

Identification of virulence and antibiotic resistance factors in *Arcobacter butzleri* isolated from bovine milk by Whole Genome Sequencing

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Abstract

Arcobacter butzleri is a pathogenic aerobic bacterium responsible for diarrhea and septicemia in humans. It is frequently isolated from food products of animal origin, including milk and dairy products. To date, few reports are currently available on the genetic characteristics and virulence profiles of *A. butzleri*. The aim of this study was to investigate the genetic diversity and to characterize the virulence and antibiotic resistance profiles of 10 *A. butzleri* strains isolated from bovine milk samples by Whole Genome Sequence (WGS). Multi-Locus Sequence Typing (MLST) revealed that three isolates belonged to the ST66, two to the ST420 and the remaining five strains to the ST627, ST629, ST630, ST633 and ST637, respectively. The 100% of the strains carried *cadF*, *pldA*, *ciaB*, *cj1349*, *mviN* and *thyA* virulence factors genes; 60% *iroE*; 50% *irgA*; 10% *hecB*. Resistome prediction showed a multidrug resistance: 100% of isolates resulted resistant to fluoroquinolones and tetracycline; 90% of strains to rifampicin and cephalosporins and a minor percentage to other antibiotics. Furthermore, the 50% of strains harbored four mutations in *Mycobacterium tuberculosis katG* gene conferring resistance to isoniazid. The study provided interesting data on the virulence characteristics and on the genetic endowment related to the antimicrobial resistance of *A. butzleri* isolates from milk. The determination of the STs also added information concerning the genetic variability of this microorganism. To date, a very limited number of studies have been published on the typing of *A. butzleri* using WGS, so this paper proposes an innovative methodological approach that allows a rapid and complete characterization of pathogenic microorganisms.

Introduction

The *Arcobacter* genus is ubiquitous in animals, in a variety of foods of animal and non-animal origin and in aquatic environments; it has been linked to animal and human illness (Collado and Figueras, 2011). *Arcobacter* spp. have been isolated from faces of numerous species of farm animals and found to contaminate different foods, including milk. In industrialized countries, the consumption of raw or undercooked food or the ingestion of *Arcobacter*-contaminated water represent the most important sources of human infection. To date, the lack of highly standardized methods for the isolation of *Arcobacter* spp. could affect the evaluation of the real prevalence of this potential pathogen in food that is believed to be underestimated (Prouzet-Mauleon *et al.*, 2006). Furthermore, the prevalence of *Arcobacter* spp. varies from species to species and from country to country, but in general *Arcobacter* spp. it is principally found in poultry followed from pigs and cattle (van Driessche *et al.*, 2003). Additionally, there are studies that displayed also the presence of *Arcobacter* spp. in other species of birds (*i.e.* ducks, turkeys, domestic geese) and in mussels (Collado *et al.*, 2009; Mottola *et al.*, 2016). Few studies investigated the presence of *Arcobacter* spp. in bovine milk and the prevalence ranged from 1 to 46% (Collado and Figueras, 2011).

A. butzleri, *A. cryaerophilus* and *A. skirrowii*, have been associated with gastrointestinal cases in humans (Collado and Figueras, 2011). *A. butzleri* is associated with enteritis and diarrhea in pigs, cattle and horses; *A. skirrowii* is usually associated with diarrhea and hemorrhagic colitis in sheep and cattle; *A. cryaerophilus* has been described as cause of abortion in animals, event rarely associated also to *A. butzleri* and *A. skirrowii* (Collado and Figueras, 2011; Mottola *et al.*, 2016; Ramees *et al.*, 2017). *A. butzleri* is the species of arcobacters most commonly reported as contaminant of foods (Collado *et al.*, 2009). *A. butzleri* represents an environmental bacterium that has developed a high level of niche adaptation (Merga *et al.*, 2013) and the ability to survive in the adverse conditions like food processing and storage (Collado and Figueras, 2011; Ferreira *et al.*, 2016). In recent years the increased number of cases of illness has raised the concern that this microorganism is a serious risk to human health (International Commission on Microbial Specification for Foods (ICMSF, 2002). The knowledge of the mechanisms of pathogenicity of *A. butzleri* is still limited (Ferreira and Glassey, 2017). Adhesion,

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invasion and cytotoxicity have been previously examined *in vitro* using different cell lines (Karadas *et al.*, 2013). Very little information is known about the genes involved in the virulence mechanisms (Collado and Figueras, 2011). It was found that this species possesses presumed virulent determinants such as the *cadF* and *cj1349* genes, encoding fibronectin-binding proteins, *ciaB* that encodes a *Campylobacter* invasion antigen, the virulence factor *mviN* encoding an essential protein required for the biosynthesis of peptidoglycan, *pldA* that encode phospholipase A, the hemolysin gene *thyA*, the *irgA* gene that encodes an iron-regulated outer membrane protein, *IroE* gene codes for a periplasmic enzyme functional for iron acquisition and required to establish and maintain infection, *hecA*, a member of the filamentous hemagglutinin (FHA) family, and *hecB* that encodes a hemolysin activation protein (Miller *et al.*, 2007).

Currently, numerous genotyping methods are used for epidemiological molecular studies and each of them shows a different discriminatory power (Giacometti *et al.*, 2018). Among them, MLST presents the advantage of the availability of an online database for *Arcobacter* spp. (<https://pubmlst.org/arcobacter/>) that allows to compare strains isolated from different studies and from various geographical areas (De Cesare *et al.*, 2016; Merga *et al.*, 2013).

Whole Genome Sequencing (WGS) of

microbial strains is becoming, due to the continuous decline of the associated costs, a powerful, very affordable and fast tool for microbial typing, allowing a much higher resolution than traditional typing methods, and an important tool for public health surveillance and outbreak investigation. Recent studies have shown that WGS analysis can potentially be a single, rapid and cost-effective approach to define resistance genotypes and predict resistance phenotypes of bacteria with great sensitivity and specificity (McDermott *et al.*, 2016)

The aim of the present study was to assess the genetic characteristics of 10 strains of *A. butzleri* isolated in raw bovine milk samples collected in Apulia Region by WGS followed by *in silico* prediction of the virulence factors and the resistome.

Materials and Methods

Strain collection and identification

Bulk tank milk samples were collected from 190 dairy farms of Apulia Region during the year 2017. Milk samples (10 mL) were homogenized with 90 mL of Arcobacter Enrichment Broth plus Cefoperazone, Amphotericin B, Teicoplanin (CAT) Selective Supplement and incubated

at 30°C for 48h in microaerophilic conditions. From each broth DNA was extracted and subjected to an *Arcobacter* genus - specific Real Time PCR as described elsewhere (Gonzalez *et al.*, 2014). The PCR positive broths were filtered using 0.45 µm pore size syringe filters and 0.2 mL were spread onto plates of Columbia Blood Agar, MCCD Agar and Karmali Agar. The plates were incubated at 30°C for 3-4 days in microaerophilic conditions and checked daily. Presumptive identification tests (Gram staining, catalase and oxidase tests) were performed on at least 5 suspected colonies among those grown on Columbia Blood Agar, MCCD Agar and Karmali Agar. Colonies of Gram-negative spiral bacteria, oxidase and catalase positive were considered presumptive *Arcobacter spp.* and subjected (one colony for each sample) to species identification using an *atpA* amplification and sequencing method (Miller *et al.*, 2014).

WGS

Ten isolates, all identified as *A. butzleri*, were characterized by WGS. Genomic DNA was extracted from the isolates using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), according to manufacturer's protocol. An indexed genomic library for each isolate was prepared using the Nextera XT DNA sample

preparation kit (Illumina, San Diego, CA), according to manufacturer's instruction. Sequencing was performed on MiSeq platform (Illumina) using the MiSeq Reagent kit v2, 2x250 paired-end cycles (Illumina). The reads obtained for each sample were trimmed, subjected to quality filtering using Trimmomatic (Bolger *et al.*, 2014), and finally assembled by SPAdes v 3.11 (Nurk *et al.*, 2013). General features of the *A. butzleri* genome sequences were collected in Table 1. For each genome, a script for the extraction of the sequences of the MLST alleles was used in Bionumerics 7.6 (Applied Maths, Belgium). Allele profiles and Sequence Types (STs) were assigned according to *Arcobacter* MLST database (<https://pubmlst.org/arcobacter/>). Assembled genomes were submitted to Virulence Factors Database (VFDB: <http://www.mgc.ac.cn/VFs/>) for the detection of virulence factors (VFs). For virulence gene identification, BLASTn method against VFDB was used considering only the genes with E Value equal to 0. Additionally, BLAST *in silico* analysis was performed for typical virulence factors of *A. butzleri* (*i.e.* *cadF*, *cj1349*, *ciaB*, *pldA*, *tlyA*, *hecA*, *hecB*, *mviN*, *irgA*, *IroE*).

In order to evaluate the presence of antimicrobial resistance (AMR) genes, assembled genomes were submitted to Resistance Gene Identifier (RGI) software

Table 1. General features of the *Arcobacter butzleri* genome sequences.

	AB141	AB142	AB143	AB144	AB147	AB149	AB151	AB155	AB157	AB158
Deep coverage	50X	40X	50X	60X	40X	35X	35X	60X	40X	35X
contigs	107	140	103	85	145	179	195	61	125	167
Largest contig	97506	96476	99956	188708	76532	78825	65537	226837	95595	87642
Total length	2296956	2279850	2389007	2395935	2119859	2392732	2470188	2359823	2268926	2340572
N50	36402	24517	35185	52313	23090	24168	21682	76682	32179	22046
N75	17051	15513	24226	24074	12615	11360	11399	48341	15033	13242
L50	20	27	21	14	30	30	33	9	22	33
L75	43	57	41	31	61	67	74	19	50	67
GC (%)	27.12	27.17	26.7	26.85	27.1	27.02	26.99	26.91	27.06	27.11

Table 2. Diversity of *A. butzleri*. Alleles and sequence types (ST).

ID Strain	<i>aspA</i>	<i>atpA</i>	<i>glnA</i>	<i>gltA</i>	<i>glyA</i>	<i>pgm</i>	<i>tkt</i>	ST
AB141	15	10	1	17	19	2	13	66
AB142	15	10	1	17	19	2	13	66
AB143	6	23	1	11	494	58	199	627
AB144	48	25	41	19	487	101	272	633
AB147	5	5	9	15	120	7	6	629
AB149	23	17	17	19	461/176	11	65	630
AB151	5	5	5	15	176/66	11	10	420
AB155	310	133	11	19	192/19	123	271	637
AB157	15	10	1	17	19	2	13	66
AB158	5	5	5	15	176/66	11	10	420

of Comprehensive Antibiotic Resistance Database (CARD) (<https://card.mcmaster.ca/>), considering the genes with 50% identity of matching region.

Results

The *Arcobacter* genus - specific PCR revealed the presence of *Arcobacter* spp. in 25/190 (13%) milk samples analyzed. The following microbiological procedures allowed to isolate suspected colonies just in 10 of the 25 (40%) PCR-positive samples. Presumptive identification tests performed on suspected colonies always identified Gram-negative spiral bacteria, oxidase and catalase positive. The presumptive *Arcobacter* spp. colonies were identified as *A. butzleri* using the partial sequencing of the *atpA* gene. Prevalence of *Arcobacter* spp. in bulk tank milk was 5% (10/190).

MLST genes revealed a high genetic diversity in *A. butzleri* isolates. The number of alleles per locus observed in the isolates were showed in Table 2. Seven different STs were identified: 30% (3/10) of strains belonged to ST66 and 20% (2/10) to ST420. One single isolate was identified as the following STs: ST627, ST629, ST630, ST633, ST637, respectively. Interestingly, the 40% (4/10) of strains showed two alleles for *glyA* genes (Table 2), previously identified as *glyA1* and *glyA2* (Miller *et al.*, 2009). Submission of genome sequences in VFDB allowed to identify more of 400 virulence factors (data not shown). Among them only 12 showed a perfect identity (E-value=0.00) with similar virulence factors of *Campylobacter* spp. Interestingly, only two of them (*cadF* and *ciaB*) have been described as virulence factors in *A. butzleri*. A further search of the determinants known to play a role in *A. butzleri* infections was performed using BLASTn with the aim to detect 10 specific virulence factors. This approach allowed to find 90% (9/10) (Table 3) of the virulence factors with the following distribution: the 100% (10/10) of them carried *cadF*, *ciaB*, *cj1349*, *mviN*, *pldA* and *tlyA* genes; the 60% of them carried the *iroE* gene; the 50% of them carried *irgA* gene; the 10% of them carried *hecB* gene. Any of them isolates carried *hecA* gene (Table 3).

The prediction of resistome by CARD showed that the 100% of strains harbored the *adeF* gene, that confers resistance to fluoroquinolone and tetracycline antibiotics; the 90% of them harbored *acrB* gene that confers resistance to rifamycin, cephalosporin, triclosan, glycylicycline, tetracycline, penam, phenicol and fluoroquinolone antibiotics; the 30% of strains harbored *pmrE* gene that confers

resistance to peptide antibiotic; the 10% of strains carried *aadA2* and *macB* genes, that confers the resistance to aminoglycoside antibiotic and macrolide antibiotic, respectively. Furthermore, the 50% of strains harbored mutations (S140N, A139V, R463L, A379T) in *Mycobacterium tuberculosis katG* gene conferring resistance to isoniazid (Table 4).

Discussion

Arcobacter are considered to be an emergent food pathogen, but a reduced number of studies is available and little is known about the pathogenic mechanism (Collado and Figueras, 2011). To date a total of 26 recognized *Arcobacter* species have been described (Vicente-Martinsa *et al.*, 2017); among these, *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have been shown to cause infection in humans. The major transmission pathway of these bacteria is represented by the ingestion contaminated

water or food, particularly raw or minimally processed foods are generally considered the main source of human infection in industrialized countries (Van Driessche and Houf, 2008). Prevalence of *Arcobacter* spp. in analyzed milk samples was 5%, although 25 samples (13%) resulted PCR positive. Despite the little number of studies that investigated the presence of *Arcobacter* spp. in bovine milk, there are evidence showing a prevalence rates of 5.8%, 15% and 46% in Malaysia, Finland and Northern Ireland, respectively; in Italy a prevalence rate of 26% (25/190) was found in bulk tank milk produced in Lombardia Region (Collado and Figueras, 2011). Although the different prevalence rates reported in literature could be due to different sampling methods and to the absence of a standardized protocol of analysis. The entire draft genome of isolates was used to predict the STs and this analysis highlight a considerable amount of genetic diversity between the *A. butzleri* isolates (De Cesare *et al.*, 2016). That result was consolidated by the identification of 5 STs (ST627; ST629; ST630; ST633; ST637) that

Table 3. Presence of main virulence genes in *A. butzleri* isolates from milk.

ID Strain	Virulence factors									
	<i>cadF</i>	<i>ciaB</i>	<i>cj1349</i>	<i>irgA</i>	<i>hecA</i>	<i>hecB</i>	<i>mviN</i>	<i>pldA</i>	<i>tlyA</i>	<i>iroE</i>
AB141	+	+	+	-	-	-	+	+	+	-
AB142	+	+	+	-	-	-	+	+	+	-
AB143	+	+	+	-	-	-	+	+	+	-
AB144	+	+	+	+	-	-	+	+	+	+
AB147	+	+	+	+	-	-	+	+	+	+
AB149	+	+	+	+	-	-	+	+	+	+
AB151	+	+	+	+	-	-	+	+	+	+
AB155	+	+	+	-	-	+	+	+	+	-
AB157	+	+	+	-	-	-	+	+	+	+
AB158	+	+	+	+	-	-	+	+	+	+

+, positive; -, negative.

Table 4. Presence of AMR genes in *A. butzleri* isolates from milk.

ID Strain	AMR genes					
	<i>adeF</i>	<i>aadA2</i>	<i>acrB</i>	<i>PmrE</i>	<i>macB</i>	<i>katG mut</i>
AB141	+	-	+	-	-	-
AB142	+	-	+	-	-	-
AB143	+	-	+	-	-	-
AB144	+	-	+	+	-	-
AB147	+	-	+	-	+	+
AB149	+	+	-	-	-	+
AB151	+	-	+	+	-	+
AB155	+	-	+	-	-	+
AB157	+	-	+	-	-	-
AB158	+	-	+	+	-	+

were previously unreported. Interestingly, these STs were represented by a single isolate. More than one isolate was identified as ST66 and ST420, both of whom were previously described as food contaminants: ST66 in ricotta cheese (De Cesare *et al.*, 2016; Giacometti *et al.*, 2018) and ST420 in raw WB milk, bulk tank valve, milk pump and mozzarella cheese molding roller (De Cesare *et al.*, 2016; Giacometti *et al.*, 2018). As more than 600 profiles are currently available in the PubMLST database, the high number of STs identified clearly shows the high genetic diversity of *A. butzleri* as previously described (De Cesare *et al.*, 2016; Miller *et al.*, 2009). The high variability of *A. butzleri* would be due to the genetic recombination causing rapid genomic changes (De Cesare *et al.*, 2016). Interestingly, ST420 has been also identified in strains isolated in nonfood contact surfaces (De Cesare *et al.*, 2016; Giacometti *et al.*, 2018). Taken together, these findings suggest that the detection of very different occurrence values in studies performed on the same sample type and using similar protocols could be related to numerous factors such as hygiene of farms, source of water, feeding, climate etc (Collado and Figueras, 2011). *A. butzleri* was the only isolated species in the analyzed bulk tank milk samples, in agreement with the results of previous studies that reported it as the most commonly recovered species both in milk, milk products, and in dairy plants (Serraino and Giacometti, 2014). In previous studies it was reported that *A. butzleri* showed the ability to grow in several substrates and in different environmental conditions and to survive to sanitizing procedures, characteristics that make the bacterium adapt to resist in the environment and to cause secondary food infections (Serraino and Giacometti, 2014). For this reason, it became very important to investigate the presence of virulence-associated genes and antibiotic resistance genes. In our study the most commonly detected genes were: *cadF*, that encodes a protein able to induce the internalization of bacterial cells by the activation of GTPases; *ciaB*, that encodes an invasion protein injected directly into the cytoplasm of the host cells by a secretion system; *cj1349*, that encodes an adhesion protein that binding specifically to fibronectin; *mviN*, that encodes an inner membrane protein required for peptidoglycan biosynthesis; *pldA*, that encodes outer membrane phospholipase A associated with lysis of erythrocytes; *tlyA*, that encodes a hemolysin. This result was in accordance with previous data (Girbau *et al.*, 2015; Mottola *et al.*, 2016) that investigated *A. butzleri* isolated from different hosts and

environments like food products, processing water, fecal samples etc.

The periplasmic enzyme gene *iroE*, which was found in 60% of isolates, and the iron-regulated outer-membrane gene *irgA*, that was found in 50% of isolates, are functional components for the iron acquisition in *E. coli* and therefore required for establishing and maintaining infections (Rashid *et al.*, 2006; Zhu *et al.*, 2005). Finally, the hemolysin activation gene *hecB* was found in a strain. The low prevalence of them were in agreement with previous studies (Ferreira *et al.*, 2014; Girbau *et al.*, 2015; Mottola *et al.*, 2016). Any of our isolates carried *hecA* gene which encodes a hemagglutinin in uropathogenic *E. coli*. This result was in accordance with a previous study (Girbau *et al.*, 2015), but it might be due to the lower number of isolates included in our study. All these genes are implicated in adhesion and invasion mechanisms that are abilities necessary for colonization and infection. These characteristics confirmed the pathogenic potential of *A. butzleri* isolates from milk. Despite the small number of analyzed isolates, the presence of these genes in the majority of *A. butzleri* isolates in our and in previous studies suggests that they represent competitive factors for the maintenance of *A. butzleri* circulation in the different host populations and consequently in different environmental sites (Piva *et al.*, 2017). Although *in vitro* cell culture assays have been performed to investigate the role of these virulence genes in adhesion, invasion and cytotoxicity, to date no correlations have been found (Karada *et al.*, 2013; Vandenberg *et al.*, 2014).

The increasing occurrence of *A. butzleri* in foods as well as the ability of these bacteria to resist in different environmental conditions and to cause human disease, put the focus on antimicrobial resistance. *A. butzleri* presents resistance to several classes of antimicrobial agents, being considered as having multidrug resistance (Ferreira *et al.*, 2016). In our study all isolates were predicted to resist to fluoroquinolone and tetracycline antibiotics, whereas 90% of isolates had determinants linked to the resistance to rifamycin, cephalosporin, triclosan, glycolcyclycline, penam, phenicol antibiotics. Only a small percentage of all isolates possessed genes responsible of resistance to peptide antibiotics (30%), aminoglycosides (10%) and macrolides (10%). Furthermore, we found, in 50% of isolates, mutations in *katG* that catalyze the activation of isoniazid. Isoniazid inhibits mycolic acid synthesis, which prevents cell wall synthesis in *Mycobacteria*. Mutations in *katG* result in inability to activate isoniazid (Tseng *et al.*, 2015). Taken

together, these findings highlight the multidrug resistance of *A. butzleri* strains. In previous studies cephalosporin resistance were commonly found in *A. butzleri*; as matter of fact, cephalosporins are added into the *Arcobacter* selective media to suppress the growth of accompanying microorganisms (Atabay and Aydin, 2001). Moreover, it has been reported a human case of infection by *A. butzleri*, which resulted resistant to chloramphenicol and ampicillin (Villalobos *et al.*, 2013). Interestingly, the majority of studies are agreeing to report that *A. butzleri* isolates are highly susceptible to tetracycline, so that it can be used for treatment of human *Arcobacter* infections (Ramees *et al.*, 2017). Based on our results, that showed a wide occurrence in the *A. butzleri* genomes of genetic determinants potentially involved in Tetracycline resistance, the sensitivity towards this class of antimicrobials should be carefully monitored to avoid therapeutic failure. These results should be confirmed by *in vitro* phenotypical assays in order to follow the evolution of resistance in this species and to allow the better choice of antimicrobials to be used in clinical therapy. Moreover, other studies are needed to understand the molecular mechanisms of antimicrobial resistance in *A. butzleri*.

According to literature, our results confirmed the importance of raw milk as a possible source of *Arcobacter* spp. infection for humans. Consumption of heat-treated milk and the use of pasteurized milk for the production of dairy products should be an effective measure to prevent the risk to human health. However, it is necessary to consider the possible risks on human health consequent to the widespread consumption of dairy products based on raw milk, especially considering that in some of them, such as mozzarella and ricotta cheese, it has been shown that *Arcobacter* spp. is able to withstand the temperatures used during production (Giacometti *et al.*, 2014).

Conclusions

In the present study, ten *A. butzleri* strains were analyzed using the WGS approach, which rapidly allowed to define the ST, revealing five new STs, to identify the presence of nine of most representative putative virulence genes and several antibiotic resistance genes. Our results confirmed the usefulness of WGS to obtain relevant information about this important food-borne pathogen in detecting virulence and antimicrobial resistant genes or for genotyping.

The availability of high-performing

online databases to get prediction analysis, based on genome sequences, will ensure to obtain ever more information in the risk assessment connected to microbial pathogens making this approach particularly suitable for genetic background analysis and to perform epidemiological investigations.

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