Thermo Fisher Scientific, Wilmington, DE, United States), and by Qubit 3.0 fluorometer (Life Technologies). DNA was then subjected to whole genome shotgun sequencing using the Ion S5™ library preparation workflow (Thermo Fisher Scientific, Waltham, MA, United States); 400 bp mate-paired reads were generated on the Ion S5™ System (Thermo Fisher Scientific). Duplicate reads were removed by FilterDuplicates (v5.0.0.0) Ionplugin. *De novo* assembly was performed by AssemblerSpades (v.5.0) Ionplugin™.

Bioinformatic Methods

Genes were predicted and annotated using PROKKA pipeline implemented in the Galaxy platform (Galaxy Tool Version 1.0.0; Afgan et al., 2016). The predicted proteins were submitted to the PFAM annotator tool within the Galaxy platform in order to predict the pfam domains. Protein ID used in the manuscript indicated those obtained by NCBI (National Center for Biotechnology Information) Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016).

Predicted proteins were assigned to Clusters of Orthologous Groups (COG) functional categories by Web CD-Search Tool (Marchler-Bauer et al., 2017) using an Expected value threshold of 0.01. COG ID were then manually mapped into functional categories³.

All the proteins sequences used in this study were retrieved from GenBank (NCBI). The homology-based relationship

of Ab 55 and Ab 6V predicted proteins toward selected proteins was determined by BLASTP algorithm on the NCBI site⁴. Gene models were manually determined, and clustering and orientation were subsequently deduced for the closely linked genes.

Antibiotic resistance genes were predicted by BLASTP search against the Antibiotic Resistance genes Database (ARDB; Liu and Pop, 2009) and beta lactamase database (Naas et al., 2017). Genes associated with antibiotic resistance were also retrieved by keywords terms search within UniProtID entry list obtained by functional annotation.

Functional annotation, subsystem prediction, and metabolic reconstruction comparison were also performed using the RAST server (Aziz et al., 2008). Genes involved in the mechanism of resistance to heavy metals were retrieved by Poole (2017) and used as queries for BLASTP search against Ab 55 and Ab 6V proteomes.

Genetic divergence was calculated by the ANI/AAI calculator (Goris et al., 2007; Rodriguez-R and Konstantinidis, 2016) which estimates the average nucleotide/aminoacid identity (ANI/AAI) using both best hits (one-way ANI) and reciprocal best hits (two-way ANI) between genomic datasets. The Genome-to-Genome Distance Calculator (GGDC) (Meier-Kolthoff et al., 2013, 2014) web service was used to report DDH for the accurate delineation of prokaryotic subspecies and to calculate differences in G+C genomic content (available at ggdc.dsmz.de). Formula 2 alone was used for analysis, providing an estimation of DDH independent from genome lengths, as recommended by the authors of GGDC for use with any incomplete genomes (Auch et al., 2010; Meier-Kolthoff et al., 2013).

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility tests for *A. butzleri* isolates were performed by disk diffusion and broth microdilution methods. The disk diffusion test was performed as described by Rathlavath et al. (2017) with modifications. Briefly, *A. butzleri* isolates were grown in 20 mL of BHI broth (Oxoid, United Kingdom) amended with 0.6% yeast extract (YE) (Biolife srl, Italy) under static condition for 48 h at 37°C and then subcultured at 1% in BHI-YE broth and incubated at 37°C for 48 h. Microbial cells were recovered after centrifugation ($16,000 \times g$ for 6 min), washed in sterile 0.9% NaCl solution, adjusting optical density (600 nm) to 0.5. One hundred microliters of this cell suspension were then plated on 4 mm thick cation adjusted Muller Hinton agar (MHIIA, Liofilchem, Italy).

Antibiotic disks, soaked with ampicillin (10 µg/disk), cefotaxime (30 µg/disk), chloramphenicol (30 µg/disk), ciprofloxacin (5 µg/disk), erythromycin (15 µg/disk), gentamicin (10 µg/disk), kanamycin (30 µg/disk), nalidixic acid (30 µg/disk), streptomycin (10 µg/disk), tetracycline (30 µg/disk), vancomycin (30 µg/disk), and penicillin G (10 units/disk) (Biolab Zrt., Hungary), were placed onto inoculated plates and incubated at 37°C under microaerophilic atmosphere (CampyGen™ Compact, Oxoid, United Kingdom),

¹ <https://pubmlst.org/arcobacter>

² www.ispa.cnr.it/Collection

³ <https://www.ncbi.nlm.nih.gov/COG/>

⁴ <http://blast.ncbi.nlm.nih.gov/Blast.cgi>

as recommended by BCCM/LMG Bacteria Collection⁵ for *A. butzleri* type strain LMG10828 (RM4018). After 48 h of incubation, inhibition zone diameters were recorded.

For those antibiotics which the tested *A. butzleri* strains did not provide inhibition zone at all, the minimal inhibitory concentration (MIC) was calculated by broth microdilution method as described by Riesenberget al. (2017). After 48 h incubation at 37°C, microtitre plates were read spectrophotometrically at 600 nm using Varioskan Flash (Thermo Fisher Scientific, United States). To determine minimal bactericidal concentration (MBC), 10 µl of broth from three replicate of all wells without microbial growth was combined in a single sample spotted on MHIIA (Liofilchem, Italy) and incubated as described above up to 72 h. Since no breakpoint values are available for *Arcobacter* spp., classification of strains as susceptible (S), resistant (R), or intermediate (I) was defined according to zone diameter and MIC interpretive standards for *Staphylococcus* spp. (erythromycin, penicillin, and vancomycin) and *Enterobacteriaceae* (ampicillin, gentamicin, cefotaxime, ciprofloxacin, tetracycline, chloramphenicol, nalidixic acid, kanamycin, and streptomycin) (CLSI, 2015), as also reported by Elmali and Can (2017).

The *A. butzleri* LMG 10828^T (RM4018) strain was used for comparison purposes.

RESULTS AND DISCUSSION

General Features of *A. butzleri* 6V and 55 Genomes

Ab 55 and *Ab* 6V genomes were sequenced using a whole genome shotgun approach on an Ion S5TM platform (Thermo Fisher Scientific) generating around 671,363 and 596,333 reads with a median length of 317 and 320 bp, respectively (Table 1). Genomes were assembled using the Spades v5.0 software for a

total of 32 and 46 large contigs (>500 bp) and a GC% of 26.79 and 26.85, respectively. The overall contiguity of the assembly is good, with a N50 of 211 and 129 kbp for *Ab* 55 and *Ab* 6V, respectively; the longest assembled fragment is 403 kbp in length for *Ab* 55 and 251 kbp for *Ab* 6V (performed by QUAST, available at <http://quast.sourceforge.net/quast>) while the total length of the assembly was around of 2.3 Mb for both genomes. These Whole Genome Shotgun projects have been deposited at DDBJ/ENA/GenBank under the accessions QXMK00000000 (*Ab* 55) and QXNB00000000 (*Ab* 6V). The versions described in this paper are QXMK01000000 (*Ab* 55) and QXNB01000000 (*Ab* 6V).

Genomic Analysis

The *in silico* MLST of the housekeeping genes retrieved from genomic sequences, confirmed *in vitro* results achieved by Mottola (2017), revealing that *Ab* 55 and *Ab* 6V belong to two novel sequence types, namely, ST675 and ST537, respectively, as they harbor 6/7 and 3/7 new alleles, respectively (Table 2).

Both *Ab* 6V and *Ab* 55 16S rRNA gene sequences show 100% identity with the type strain *Ab* RM4018 (Miller et al., 2007). ANI, AAI, and DDH analyses were performed with 22 strains within the *Arcobacter* group (Supplementary Table S1). *Campylobacter jejuni* subsp. *jejuni* NCTC 11168 and *Helicobacter pylori* 26695 were included as outgroups.

Ab 55 and *Ab* 6V have 98.06% nucleotide identity (Supplementary Table S2) and are comprised in the clustering including all the *A. butzleri* species. According to ANI, the closest relatives for both *Ab* 55 and *Ab* 6V are *Ab* NCTC 12481 (97.80% and 97.81 ANI, respectively) and *Ab* RM4018 (97.79% and 97.80 ANI). The same clustering is obtained by using AAI (Supplementary Table S3) with 97.60% between *Ab* 55 and *Ab* 6V, and 97.67 and 97.36% with *Ab* RM4018, respectively. DDH analysis confirmed the clustering obtained by ANI and AAI analysis, with values of 83.50 between *Ab* 6V and *Ab* 55, 81.00% between *Ab* 6V and *Ab* RM4018, and 81.30% between *Ab* 55 and *Ab* NCTC 12481 (Supplementary Table S4). As proposed by Chun et al. (2018) and, more specifically for *Arcobacter* spp., by On et al. (2017), these ANI and DDH values are within the range suggested to include *Ab* 55 and *Ab* 6V into the *A. butzleri* species. Moreover, our results support those achieved by Pérez-Cataluña et al. (2018a). Therefore, *Ab* 55 and *Ab* 6V should be placed within the *Aliarcobacter* gen. nov. as *Al. butzleri* comb. nov. (Pérez-Cataluña et al., 2018a,b), while for the definition of subspecies, a phenotypic comparison should support the *in silico* analyses (On et al., 2017).

Protein Functional Classification

For *Ab* 55, 1173 UniProtKB AC/ID identifiers retrieved by PFAM annotator tools (Galaxy Tool Version 1.0.0) were successfully mapped to 1190 UniProtKB IDs (The UniProt Consortium, 2017). In *Ab* 55, the retrieved list included 33 genes associated with antibiotic resistance, including beta-lactamase, multidrug efflux pump, DNA gyrase, and resistance protein, and three with antibiotic biosynthesis related to bacteriocin, six associated with drug transmembrane transporter activity, 19 with virulence, four with hemolysis, and two with quorum sensing (*luxS* and *tqsA*).

⁵<http://bccm.belspo.be>

TABLE 1 | Summary of *A. butzleri* 55 and 6V genome sequencing and assembly results.

	<i>A. butzleri</i> 55	<i>A. butzleri</i> 6V
Total sequenced bases	212,850,706	190,979,223
Mean read length	317	320
Total length	2,330,339	2,303,554
Number of scaffolds	47	61
Largest contig	403,569	251,748
Number reads	671,363	596,833
N50 reads	211,847	129,283
Genome size	2,325,213	2,297,763
GC content	26.79	26.85
Predicted genes	2395	2338
CDS	2344	2289
tRNA	46	44
ncRNA	2	2
rRNA	1, 1, 1 (5S, 16S, 23S)	1, 1, 1 (5S, 16S, 23S)

TABLE 2 | Allelic profiles of *A. butzleri* isolates.

ID	Isolate	Species	Source	MLST							ST
				<i>aspA</i>	<i>atpA</i>	<i>glnA</i>	<i>gltA</i>	<i>glyA</i>	<i>pgm</i>	<i>tkt</i>	
717	6V	<i>Arcobacter butzleri</i>	clam	236	161	1	183	521	296	207	537
839	55	<i>Arcobacter butzleri</i>	mussel	332	8	1	166	685	367	292	675

Novel alleles and novel sequence type (ST) are indicated in bold.

For *Ab* 6V, 1189 out of 1189 UniProtKB AC/ID identifiers were mapped to 1207 UniProtKB. Based on their classification, we counted three genes associated with antibiotic biosynthesis, 32 related to antibiotic resistance, including beta-lactamase, multidrug efflux pump, DNA gyrase and resistance protein, six referred to drug transmembrane transporter activity, 22 associated with virulence, three with hemolysis, and two with quorum sensing (*luxS* and *tqsA*).

One of the few differences between these strains is the presence in *Ab* 6V of the hemolysin *tylC* gene which codes for a protein with a conserved protein/cyclin M (CNNM) transmembrane domain, while, in *Ab* 55, we found only a precursor of hemolysin C. In both genomes, we identified the RNA methyltransferase hemolysin A (*tylA*), which encodes for a 16S/23S rRNA (cytidine-2'-O)-methyltransferase that is considered a virulence factor in *H. pylori* infection and probably acts as a pore-forming toxin (Javadi and Katzenmeier, 2016).

Overall, 1551 for *Ab* 55 and 1592 for *Ab* 6V predicted genes were assigned to the COG classification (Figure 1). The few differences in the distribution of genes into clusters of COG functional categories across *Ab* 55 and *Ab* 6V genomes, which emerged from Figure 1, supported the limited functional variability among these two strains, even though isolated from different samples and in different harvest seasons (Mottola et al., 2016).

Among COG categories, the cluster signal transduction mechanisms represented the largest group for both organism (172 genes, 11.09% for *Ab* 55 and 187 genes, 11.75% for *Ab* 6V), followed by amino acid transport and metabolism cluster (147 genes, 9.48% for *Ab* 55 and 147 genes, 9.23% for *Ab* 6V), Cell wall/membrane/envelope biogenesis cluster (140 genes, 9.03% for *Ab* 55 and 147 genes, 9.23% for *Ab* 6V) and energy production and conversion cluster (133 genes, 8.58% for *Ab* 55 and 135 genes, 8.48% for *Ab* 6V). These findings suggest that our *Arcobacter* strains have a versatile sensory transduction system and that for energy and carbon mainly rely on amino acid catabolism rather than on sugar fermentation, which is consistent with the ecological niches they have been isolated from.

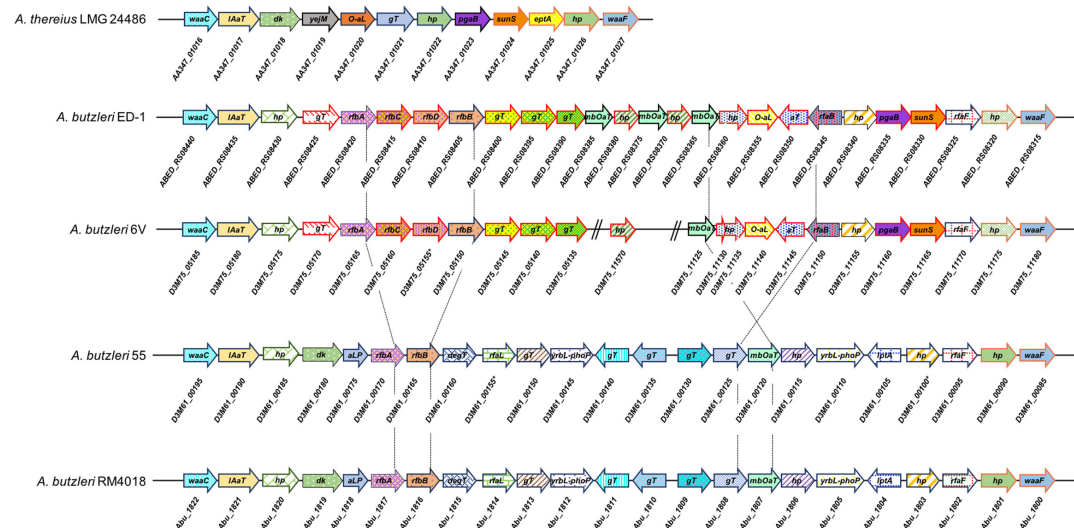
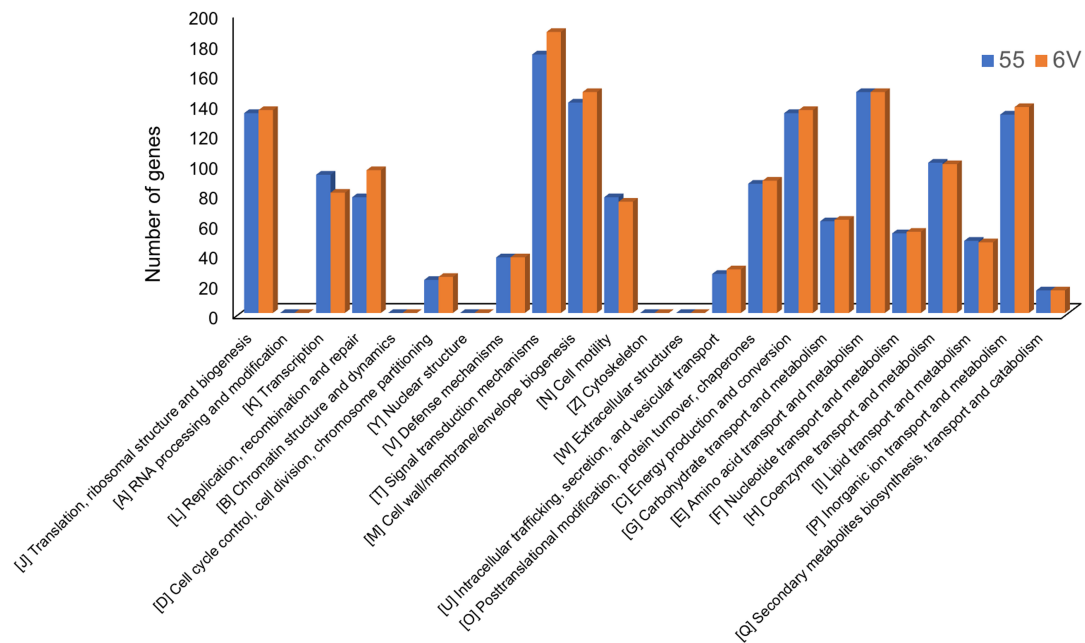
Virulence Determinants

The ability to adhere to various surfaces as well as chemotaxis, motility, and signal transduction play a pivotal role in the microbial survival and colonization of diverse ecological niches and can be involved in pathogenesis and antibiotic resistance (Richards et al., 2013; Chaban et al., 2015; Tiwari et al., 2017; Matilla and Krell, 2018).

In both genomes, we identified orthologues of *waaC* and *waaF* genes, which are described as virulence determinant in *A. theaeus* (Rovetto et al., 2017), but also in *Pseudomonas aeruginosa*, *E. coli*, *Klebsiella pneumonia*, and other *Campylobacteraceae* (Oldfield et al., 2002; DeLucia et al., 2011; Nilsson et al., 2018). *waaF* codes for a predicted ADP-heptose-LPS heptosyltransferase (*Ab* 55 D3M61_00085; *Ab* 6V D3M75_11180), involved in the biosynthesis of lipooligosaccharide (LOS); *waaC* codes for a lipopolysaccharide heptosyltransferase (*Ab* 55 D3M61_00195; *Ab* 6V D3M75_05185), which catalyzes the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds. As in *Ab* RM4018 (Miller et al., 2007), which it shares the same content and organization with, in *Ab* 55, *waaC* and *waaF* genes are closely located suggesting the presence of a genetic cluster, whose composition is, however, different from *A. theaeus* (Figure 2). In *Ab* 6V, the genetic locus comprised by *waaC* and *waaF* is similar to that of *Ab* ED-1 (26 genes) and contains several glycosyltransferases with no orthologues in *Ab* 55 and *Ab* RM4018. The outer genes of this locus are similar to *A. theaeus*. However, in *Ab* 6V, these genes are located in three different contigs, which made only provisional, although likely, the reconstruction of the organization of the entire locus.

Arcobacter butzleri, as the other *Arcobacter* species, is motile by means of one single polar flagellum (Miller et al., 2007). The bacterial flagellar motor is comprised by a core structure (inner membrane stator complexes MotA4B2 and the C-ring), a dedicated type III secretion system (T3SS) export apparatus, the inner membrane MS-ring and the P- and L-rings. These core components are conserved across bacterial genera. Nevertheless, the architecture of flagellar motors in *A. butzleri* has a diverse (Rossmann and Beeby, 2018), “intermediate” (Chaban et al., 2018) motor structure. According to the analysis performed by Chaban et al. (2018), we retrieved homologous proteins of the flagellar system in *Ab* 55 and *Ab* 6V genomes (Table 3). In both strains, loci share the same genomic content and most of the flagellar genes are located within the same genomic locus. As reported by Chaban et al. (2018), the *Arcobacter*-type motor accessory proteins did not contain homologues of the accessory proteins FlgP, FlgQ, FlgT, while we identified the homologue FlgO, an outer membrane protein required for flagellar motility in *Vibrio cholerae*, highly conserved in *Vibrio* spp. (Zhu et al., 2017).

In *Ab* 55, the flagellar biosynthetic protein FlpI (an integral membrane component) (Ferris and Minamino, 2006) was predicted as a pseudogene (frameshifted) (D3M61_02925), as the flagellar biosynthetic protein FlhB (a flagellar export component responsible for substrate specificity switching) (Minamino and



in both the genomic loci which might have affected the base calling of the sequencing.

TABLE 3 | Flagellum proteins in *Arcobacter butzleri* genomes.

			Organism	<i>A. butzleri</i> RM4018	<i>A. butzleri</i> 55	<i>A. butzleri</i> 6V
			Whole genome record	NC_009850.1	QXMK00000000	QXNB00000000
			Class	Epsilon	Epsilon	Epsilon
				GenBank accession	Protein ID	
T3SS	Flagellar biosynthesis protein FlhA	FlhA		ABV68160.1	D3M61_10075	D3M75_09250
	Flagellar biosynthetic protein FlhB	FlhB		ABV68164.1	D3M61_10055	D3M75_09230*
	Flagellum-specific ATP synthase	FliI		ABV68162.1	D3M61_10065	D3M75_09240
	Flagellar biosynthetic protein FliP	FliP		ABV67255.1	D3M61_02925*	D3M75_03525
	Flagellar biosynthetic protein FliQ	FliQ		ABV66480.1	D3M61_08725	D3M75_07160
	Flagellar biosynthesis protein FliR	FliR		ABV68165.1	D3M61_10050	D3M75_09225
C-ring	Flagellar motor switch protein FliG	FliG		ABV68184.1	D3M61_09955	D3M75_09130
	Flagellar motor switch protein FliM	FliM		ABV66477.1	D3M61_08740	D3M75_07145
	Flagellar motor switch protein FliN	FliN		ABV68182.1	D3M61_09965	D3M75_09140
MS-ring	Flagellar M-ring protein	FliF		ABV68185.1	D3M61_09950	D3M75_09125
Proximal rod	Flagellar hook-basal body protein FliE	FliE		ABV68168.1	D3M61_04895	D3M75_09210
	Flagellar basal body rod protein FlgB	FlgB		ABV68186.1	D3M61_09945	D3M75_09120
	Flagellar basal-body rod protein FlgC	FlgC		ABV68167.1	D3M61_10040	D3M75_09215
P-ring	Flagellar hook-associated protein	FlgI		A8ERA4.1	D3M61_08735	D3M75_07150
L-ring	Flagellar L-ring protein precursor	FlgH		ABV66485.1	D3M61_08700	D3M75_07185
Campy-specific	Flagellar lipoprotein	FlgP		n/a	n/a	n/a
	Hypothetical protein	FlgQ		n/a	n/a	n/a
	Tetratricopeptide repeat protein	PflA		WP_012147853.1	D3M61_10060	D3M75_09235
	Tetratricopeptide repeat protein	PflB		WP_012147861.1	D3M61_09990	D3M75_09165
Vibrio-specific	Flagellar assembly protein	FlgT		n/a	n/a	n/a
	Hypothetical protein	FlgO		WP_004511148.1	D3M61_08900	D3M75_07635

*Pseudogene (frameshifted).

Both genomes harbor the genes *cj1349* and *cadF* (coding for fibronectin-binding proteins CadF and Cj1349), *mviN* (encoding a protein essential for the peptidoglycan biosynthesis) and *pldA* (encoding phospholipase A), the *gyrA*, *iroE*, and *irgA* (iron-regulating outer membrane protein), *ciaB* (encoding the *C. jejuni* invasion antigen B) genes and the hemolysin gene *tylA*, while only *Ab* 55 harbors *hecA* [a member of the filamentous hemagglutinin (FHA) family], and *hecB* (encoding a hemolysin activation protein), as occurs in the *Ab* RM4018 (Miller et al., 2007).

Cia proteins (including CiaB, CiaC, and CiaD) have been suggested to be involved in promoting internalization of *C. jejuni* for host invasion and require a full-length flagellar filament for proper secretion (Chaban et al., 2015).

Several authors have screened *A. butzleri* strains for the presence of virulence genes such as *ciaB*, *cadF*, *cj1349*, *hecA*, and *irgA*, finding diverse prevalence of these genes in isolates from the same or different ecological niches (Collado et al., 2014; Ferreira et al., 2017; Rathlavath et al., 2017; Vicente-Martins et al., 2018). However, nucleotide sequence heterogeneity as well as PCR biases may provide false negative results thus underestimation of the actual prevalence of these genes. These drawbacks may be overcome by a genomic-based assessment, which, moreover, may allow the detection of novel (acquired) virulence genes.

Indeed, apart from the above-mentioned genes, recognized as putative virulence determinants in *A. butzleri* (Miller et al., 2007), we identified other genes coding for additional

virulence associated protein in *Ab* 55 and *Ab* 6V genomes. Among these and present in *Ab* 55, *Ab* 6V, and *Ab* RM4018, we distinguished a DNA binding protein of the virulence factor B family, which contributes to the expression of virulence factors and to pathogenicity in *Staphylococcus aureus* (Matsumoto et al., 2010; Junecko et al., 2012), a VOC family virulence protein (glyoxalase/bleomycin resistance protein/dioxygenase superfamily domain), a conserved virulence factor B (DNA binding protein), and VirF of the AraC family of transcriptional regulators.

Only in *Ab* 55, with no orthologs in *Ab* 6V and *Ab* RM4018, we identified a virulence associated protein VirE (D3M61_07785), which has an 87% identity with a hypothetical protein of *Ab* L353 (WP_080952707.1); this was the only hit retrieved within *Arcobacter* genus (taxid: 28196) by BLASTP search in the NCBI database, while other results indicated identity of about 32% with a hypothetical protein of *Dyella* sp. 4G-K06 (WP_115495989.1) and with a primase from the *Escherichia* phage vB_EcoM-ep3. This sequence was also compared to metagenomic sequences comprised in the microbiome database MGNIFY (EMBL-EBI®; Mitchell et al., 2017) by BlastP analysis. Analysis showed 91% of identity with metagenomic sequence of a sample retrieved from Charlotte area wastewater treatment plants (North Carolina, United States). InterPro functional classification (Finn et al., 2017) assigned the protein the Virulence-associated E (IPR007936) family membership with a D-loop motif (Pfam:PF05272.5) and a related COG: 5545

Mobilome: prophage transposone category. The genomic locus in which the gene is located also comprises several hypothetical proteins, one Prophage CP4-57 integrase, several tRNA, one site-specific tyrosine recombinase XerC, putative DNA-invertase from lambdoid prophage Rac. The structure of this genomic locus is compatible with the presence of a genomic island (Juhas et al., 2009) and suggests the acquisition of this virulence element by a mechanism of horizontal gene transfer.

Furthermore, in *Ab* 6V genome, we also found one virulence sensor protein BvgS precursor and one virulence sensor histidine kinase PhoQ, which both have orthologues in *Ab* RM4018 (WP_012012740.1 and WP_012012731.1, respectively) but not in *Ab* 55.

Unique to *Ab* 6V, we identified two putative hemolysin activation/secretion proteins (D3M75_11460 and D3M75_03300), which have orthologues in *A. butzleri* ED-1, with a ShlB/FhaC/HecB family hemolysin secretion/activation domain.

All together these findings lead to hypothesize a relatively high virulence of *A. butzleri* isolated from shellfish and prompt the need for a genomic analysis of more strains from this ecological niche, as well as for *in vitro* and *in vivo* studies, to unravel the mechanism of pathogenicity of this species.

Antibiotic Susceptibility and Genetic Determinants

As shown in **Table 4**, both genomes harbor a wide endowment of genes involved in antibiotic resistance, including transporter, efflux pump, multidrug resistance protein and methyltransferase, although with some differences. As example, *Ab* 55 harbors a protein predicted as a bifunctional polymyxin resistance protein ArnA (D3M61_11465), which is not present neither in *Ab* 6V nor in the *A. butzleri* RM4018. BLASTP analysis retrieved as best hit a hypothetical protein of *Campylobacter hyointestinalis* (WP_111949105.1).

The UDP-4-amino-4-deoxy-L-arabinose-oxoglutarate aminotransferase *arnB* gene was instead identified in both *Ab* 55 and *Ab* 6V, while it is not present in *A. butzleri* RM4018. The coded protein belongs to the DegT/DnrJ/EryC1/StrS aminotransferase family protein, and it is required for resistance to polymyxin and cationic antimicrobial peptides (Lee and Sousa, 2014).

The predicted serine/threonine-protein kinase HipA belonging to the type II toxin-antitoxin system was only retrieved in *Ab* 6V predicted proteome (D3M75_03575), and it is involved in the methicillin resistance. Only in *Ab* 55, we identified the mRNA interferase toxin RelE (D3M61_04745), which is involved in ciprofloxacin and ampicillin resistance in *E. coli* (Harms et al., 2017).

Table 5 shows metal resistance genes annotated in *Ab* 55 and *Ab* 6V genomes.

Observational and experimental studies have highlighted that exposure of bacteria to heavy metals (mainly zinc and copper), mainly due to anthropogenic environmental contamination, can induce or co-select resistance to them and to one or more antibiotics. In particular, resistance may be induced by metals

(i) via co-selection resistance, when different genes coding for antibiotic and metal resistance share a close location (as in mobile genetic elements, such as integrin, plasmid, or transposon), (ii) via cross-selection, when the same genetic element encodes for both antibiotic and metal resistance, and (iii) via co-regulation, when antibiotic and metal resistance genes share the same regulatory system (Lupo et al., 2012; Seiler and Berendonk, 2012; Chenia and Jacobs, 2017; Li et al., 2017; Poole, 2017; Yu et al., 2017; Wu et al., 2018). Nevertheless, as far as we know, only Otth et al. (2005) investigated susceptibility of *A. butzleri* to heavy metals, finding that all the 50 tested strains were susceptible to mercury, silver, and chrome salts, whereas all were resistant to molybdenum, manganese, nickel, cobalt, lead, and iron.

Metal resistance genes share the same location in both *Ab* 55 and *Ab* 6V genomes. *cadA* is close to genes coding for the ferrous iron transport protein FeoA and FeoB, outer membrane efflux proteins, transcriptional regulators, and several tRNA genes, suggesting the presence of a genomic island (Juhas et al., 2009). *czcB* is close to gene coding for the multidrug resistance protein MstB, one permease and the gene coding for the sensor protein ZraS. One *copA* gene is close to the gene coding for outer membrane porin precursor and the copper sensing transcriptional repressor CsoR, while the other *copA* is close to the ferrous iron transport proteins FeoA and FeoB. *cusS* is close to the transcriptional activator protein *czcR*, *macB*, and several chaperonine, whereas *arsC* is located near to genes coding for plasmid stabilization system protein and genes coding for flagellum biosynthesis (*fliK* and *fliH*), and cell division protein FtsA and FtsZ. *modA* gene is located within a hypothetical operon including a transcriptional regulator GltR, a gene coding for a molybdenum-pterin binding protein, the regulator ModE, and the transport system permease protein ModB. *copZ* is close to *merT* gene, coding for a mercuric transport protein, a gene coding for a natural resistance-associated macrophage protein, a transcriptional regulatory protein ZraR and a sensor protein FixL.

The antimicrobial susceptibility of *Arcobacter* spp. isolated from various ecological niches has been investigated by several authors (Kabeya et al., 2004; Kayman et al., 2012; Ferreira et al., 2013, 2017; Scanlon et al., 2013; Collado et al., 2014; Rahimi, 2014; Yesilmen et al., 2014; Zacharow et al., 2015b; Aski et al., 2016; Van den Abeele et al., 2016; Elmali and Can, 2017; Rathlavath et al., 2017; Šilha et al., 2017; Soma et al., 2017; Vicente-Martins et al., 2018), but the lack of standardized protocols and interpretive criteria for the antimicrobial susceptibility testing (AST) of *Arcobacter* spp. is the major limitation for a comparable and univocal evaluation of antimicrobial resistance and susceptibility for these microorganisms (Ferreira et al., 2016). Results of the disk diffusion test are shown in **Table 6**, whereas in **Table 7**, are reported the MICs and MBCs assessed by broth microdilution method (section “Antimicrobial Susceptibility Testing”). The antimicrobial susceptibility pattern of *Ab* type strain LMG 10828^T (ATCC 49616, RM4018), used as a reference strain for the AST in our study, was comparable to that reported by Miller et al. (2007), for 10 out of 12 tested antibiotics, i.e., gentamicin, kanamycin, streptomycin, ciprofloxacin, chloramphenicol, erythromycin,

TABLE 4 | Antibiotic resistance genes in *Ab* 55 and *Ab* 6V genomes.

Gene name	Product	UniProt KB	Present in
<i>arnA</i>	Bifunctional polymyxin resistance protein ArnA	O52325	<i>Ab</i> 55
<i>arnB</i>	UDP-4-amino-4-deoxy-L-arabinose-oxoglutarate (polymyxin resistance)	Q8ZNF3	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>bcr</i>	Bicyclomycin resistance protein	P28246	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>bepD</i>	Efflux pump periplasmic linker BepD precursor	Q8G2M7	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>bepE</i>	Efflux pump membrane transporter BepE	Q8G2M6	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>bla</i>	Beta-lactamase OXA-15 precursor	Q51574	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>cat3</i>	Chloramphenicol acetyltransferase 3	P00484	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>eptA</i>	Phosphoethanolamine transferase EptA (polymyxin resistance)	P30845	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>fsr</i>	Fosmidomycin resistance protein	P52067	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>hcpC</i>	Putative beta-lactamase HcpC precursor	O25728	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>hlpA</i>	Serine/threonine-protein kinase HipA (methicillin resistance)	P23874	<i>Ab</i> 6V
<i>ileS</i>	Isoleucine-tRNA ligase (mupirocine resistance)	P00956	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>lmrA</i>	Multidrug resistance ABC transporter ATP-binding and permease protein	Q9CHL8	<i>Ab</i> 6V
-	putative metallo-hydrolase (metallo Beta-lactamase)	Q5XD24	<i>Ab</i> 55
<i>macA</i>	Macrolide export protein MacA	P75830	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>macB</i>	Macrolide export ATP-binding/permease protein MacB	P75831	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>bla2</i>	Beta-lactamase 2 precursor	P10425	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>mdtB</i>	Multidrug resistance protein MdtB	B7NCB1	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>mdtE</i>	Multidrug resistance protein MdtE precursor	P37636	<i>Ab</i> 55
<i>mexA</i>	Multidrug resistance protein MexA	P52477	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>mexB</i>	Multidrug resistance protein MexB	P52002	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>oprM</i>	Outer membrane protein OprM	Q51487	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>pbp</i>	Beta-lactam-inducible penicillin-binding protein	P07944	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>relE</i>	mRNA interferase toxin RelE (ciprofloxacin and ampicillin)	P0C077	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>rlmN</i>	Putative dual-specificity RNA methyltransferase RlmN (ribosome target antibiotics)	Q7A600	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>sttH</i>	Streptothricin hydrolase	Q1MW86	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>tetA</i>	Tetracycline resistance protein, class C	P02981	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>uppP</i>	Undecaprenyl-diphosphatase (bacitracin resistance)	P60932	<i>Ab</i> 55 – <i>Ab</i> 6V
<i>wbpD</i>	Group B chloramphenicol acetyltransferase	G3XD01	<i>Ab</i> 55 – <i>Ab</i> 6V

TABLE 5 | Metal resistance genes in *Ab* 55 and *Ab* 6V genomes.

Genes	Product	Metal resistance	Present in
<i>cadA</i>	Cadmium, zinc, and cobalt-transporting ATPase	Cadmium, zinc, cobalt	<i>Ab</i> 6V – <i>Ab</i> 55
<i>czcB</i>	Cobalt-zinc-cadmium resistance protein CzcB	Cadmium, zinc, cobalt	<i>Ab</i> 6V
<i>czcD</i>	Cadmium, cobalt and zinc/H(+)-K(+) antiporter	Cadmium, zinc, cobalt	<i>Ab</i> 6V – <i>Ab</i> 55
<i>copA_1</i>	Copper-exporting P-type ATPase A	Copper	<i>Ab</i> 6V – <i>Ab</i> 55
<i>copA_2</i>	Putative copper-importing P-type ATPase A	Copper	<i>Ab</i> 6V – <i>Ab</i> 55
<i>cusS</i>	Sensor kinase CusS	Copper	<i>Ab</i> 6V – <i>Ab</i> 55
Additional genes			
<i>arsC</i>	Arsenate reductase	Arsenic	<i>Ab</i> 6V – <i>Ab</i> 55
<i>modA</i>	Molybdate-binding periplasmic protein precursor	Molybdate chromate	<i>Ab</i> 6V – <i>Ab</i> 55
<i>copZ</i>	Copper chaperone CopZ	Copper	<i>Ab</i> 6V – <i>Ab</i> 55

vancomycin, ampicillin, cefotaxime, and penicillin G. Slight differences were related to susceptibility and resistance toward tetracycline and nalidixic acid, reported by Miller et al. (2007);

in particular, in our test, the type strain resulted intermediate resistant toward both antibiotics. As concerns MIC values, the type strain *Ab* LMG10828 showed results consistent with those find by Riesenberger et al. (2017). Additionally, in our study, using a wider range of concentrations for penicillin and vancomycin, we were able to assess MIC values for *Ab* LMG10828^T toward these two antibiotics (i.e., 128 and 2048 µg/ml, respectively) that were previously reported by Riesenberger et al. (2017) as ≥64 µg/ml. According to MIC interpretive standards, *A. butzleri* LMG 10828^T, *Ab* 55 and *Ab* 6V have been classified as resistant toward vancomycin and the three β-lactam antibiotics used in this study, i.e., cefotaxime (β-lactam cephalosporin), ampicillin, and penicillin G (β-lactam penicillins), confirming the results obtained by disk diffusion test (Tables 6, 7).

For *A. butzleri* type strain, *Ab* 55 and *Ab* 6V, the highest MIC and MBC values were observed for vancomycin, which were ≥2048 µg/ml (Table 7).

As recently reviewed by Ahmed and Baptiste (2018) in enterococci, molecular basis for vancomycin resistance phenotypes are determined by the presence of the vancomycin resistance (Van) operons (described as vanA, -B, -C, -D, -E, -G, -L, -M, and N) which may be located on the chromosome or on

TABLE 6 | Disk diffusion susceptibility test for *A. butzleri*.

Class	Antibiotics	<i>A. butzleri</i> strain		
		LMG 10828	55	6V
β -Lactams	Ampicillin 10 μ g/disk	R*	R*	R*
	Penicillin G 10 units/disk	R*	R*	R*
	Cefotaxime 30 μ g/disk	R*	R*	R*
Glycopeptides	Vancomycin 30 μ g/disk	R*	R*	R*
Quinolones	Ciprofloxacin 5 μ g/disk	S	S	S
	Nalidixic acid 30 μ g/disk	I	R	I
Aminoglycosides	Gentamicin 10 μ g/disk	S	S	S
	Kanamycin 30 μ g/disk	S	S	S
	Streptomycin 10 μ g/disk	S	S	S
Phenicol	Chloramphenicol 30 μ g/disk	R	I	I
Tetracycline	Tetracycline 30 μ g/disk	I	R	R
Macrolide	Erythromycin 15 μ g/disk	I	R	R

Classification as S (susceptible), I (intermediate), and R (resistant) was carried out according to zone diameter interpretive standards for *Staphylococcus* spp. (erythromycin and penicillin G) and *Enterobacteriaceae* (ampicillin, gentamicin, cefotaxime, ciprofloxacin, tetracycline, chloramphenicol, nalidixic acid, kanamycin, and streptomycin) (CLSI, 2015; Elmali and Can, 2017). To our knowledge, no vancomycin reference interpretive criteria are reported for *A. butzleri*. *No inhibition zone detected.

a plasmid. No element of the described operons was found in *Ab* 55 and *Ab* 6V genomes, as well as in *Ab* RM4018. However, the resistance of Gram-negative bacteria toward vancomycin may be intrinsic due to the inability of glycopeptides [molecules with high molecular weight (1450–1500 Da) and size] to pass through porins, which govern the movement of hydrophilic molecules across their outer membrane (Quintiliani and Courvalin, 1995) to reach their site of action, i.e., the cell wall (Nicolosi et al., 2010). Indeed, 100% of the *A. butzleri* isolates tested by Aski et al. (2016), Rathlavath et al. (2017), and Soma et al. (2017) were resistant to vancomycin.

Both strains are resistant to all the β -lactam antibiotics tested (ampicillin, penicillin, and cefotaxime) (Table 6). The resistance of *A. butzleri* isolates to β -lactams is widespread in seafood and water sources (Collado et al., 2014; Rathlavath et al., 2017; Šilha et al., 2017) as well as in other environments (Kayman et al., 2012; Ferreira et al., 2013; Rahimi, 2014; Yesilmen et al., 2014; Zacharow et al., 2015b; Aski et al., 2016; Van den Abeele et al., 2016; Elmali and Can, 2017; Vicente-Martins et al., 2018). Rathlavath et al. (2017) found that on 40 *A. butzleri* isolated

from shellfish, 100% resulted resistant to cefotaxime and 70% to ampicillin, while among 81 *A. butzleri* isolated from fish, 98.7% was resistant to cefotaxime and 72.8% to ampicillin. Moreover, of 26 *A. butzleri* isolated from coastal water, 100% was resistant to cefotaxime and 73% to ampicillin (Rathlavath et al., 2017). Šilha et al. (2017) found that 94.4% of 18 *A. butzleri* isolated from water sources were resistant to ampicillin and 100% were resistant to penicillin G, while Collado et al. (2014) found that 45.2% of 62 *A. butzleri* isolated from bivalve molluscs were resistant to ampicillin.

The β -lactam resistance is generally due to the combined effects of the presence and activity of β -lactamase genes, of the binding to targets (penicillin-binding proteins) and, in Gram-negative bacteria, of the outer-membrane permeability (Georgopapadakou, 1993). In the genomes of *Ab* 55 and *Ab* 6V, we identified three putative β -lactamases orthologues to that of *A. butzleri* RM4018 (Miller et al., 2007) [MBL fold metallo-hydrolase D3M61_00510 and D3M61_04375 (*Ab* 55), D3M75_05505 and D3M75_08430 (*Ab* 6V); class D beta-lactamase D3M61_10735, D3M75_10375 (*Ab* 6V)] as well as penicillin binding proteins (*Ab* 6V D3M75_03520, D3M75_10675, D3M75_10720; *Ab* 55: D3M61_02930, D3M61_10940, D3M61_10985).

Furthermore, in the genomes of both strains, we retrieved the *lrgAB* operon, which modulates penicillin tolerance in *Staphylococcus* (Bayles, 2000; Groicher et al., 2000) and was previously suggested as the β -lactam resistance enhancer in *A. butzleri* RM4018 (Miller et al., 2007).

Figure 3 shows the multialignment of beta-lactamase protein in *Ab* 55 and *Ab* 6V and other *Arcobacter* species. In *Ab* 6V the predicted protein (D3M75_10375) is truncated at N-terminal due to a nucleotide mutation in the genomic locus which leads to a premature stop codon. The DNA sequence translated by EMBOSS® Sixpack shows that changing the reading frame would recover the entire protein, identical to *Ab* 55 D3M61_10735. The sequence of *Ab* 55 D3M61_10735 share 100% of identity with OXA-464, which differs with the β -lactamase of the type strain *A. butzleri* RM4018 for a glutamine instead of glutamic acid in position 177.

Ab 55 and *Ab* 6V as well as the *Ab* type strain LMG 10828 (RM4018) were sensitive to the three aminoglycoside antibiotics used in this study namely gentamicin, kanamycin, and streptomycin (Table 6). Our results are in agreement with those obtained by Rathlavath et al. (2017) on the 147

TABLE 7 | Cefotaxime, ampicillin, penicillin G, and vancomycin MIC and MBC values for *A. butzleri*.

<i>A. butzleri</i> strain	β -Lactams						Glycopeptide	
	Cefotaxime μ g/ml		Ampicillin μ g/ml		Penicillin G μ g/ml		Vancomycin μ g/ml	
	MIC ^a	MBC ^b	MIC ^a	MBC ^b	MIC ^a	MBC ^b	MIC ^a	MBC ^b
LMG 10828	16	16	32	32	128	256	2, 048	>2, 048
55	128	128	256	256	1, 024	1, 024	>2, 048	>2, 048
6V	64	64	64	64	256	256	2, 048	>2, 048

^aMinimal inhibitory concentration. ^bMinimal bactericidal concentration.

Ab 55	1	MNKKIKLIFILIFSINLFANDVELENLFKKYQVEGTLVLESINTKKVDIYNEKRANTSFS
Ab 6V	1	MNKKIKLIFILIFSINLFANDVELENLFKKYQVEGTLVLESINTKKVDIYNEKRANTSFS
OXA-464	1	MNKKIKLIFILIFSINLFANDVELENLFKKYQVEGTLVLESINTKKVDIYNEKRANTSFS
OXA-490	1	MNKKIKLIFILIFSINLFANDVELENLFKKYQVEGTLVLESINTKKVDIYNEKRANTSFS
Ab RM4018	1	MNKKIKLIFILIFSINLFANDVELENLFKKYQVEGTLVLESINTKKVDIYNEKRANTSFS
Ab S2_012_000_R	1	MNKKIKLIFILIFSINLFANDVELENLFKKYQVEGTLVLESINTKKVDIYNEKRANTSFS
OXA-491	1	MNKKIKLIFILIFSINLFANDVELENLFKKYQVEGTLVLESINTKKVDIYNEKRANTSFS
Ab L348	1	MNKKIKLIFILIFSINLFANDVELENLFKKYQVEGTLVLESINTKKVDIYNEKRANTSFS
Ab L353	1	MNKKIKLIFILIFSINLFANDVELENLFKKYQVEGTLVLESINTKKVDIYNEKRANTSFS
Ab ED-1	1	MNKKIKLIFILIFSINLFANDVELENLFKKYQVEGTLVLESINTKKVDIYNEKRANTSFS
Ab L355	1	MNKKIKLIFILIFSINLFANDVELENLFKKYQVEGTLVLESINTKKVDIYNEKRANTSFS
Ab JV22	1	MNKKIKLIFILIFSINLFANDVELENLFKKYQVEGTLVLESINTKKVDIYNEKRANTSFS
Ab L349	1	MNKKIKLIFILIFSINLFANDVELENLFKKYQVEGTLVLESINTKKVDIYNEKRANTSFS
Ab L.	1	MNKKIKLIFILIFSINLFANDVELENLFKKYQVEGTLVLESINTKKVDIYNEKRANTSFS
As_CCUG 10374	1	MNKKIKLIFILIFSINLFANDVELENLFKKYQVEGTLVLESINTKKVDIYNEKRANTSFS
consensus	1	*****
Ab 55	61	PASTFKIPNTLIALNEGTVNKDSIIVWDKKVREFDAWNKDQTLQSAFKSSCVWCYKEFAS
Ab 6V	61	PASTFKIPNTLIALNEGTVNKDSIIVWDKKVREFDAWNKDQTLQSAFKSSCVWCYKEFAS
OXA-464	61	PASTFKIPNTLIALNEGTVNKDSIIVWDKKVREFDAWNKDQTLQSAFKSSCVWCYKEFAS
OXA-490	61	PASTFKIPNTLIALNEGTVNKDSIIVWDKKVREFDAWNKDQTLQSAFKSSCVWCYKEFAS
Ab RM4018	61	PASTFKIPNTLIALNEGTVNKDSIIVWDKKVREFDAWNKDQTLQSAFKSSCVWCYKEFAS
Ab S2_012_000_R	61	PASTFKIPNTLIALNEGTVNKDSIIVWDKKVREFDAWNKDQTLQSAFKSSCVWCYKEFAS
OXA-491	61	PASTFKIPNTLIALNEGTVNKDSIIVWDKKVREFDAWNKDQTLQSAFKSSCVWCYKEFAS
Ab L348	61	PASTFKIPNTLIALNEGTVNKDSIIVWDKKVREFDAWNKDQTLQSAFKSSCVWCYKEFAS
Ab L353	61	PASTFKIPNTLIALNEGTVNKDSIIVWDKKVREFDAWNKDQTLQSAFKSSCVWCYKEFAS
Ab ED-1	61	PASTFKIPNTLIALNEGTVNKDSIIVWDKKVREFDAWNKDQTLQSAFKSSCVWCYKEFAS
Ab L355	61	PASTFKIPNTLIALNEGTVNKDSIIVWDKKVREFDAWNKDQTLQSAFKSSCVWCYKEFAS
Ab JV22	61	PASTFKIPNTLIALNEGTVNKDSIIVWDKKVREFDAWNKDQTLQSAFKSSCVWCYKEFAS
Ab L349	61	PASTFKIPNTLIALNEGTVNKDSIIVWDKKVREFDAWNKDQTLQSAFKSSCVWCYKEFAS
Ab L.	61	PASTFKIPNTLIALNEGTVNKDSIIVWDKKVREFDAWNKDQTLQSAFKSSCVWCYKEFAS
As_CCUG 10374	61	PASTFKIPNTLIALNEGTVNKDSIIVWDKKVREFDAWNKDQTLQSAFKSSCVWCYKEFAS
consensus	61	*****
Ab 55	121	KIGVEKYKKYLKELNYGNKTIGKDVTFWLDESRLITAFEEIRFLKQLQANNLAFKQEDI
Ab 6V	121	KIGVEKYKKYLKELNYGNKTIGKDVTFWLDESRLITAFEEIRFLKQLQANNLAFKQEDI
OXA-464	121	KIGVEKYKKYLKELNYGNKTIGKDVTFWLDESRLITAFEEIRFLKQLQANNLAFKQEDI
OXA-490	121	KIGVEKYKKYLKELNYGNKTIGKDVTFWLDESRLITAFEEIRFLKQLQANNLAFKQEDI
Ab RM4018	121	KIGVEKYKKYLKELNYGNKTIGKDVTFWLDESRLITAFEEIRFLKQLQANNLAFKQEDI
Ab S2_012_000_R	121	KIGVEKYKKYLKELNYGNKTIGKDVTFWLDESRLITAFEEIRFLKQLQANNLAFKQEDI
OXA-491	121	KIGVEKYKKYLKELNYGNKTIGKDVTFWLDESRLITAFEEIRFLKQLQANNLAFKQEDI
Ab L348	121	KIGVEKYKKYLKELNYGNKTIGKDVTFWLDESRLITAFEEIRFLKQLQANNLAFKQEDI
Ab L353	121	KIGVEKYKKYLKELNYGNKTIGKDVTFWLDESRLITAFEEIRFLKQLQANNLAFKQEDI
Ab ED-1	121	KIGVEKYKKYLKELNYGNKTIGKDVTFWLDESRLITAFEEIRFLKQLQANNLAFKQEDI
Ab L355	121	KIGVEKYKKYLKELNYGNKTIGKDVTFWLDESRLITAFEEIRFLKQLQANNLAFKQEDI
Ab JV22	121	KIGVEKYKKYLKELNYGNKTIGKDVTFWLDESRLITAFEEIRFLKQLQANNLAFKQEDI
Ab L349	121	KIGVEKYKKYLKELNYGNKTIGKDVTFWLDESRLITAFEEIRFLKQLQANNLAFKQEDI
Ab L.	121	KIGVEKYKKYLKELNYGNKTIGKDVTFWLDESRLITAFEEIRFLKQLQANNLAFKQEDI
As_CCUG 10374	121	KIGVEKYKKYLKELNYGNKTIGKDVTFWLDESRLITAFEEIRFLKQLQANNLAFKQEDI
consensus	121	*****
Ab 55	241	LTLEALKTKGIID
Ab 6V	241	LTLEALKTKGIID
OXA-464	241	LTLEALKTKGIID
OXA-490	241	LTLEALKTKGIID
Ab RM4018	241	LTLEALKTKGIID
Ab S2_012_000_R	241	LTLEALKTKGIID
OXA-491	241	LTLEALKTKGIID
Ab L348	241	LTLEALKTKGIID
Ab L353	241	LTLEALKTKGIID
Ab ED-1	241	LTLEALKTKGIID
Ab L355	241	LTLEALKTKGIID
Ab JV22	241	LTLEALKTKGIID
Ab L349	241	LTLEALKTKGIID
Ab L.	241	LTLEALKTKGIID
As_CCUG 10374	240	LTLEALKTKGIID
consensus	241	*****

FIGURE 3 | Multialignment of OXA beta-lactamase from *Arcobacter butzleri* obtained by using T-Coffee web server (Di Tommaso et al., 2011). Ab 55 protein ID: D3M61_10735; Ab 6V: D3M75_10375; OXA-491, Accession: ANW35665.1; OXA-464: ANW35663.1; OXA-490: ANW35664.1. *A. butzleri* RM4018: WP_012013127.1; *A. butzleri* S2_012_000_R2_80: PZP12670.1; *A. butzleri* L348: WP_046997374.1; *A. butzleri* L353: WP_050071304.1; *A. butzleri* ED-1: WP_014468976.1; *A. butzleri* L355: WP_046997672.1; *A. butzleri* JV22: EFU68937.1; *A. butzleri* L349: WP_046993700.1; *A. butzleri* L.: WP_014474670.1; *A. skirrowii* CCUG 10374: WP_115588490.1.

A. butzleri isolates from seafood (40 isolates were from shellfish) and coastal water, which were all sensitive to these three antibiotics. Susceptibility to these antibiotics is also consistent to that found by other authors who reported susceptibility to streptomycin and/or gentamicin and/or kanamycin for the great majority (96.4–100%) of *A. butzleri* isolated from shellfish [gentamicin (Collado et al., 2014)], water sources [gentamicin and streptomycin (Šilha et al., 2017)] and other various sources [gentamicin (Kayman et al., 2012; Ferreira et al., 2013, 2017; Van den Abeele et al., 2016); gentamicin and streptomycin (Šilha et al., 2017); kanamycin and streptomycin (Kabeya et al., 2004); gentamicin, kanamycin and streptomycin (Rahimi, 2014)]. Aminoglycosides are proposed as antibiotics to be used in *Arcobacter* infections (Rahimi, 2014; Ferreira et al., 2016; Rathlavath et al., 2017). Nevertheless, a study reported high percentage (80%) of resistance to gentamicin and kanamycin in *A. butzleri* isolates from porcine samples (Scanlon et al., 2013) but only five isolates were tested. Mechanisms of bacterial resistance to this class of antibiotics are diverse including the inactivation by aminoglycoside modifying enzymes, mutation of the ribosome target, and modification of the ribosome by methyltransferase enzymes (Wilson, 2014; Garneau-Tsodikova and Labby, 2016). Resistance may also arise from mutation in the *rrs* encoding for 16S rRNA, even if these mutations are quite rare, as they would interfere with the vital cellular machinery.

Susceptibility to the hydrophilic fluoroquinolone ciprofloxacin or very low percentage of resistant isolates (0–3.2%) are widely reported in literature (Kayman et al., 2012; Collado et al., 2014; Rahimi, 2014; Ferreira et al., 2017; Rathlavath et al., 2017; Šilha et al., 2017; Soma et al., 2017), although some authors reported percentages ranging from 12.7 to 55.8% of *A. butzleri* resistant to ciprofloxacin isolated from patients with gastroenteritis, retail food products, and poultry slaughterhouse (Ferreira et al., 2013; Zacharow et al., 2015b; Van den Abeele et al., 2016; Vicente-Martins et al., 2018). The acquisition of fluoroquinolone resistance may represent a serious issue for the health care system since they are the first-choice antibiotic for treating *Campylobacter* infection in humans and they were suggested to be used in *Arcobacter* enteritis (Vandenberg et al., 2006; Collado et al., 2014; Ferreira et al., 2016).

Our strains are susceptible to ciprofloxacin. Indeed, we did not find in *A. butzleri* Ab 55 and Ab 6V the mutation in the quinolone resistance determining region in position 254 of the *gyrA* gene which causes a transition from cytosine to thymine leading to the substitution of a threonine to isoleucine (Abdelbaqi et al., 2007).

Ab LMG10828^T (RM 4018) and Ab 6V were intermediate resistant to the hydrophobic quinolone nalidixic acid, whereas Ab 55 was resistant to this antibiotic. However, the absence of *gyrA* mutations in these strains suggests that the putative resistance could be due to the mechanisms of hydrophobic quinolones uptake as suggested by Miller et al. (2007). High percentage of resistance toward the nalidixic acid was reported for 77.5 and 83.4% of *A. butzleri* isolated from shellfish by Rathlavath et al. (2017) and Collado et al. (2014), respectively, for 71.6% of *A. butzleri* isolates from fish (Rathlavath et al., 2017) and for 57.6 and 88.9% of *A. butzleri* isolates from water sources in

two different studies (Rathlavath et al., 2017; Šilha et al., 2017). One hundred percent of *A. butzleri* isolated from other various sources, namely, retail food products, porcine, slaughterhouse, and dairy plant samples, by different authors, were resistant to nalidixic acid (Scanlon et al., 2013; Elmali and Can, 2017; Ferreira et al., 2017; Vicente-Martins et al., 2018). By contrast, high percentage of susceptibility toward nalidixic acid, ranging from 50 to 77.8%, was reported by Yesilmen et al. (2014) and Kayman et al. (2012) for *A. butzleri* isolated from milk and dairy products and human gastroenteritis stool samples, respectively. However, no more than 10 *A. butzleri* isolates were tested in both studies.

Arcobacter butzleri Ab 55 and Ab 6V are intermediate resistant to chloramphenicol (Table 6). Differences about susceptibility against this antibiotic were reported among several studies (Ferreira et al., 2016); e.g., Rathlavath et al. (2017) reported high susceptibility rates ranging from 65.3 to 77.7% for 147 *A. butzleri* isolates from shellfish, fish, and coastal water whereas Šilha et al. (2017) reported that 66.6% of *A. butzleri* isolated from water sources was resistant. Also for *A. butzleri* isolated from various sources (poultry meat and other retail food products, poultry slaughterhouse, and animal and human stool samples) variable resistance percentages were reported ranging from 2.3 to 87.7% (Ferreira et al., 2013; Rahimi, 2014; Aski et al., 2016; Šilha et al., 2017; Soma et al., 2017; Vicente-Martins et al., 2018). Otth et al. (2004) suggested that it may depend on local differences in the usage of this antibiotic.

Chloramphenicol resistance commonly consists of its enzymatic inactivation mainly by acetyltransferases or occasionally by phosphotransferases, but additional mechanisms involve the presence of efflux pump which act as extrusion transporters, mutation or modification of the target site, and decreased outer membrane permeability. In the genomes of Ab 55 and Ab 6V, we retrieved the *cat3* gene encoding for a type A chloramphenicol O-acetyltransferase (Ab 55 D3M61_09345; Ab 6V D3M75_06440), which catalyzes the acetyl-CoA-dependent acetylation of chloramphenicol and they resulted as intermediate resistant.

Our *A. butzleri* strains Ab 55 and Ab 6V are resistant to tetracycline (Table 6). As for chloramphenicol, tetracycline susceptibility results vary among studies, even if tetracycline is proposed for treating *Arcobacter* infections by different authors (Ferreira et al., 2016; Rathlavath et al., 2017). Rathlavath et al. (2017) and Šilha et al. (2017) reported that 100 and 77.8% of *A. butzleri* isolated from shellfish, fish, coastal water, and water sources, respectively, were susceptible to tetracycline and high susceptibility rates, ranging from 78.2 to 100% were reported also for *A. butzleri* isolated from poultry meat, human and animal stool samples (Rahimi, 2014; Aski et al., 2016; Šilha et al., 2017). Conversely Vicente-Martins et al. (2018) reported that 95.4% of 65 *A. butzleri* strains isolated from retail food products was resistant to tetracycline and also Yesilmen et al. (2014) reported that 100% of *A. butzleri* isolated from milk and cheese was resistant toward this antibiotic, even if only 10 isolates were tested.

Tetracycline resistance may be due to several mechanisms: efflux, modification, protection from the ribosome binding, modification of 16S rRNA at the tetracycline binding site.

These mechanisms are mediated by different proteins, among which Tet(O) and Tet(M) are the most important. These proteins are paralogues of the translational GTPase EF-G which removes tetracycline from its inhibitory site on the ribosome through a GTP-dependent hydrolysis. Both are part of a larger group of proteins called ribosomal protection proteins (RPPs), which also includes Tet(Q), Tet(S), Tet(T), Tet(W), and OtrA (Chopra and Roberts, 2001). In the genomes of our strains, we retrieved proteins predicted as tetracycline resistance protein of class C (MFS transporter-multidrug efflux pump) with 27% identity with the orthologues in *E. coli* and elongation factors with the same domain found in the C terminus of RPPs Tet(M) and Tet(O), with 65% identity with EF-G of *E. coli*.

Concerning erythromycin, *A. butzleri* Ab 55 and Ab 6V are resistant to this antibiotic (Table 6). However, different percentages of erythromycin resistance were reported in several studies. In particular, *A. butzleri* isolated from seafood and water sources, as well as from poultry meat, animal and human stool samples, dairy plant, and cheese were found to be susceptible to this antibiotic at percentages ranging from 65 to 100% (Kayman et al., 2012; Collado et al., 2014; Aski et al., 2016; Ferreira et al., 2017; Rathlavath et al., 2017; Šilha et al., 2017). Zacharow et al. (2015b) and Soma et al. (2017) reported that 62 and 50%, respectively, of *A. butzleri* isolated from animals, humans, and foods of animal origin were resistant toward this antibiotic whereas Yesilmen et al. (2014) and Scanlon et al. (2013) reported that 80% of *A. butzleri* isolated from milk, cheese, and porcine samples, respectively, were resistant too, but no more than 10 isolates were tested.

Erythromycin resistance is due, besides the less common mutation in 23S rRNA or ribosomal proteins, to post-transcriptional methylation of an adenine residue in 23S caused by the action of erm class gene-coded methylases in Gram-positive bacteria (Kurincic et al., 2007). Ribosomal RNA small subunit methyltransferases were also present in Ab 55 (D3M61_05905) and Ab 6V (D3M75_01835) genomes with 26 and 27% identity with reference sequence of *Enterococcus faecium* (Accession: YP_004172630.1), respectively; this protein is also present in the genome of *A. butzleri* RM4018, although it results intermediate resistant in response to erythromycin. Furthermore, we identified *macA* and *macB* genes encoding for macrolide exporter proteins in both Ab 55 and Ab 6V. The single copy of 23S rRNA of both Ab 55 and Ab 6V does not present any of the identified mutation responsible for erythromycin resistance.

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CONCLUSION

Genomic analyses herein performed allowed us to confirm the recently (Pérez-Cataluña et al., 2018a,b) suggested amendment of *A. butzleri* as *Al. butzlerii*, comb. nov.

Antimicrobial susceptibility tests defined Ab 55 and Ab 6V strains as resistant to vancomycin, tetracycline, nalidixic acid (only Ab 55 whereas Ab 6V is intermediate resistant), erythromycin, and β -lactam antibiotics. Moreover, in our strains isolated from shellfish, we identified numerous virulence, antibiotic, and heavy metal resistance determinants, also additional to those previously found in the genome sequenced *A. butzleri* ED-1, isolated from fuel cell, and in the *A. butzleri* type strain RM 4018, isolated from human gastroenteritis (Miller et al., 2007; Pérez-Cataluña et al., 2018a), leading to hypothesize that shellfish strain may be potentially more virulent.

The findings of food-related *A. butzleri* support both epidemiological surveillance and food safety risk assessment and management in the shellfish industry.

Further analysis in our laboratories is ongoing to sequence and characterize other *A. butzleri* strains isolated from shellfish and from other food matrices, in order to obtain an updated description of the species and to clarify the role of genetic endowment, as well as of the ecological niches the strain come from, in the pathogenesis of *A. butzleri*. The genomic sequences here presented, and the novel insights obtained in the present study appreciably contribute to achieve these goals.

AUTHOR CONTRIBUTIONS

VF conceived the work, interpreted the data, and organized the bioinformatic work. FF performed the genomic sequencing and the bioinformatic work. DC and FB performed the antimicrobial susceptibility testing. VF and FF wrote the manuscript. All the authors contributed to the revision of the manuscript and read and approved the submitted manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.00670/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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