Large genetic diversity of *Arcobacter butzleri* isolated from raw milk in Southern Italy

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Abstract

Arcobacter butzleri is a zoonotic foodborne pathogen able to cause enteric and extraintestinal diseases. Its occurrence in foodstuff is well recognized worldwide but data on its presence in foods from Southern Italy are scarce. In this study the results on the occurrence and genotyping of *Arcobacter* spp. in bulk milk samples collected in Southern Italy are reported. Out of 484 samples, 64 (13.2%) resulted positive for the presence of *Arcobacter* spp. using Real Time PCR but as few as 31.2% of these samples turned out as positive by using the cultural method, showing an overall prevalence of 4.1%. All isolates were identified as *A. cryaerophilus* using the biochemical identification whilst the sequencing of the *atp*A gene revealed that all the isolates were *A. butzleri*.

Among the confirmed isolates, 16 different Sequence Types (ST) were identified using the Multi Locus Sequence Typing (MLST), 14 (87.5 %) of which were previously unreported. Our survey reveals the presence of *A. butzleri* in bulk tank milk from Southern Italy and highlights the discrepancy between the two approaches used both for the detection (i.e., real time PCR vs cultural method) and the identification (i.e., biochemical test vs aptA sequencing) of *Arcobacter* spp In addition, a large genetic diversity among the isolates was detected and this makes the identification of source of the infections very challenging in outbreaks investigation.

Key Words: *Arcobacter*, Genotyping, Multi Locus Sequence Typing (MLST), Real-time PCR**,** Bulk Tank Milk

Introduction

Arcobacter spp. has been associated with human and animal disease and it is considered an important foodborne pathogen (Collado and Figueras, 2011; Ho *et al.*, 2006). The true incidence of human infections associated with *Arcobacter* is unknown, because these bacteria are not routinely investigated during human diarrheal diseases. In addition to this, a standardized protocol for their detection and characterization is not available (Collado and Figueras, 2011; Figueras *et al*., 2014). Among the several known *Arcobacter* species, *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have been recognized as those of clinical importance for animals and humans (Collado *et al*., 2011; Figueras at *al*., 2014; Hsu and Lee, 2015; Peréz-Cataluña *et al*., 2018; Ramees *et al*., 2017; Whiteduck-Léveillée *et al.*, 2015). In humans, these species have been associated with enterocolitis, peritonitis and bacteremia (Jiang *et al*., 2010; Lappi *et al*., 2013; Mottola *et al*., 2016a; Webb *et al*., 2016), while in animals they can cause gastroenteritis, mastitis, bacteremia and reproductive disorders (Arguello *et al*., 2015; Ho *et al*., 2006; Logan *et al*., 1982; Van Driessche and Houf, 2008; Whiteduck-Léveillée *et al*., 2016). Although the infectious dose has not yet been established, point-source outbreaks caused by *Arcobacter* spp. have been associated with well water ingestion, or with

the handling or consumption of contaminated raw or poorly-cooked animal food products. Also, direct contact with infected animals has been reported as a potential source of human infection (Fernandez *et al.*, 2015). In fact, the presence of *Arcobacter* has been documented worldwide from a wide range of sources and hosts with *A. butzleri* as the most prevalent species, followed by *A. cryaerophilus* and *A. skirrowii* (Collado and Figueras, 2011; Fallas-Padilla *et al*., 2014; Ramees *et al.*, 2017). *Arcobacter* spp. have also been isolated from faeces of healthy and sick humans and animals, including cattle, poultry, small ruminants, pigs and wild-living birds (Bogantes *et al*., 2015; Collado *et al*., 2009; Van Driessche *et al.*, 2003; Ottaviani *et al.*, 2017). In addition, *Arcobacter* have been detected from different foods such as fresh and ready to eat vegetables (González and Ferrús, 2011; González *et al*., 2017; Mottola *et al.*, 2016b), meat and meat products (Rivas *et al*., 2004; Rahimi, 2014, Lehmann *et al*., 2015), shellfish (Leoni *et al*., 2017; Levican *et al.*, 2014; Mottola *et al.*, 2016a), fish (Laishram *et al.*, 2016), eggs (Lee *et al.*, 2016) and drinking water (Ertas *et al*., 2010; Jalava *et al*., 2014; Jacob *et al.*, 1998). However, for better evaluating the foodborne risk linked to *Arcobacter* spp., more information on its occurrence in foods is needed (Lappi *et al.*, 2013). Regarding milk and dairy products, the detection of *Arcobacter* from these foodstuffs has been also reported (Logan *et al*., 1982; Pianta *et al*., 2007; Scullion *et al.*, 2006) but data on the occurrence of *Arcobacter* spp. in raw milk are still scarce (Shah *et al*., 2011). In Italy, 19 million tons of cow's milk are produced every year (www.agri.istat.it), mostly intended for cheese making or direct consumption as pasteurized or sterilized milk. However, in the last few years, the sale of raw milk for direct consumption via vending machines could have increased the risk of contact between humans and zoonotic agents (Haran *et al.*, 2012). Our work aims at improving the knowledge on the occurrence of *Arcobacter* spp. and its molecular characterization in bulk tank milk (BTM) samples collected in Southern Italy.

2. Matherials and methods

2.1 Sampling

The survey investigated two Italian Regions, Apulia and Basilicata, located in Southern Italy. On the whole, in these Regions are located 1.230 dairy farms with approximate 130.000 animals (www.vetinfo.it). A total of 484 BTM samples, corresponding to 39.4% of the total number of farms, were collected during 2014 to 2015. Specifically, the samples were from 396 dairy farms in Apulia and from 88 in Basilicata. The samples were aseptically collected in 500-mL sterile plastic containers, carried to laboratory in cooled containers within 24 hours after the of sampling. Samples were stored at -80 °C until analyzed.

2.2. Samples processing

BTM samples were defrosted and mixed using an agitator; then, 10 mL of milk were added to 90 mL of *Arcobacter* broth (Oxoid, Milan, Italy) supplemented with Cefoperazone, Amphotericin B and Teicoplanin (CAT selective supplement SR0174E; Oxoid) in sterile bags and homogenized using a stomacher (PBI International, Milan, Italy) at 11.000 rev min $^{-1}$ for 1 min. Then, the bags were incubated at 30 °C under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) by means of the CampyGen gas generating system (Oxoid) for 48 h.

2.3 Molecular screening

2.3.1 DNA extraction from enrichment broth

After incubation, 1 mL of enrichment broth was centrifuged at 13.000 rpm for 5 min at room temperature. The supernatant was discarded and the pellet was subjected to DNA extraction using the heat lysis and snap chilling method as described by Rasmussen et al., 2013 with some modifications. Briefly, 200 µL of sterile distilled water was added to the pellet and boiled in a water

bath at 100 °C for 15 minutes. The cell lysate was immediately transferred into ice and centrifuged at 13.000 rpm for 2 minutes. Supernatant was collected and used as DNA template for direct real-time PCR detection.

2.3.2 Real-time PCR

Genus specific real-time PCR was performed in order to screen the presence of *Arcobacter* spp. directly on the bacterial lysate. The reactions were performed in a final volume of 25 µL, using 1.25 µL EvaGreen 20X (Biotium, Hayward, USA), 0.2 nM of each dNTP, 2.5 µL of HotMaster Taq Buffer 10X (5PRIME, Hilden, Germany), 1 U of HotMaster Taq DNA Polymerase (5PRIME, Hilden, Germany), 5 pmol of each oligonucleotide primer and 2 µL of DNA template. The oligonucleotide primers used in this study (Arco-Fw and Arco-Rv), described by Gonzàlez et al., 2014. The amplification profile was carried out as follows: 95 °C for 3 min, followed by 40 cycles consisting of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. *A. butzleri* ATCC 46916T, *A. cryaerophilus* ATCC 43158T and *A. skirrowii* ATCC 51132 were used as positive controls. In order to identify nonspecific products, the melting curve was generated at the end of each run, thus exposing the final PCR product to a temperature gradient from about 60 °C to 90 °C in 20 min. The PCR reactions were processed in Applied Biosystems® 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, USA). All reactions were performed in duplicate.

2.4 Isolation and biochemical identification of Arcobater *spp.*

Ten mL of real-time PCR *Arcobacter* spp. positive enrichment broths were filtered using 0.45 µm membrane filters (Sartorius Stedim Biotech GmbH, Germany). Then, 200 µL of each filtered sample were streaked in parallel on Columbia Blood, Modified Charcoal Cefoperazone Deoxycholate (MCCD) and Karmali Agar plates (Oxoid). Plates were incubated at 30 °C under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) as described above, for 3-4 days. After

incubation, five typical *Arcobacter* spp. colonies for each sample were picked, subcultured onto Columbia Blood Agar and incubated for 48 h at 30 °C under microaerophilic conditions.

The colonies were confirmed morphologically by Gram staining and by determination of oxidase (Oxidase strips, Oxoid Microbact, Basingstoke, UK) and catalase activity (Mottola et al., 2016a; Mottola et al, 2016b). In addition, presumptive *Arcobacter* spp. colonies were further subjected to biochemical identification using API Campy® bioMerièux. The colonies identified as *Arcobacter* spp. were transferred onto *Arcobacter* broth (Oxoid, Basingstoke, UK) and incubated at 30 °C for 48 h.

2.5 Molecular identification and MLST typing of Arcobacter isolates

The extraction of DNA of isolates previously identified as *Arcobacter* spp. was performed using the GenomicPrep® kit (GE Helthcare. Illinois, USA) following the manufacturer's instructions. The identification of the *Arcobacter* isolates was determined using the *atp*A gene sequencing as described by Miller at al., 2014.

MLST was carried out on one identified isolate per positive sample using primers and conditions described by Miller et al., 2009. Specifically, the amplification and the sequencing of the seven housekeeping genes (*asp*A, *atp*A, *gln*A, *glt*A, *gly*A, *pgm* and *tkt*) included in the *Arcobacter* scheme of the PubMLST database were performed (http://pubmlst.org/arcobacter/).

The PCR products were purified using ExoSAP-IT according to supplier recommendations (GE Healthcare). Sequence reactions were carried out using BigDye 3.1 Ready reaction mix (Life Technologies) according to the manufacturer's instructions. The sequenced products were separated with a 3130 Genetic Analyzer (Life Technologies). Sequences were imported and assembled with Bionumerics 7.6 software (Applied Maths, Belgium). Any new alleles and STs were assigned by submitting the DNA sequences to the *Arcobacter* MLST database (https://pubmlst.org/arcobacter/).

3. Results

3.1 Molecular screening

The Real-Time PCR performed on enrichment broth from each BTM sample gave positive results for *Arcobacter* spp. in 64/484 (13.2 %) BTM samples. Specifically, all the positive samples were from Apulia (64/396) and none of Basilicata samples were positive for *Arcobacter* spp.

3.2 Confirmation of Real-Time PCR screening by cultural methods and identification of Arcobacter isolates

The cultural analysis carried out on the 64 Real-Time PCR positive enrichment broth showed typical *Arcobacter* colonies in 20 (31.2%) samples. On the whole, 4.1 % of BTM samples were positive for *Arcobacter* spp. Biochemical tests identified all isolates as *A. cryaerophilus*. Since the API Campy® test misidentifies all *Arcobacter* species as *A. cryaerophilus,* all the isolates identified as *A. cryaerophilus* were considered *Arcobacter* spp. The sequencing of the *atp*A gene revealed that all the isolates were *A. butzleri.*

3.3 Multi-Locus Sequence Typing

All the 20 *A. butzleri* isolates were successfully typed by MLST allowing the identification of 81 alleles of which 15 (18.5%) were previously unreported. A total of 16 STs were identified of which 14 (87.5 %) STs were previously unreported and resulted from new allele's sequences (**Table 1**).

4. Discussion

Arcobacter spp., is an important pathogen with an increasing interest for public health and food safety because of its frequent detection in different foods and its link to gastrointestinal diseases in humans (Fong *et al*., 2007). In addition, it represents a common pathogen isolated from fecal samples from people with acute enteric disease and it is responsible for traveler's diarrhea

(Collado and Figueras, 2011; Figueras *et al.*, 2014; Van den Abeele *et al.*, 2014). In order to perform proper food safety risk assessments, data on the presence of *Arcobacter* spp. and related genotypes circulating in foods are needed. Given the identification of *Arcobacter* spp. (especially *A. butzleri*) in raw milk and the evidence of *Arcobacter* spp. transmission to human that is also possible through the consumption or handling of contaminated raw milk led researchers to have more attention in this subject. Therefore, many authors focused their research on the topic through publication of data on *Arcobacter* spp. prevalences in Europe, Asia and Southern America (Ertas *et al.*, 2010; Milesi, 2010; Pianta *et al*., 2007; Revez *et al.*, 2013; Scullion *et al*., 2006; Serraino *et al*., 2013a; Shah *et al*., 2012; Yesilmen *et al.*, 2014). Surveys on BTM detected prevalence rates of 5.8%, 15% and 46% in Malaysia, Finland and Northern Ireland, respectively (Revez *et al*., 2013; Scullion *et al.*, 2006; Shah *et al*., 2012). In our survey the prevalence of *Arcobacter* spp. in BTM samples was low (4.1%) if compared to other Italian studies reporting a prevalence rate of 26% in BTM produced in Nothern Italy and of 57% as a result of an on in-line milk filters survey of dairy farms authorized to produce raw milk for direct human consumption (Milesi, 2010; Serraino *et al*., 2013a). Many factors could explain the remarkable difference between the prevalence rates reported in literature, such as, the different sampling methods, the absence of a standardized protocol for the detection of *Arcobacter*, but also the hygienic standard protocols adopted on farms, the feeding type, the climate, etc. (Collado and Figueras 2011; Hsu and Lee, 2015). In our study, all the isolates were identified as *A. butzleri* by molecular methods; these results were in agreement with other studies where *A. butzleri* was the main species isolated from raw milk and dairy plants (Ertas *et al.*, 2010; Giacometti *et al.*, 2015a; Ferreira *et al*., 2017; Milesi, 2010; Nieva-Echevarria *et al*., 2013; Pianta *et al.*, 2007; Shah *et al.* 2012; Revez *et al.*, 2013: Scuillon *et al*., 2006; Yesilmen *et al*., 2014). On the other hand, *A. butzleri* was the only species isolated probably because of the lack of standardized isolation protocols for *Arcobacter* spp. other than *A. butzleri*. In fact, our isolation procedure requires the use of an enrichment step that promotes the growth of *A. butzleri* which

could mask the presence of other *Arcobacter* species (Levican *et al*. 2016). This could represent a procedure's drawback.

Furthermore, the difference in findings between the molecular screening and cultural analysis are probably due to the viable but non-culturable (VNC) state of *Arcobacter* spp. in response to adverse environmental conditions (Mottola *et al.*, 2016a) or to the presence of free DNA deriving from dead bacterial cells. Notably, our study highlighted that a strong discrepancy between the biochemical and the molecular identification of *Arcobacter* exists. In fact, all the isolates were identified as *A. cryaerophilus* using a miniaturize biochemical identification kit and as *A. butzleri* when using the molecular approach. This could be due to the difficult of identification of *Arcobacter* at species level by biochemical tests; in fact the API Campy® test misidentifies all *Arcobacter* species as *A. cryaerophilus* These findings are noteworthy because they show that the epidemiological studies carried out using one or other identification method could have been affected by the chosen technique.

The ability of *A. butzleri* to grow between 4 and 10 °C, to survive to sanitizing procedures and adhere to glass, stainless steel and plastic surfaces and to form biofilm, could promote its survival, colonization and persistence in farms, milking equipment and dairy plants, becoming a source of contamination for milk and dairy products (Assanta *et al.,* 2002; Kjeldgaard *et al.,* 2009; Rasmussen *et al*., 2013; Mottola *et al.*, 2016 a,b; Giacometti *et al*., 2014;2015 a,b; Badilla-Ramírez *et al*. 2016; Serraino *et al*., 2013 a,b; Serraino and Giacometti, 2014). Contaminated raw milk and dairy products represent a potential source of human infections, having significant food safety and human health implications, especially for immunocompromised people for which the consumption of cheese manufactured from unpasteurized milk in small processing facilities employing traditional production technologies could represent a risk factor (Giacometti *et al*., 2015 b; Serraino *et al*., 2013 a).

It is well known that *Arcobacter* spp. population show a great genetic diversity hindering the epidemiologic studies, especially when the source of infection must be traced. In our study, among

20 genotyped isolates, five belonged to the already known ST66 and ST420. Both genotypes ST66 and ST420 were reported in a previous survey on dairy plants in Italy (De Cesare *et al*., 2016) . The detection of the genotypes ST66 and ST420 from our samples, supports the hypothesis that some genotypes could be associated with specific foods. Our study led to the identification of new alleles and new STs, confirming that the *A. butzleri* population has a great genetic diversity (Alonso *et al*., 2014; De Cesare *et al*., 2015; Merga *et al*., 2011; Merga *et al.,* 2013; Miller *et al.,* 2009; Perez-Cataluna *et al.,* 2017; Rasmussen *et al.,* 2013). In fact, in the present study the 87.5 % of the detected STs were unreported; the presence of new alleles among the seven analysed loci, or from new combinations of known alleles, highlights a high diversity among the strains and confirms that recombination is possible in *A. butzleri* (Alonso *et al.,* 2014). Among new alleles, the gene *gly*A was the most diverse, confirming the diversity observed by Pérez-Cataluña *et al*. (2017).

On the other hand, other authors have also reported a high heterogeneity among isolates using different genotyping techniques such as Pulsed-Field Gel Electrophoresis (PFGE), Multiple Locus Variable-Number Tandem Repeat Analysis (MLVA), Amplified Fragment Length Polymorphism (AFLP), Random Amplification of Polymorphic DNA (RAPD), and Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) (Forsythe, 2006; Douidah *et al*., 2014; Ramees *et al*., 2014).

However, in comparison with other genotyping methods, MLST is a good typing method because it gives fast and comparable results, and has been used as a routine molecular typing procedure for *Arcobacter* spp. in several studies (Ramees *et al*., 2014).

In conclusion, our study clearly shows the presence of *A. butzleri* in BTM in Southern Italy and a large genetic diversity between the isolates, contributing effectively to fill up the knowledge gap on this foodborne pathogen. The presence of *A. butzleri* in raw milk, could represent a hazard for consumers; thus, its presence should be carefully taken into account by both dairy food business operators and competent authority for reducing the foodborne risk linked to this pathogen.

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Table 1 *A. butzleri* MLST analysis results.

Isolate	Herd	*aspA*	*atpA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	ST
1	Herd 27	15	10	1	17	19	2	13	66
2	Herd 34	15	10	1	17	19	2	13	66
3	Herd 38	6	23	1	11	494	58	199	**627**
4	Herd 63	48	25	41	19	487	101	**272**	**633**
5	Herd 64	15	10	1	17	186	102	13	**628**
6	Herd 70	77	**209**	1	17	**637**	**339**	199	**634**
7	Herd 155	5	5	9	15	120	7	6	**629**
8	Herd 166	20	39	34	19	104	**340**	51	**635**
9	Herd 167	23	17	17	19	461	11	65	**630**
10	Herd 184	209	15	**186**	48	**638**	74	86	**646**
11	Herd 227	5	5	5	15	66	11	10	420
12	Herd 241	**309**	**210**	4	146	467	58	14	**636**
13	Herd 242	20	20	11	19	**639**	255	11	**647**
14	Herd 244	13	12	1	**208**	**640**	290	165	**648**
15	Herd 261	**310**	133	11	19	19	123	**271**	**637**
16	Herd 271	20	12	11	19	458	11	10	**631**
17	Herd 274	15	10	1	17	19	2	13	66
18	Herd 312	5	5	5	15	66	11	10	420
19	Herd 344	48	25	41	19	487	101	**272**	**633**
20	Herd 351	17	15	15	12	66	102	17	**632**

Highlights

Bulk tank milk produced in southern Italy is contaminated by *Arcobacter butzleri*.

Large genetic diversity of *A. butzleri* isolated from bulk tank milk in southern in Italy and identification of 14 previously unreported sequence-types.

Great discrepancy of the two technical approaches used for the identification of *Arcobacter* spp.

Large genetic diversity of *Arcobacter butzleri* isolated from raw milk in Southern Italy

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Abstract

Arcobacter butzleri is a zoonotic foodborne pathogen able to cause enteric and extraintestinal diseases. Its occurrence in foodstuff is well recognized worldwide but data on its presence in foods from Southern Italy are scarce. In this study the results on the occurrence and genotyping of

40 *Arcobacter* spp. in bulk milk samples collected in Southern Italy are reported. Out of 484 samples,
41 64 (13.2%) resulted positive for the presence of *Arcobacter* spp. using Real Time PCR but as few as
42 31.2% of these samples turned out as positive by using the cultural method, showing an overall
43 prevalence of 4.1%. All isolates were identified as *A. cryaerophilus* using the biochemical
44 identification whilst the sequencing of the *atp*A gene revealed that all the isolates were *A. butzleri.*

Among the confirmed isolates, 16 different Sequence Types (ST) were identified using the Multi Locus Sequence Typing (MLST), 14 (87.5 %) of which were previously unreported. Our survey reveals the presence of *A. butzleri* in bulk tank milk from Southern Italy and highlights the discrepancy between the two approaches used both for the detection (i.e., real time PCR vs cultural method) and the identification (i.e., biochemical test vs aptA sequencing) of *Arcobacter* spp In addition, a large genetic diversity among the isolates was detected and this makes the identification of source of the infections very challenging in outbreaks investigation.

Key Words: *Arcobacter*, Genotyping, Multi Locus Sequence Typing (MLST), Real-time PCR**,** Bulk Tank Milk

Introduction

Arcobacter spp. has been associated with human and animal disease and it is considered an important foodborne pathogen (Collado and Figueras, 2011; Ho *et al.*, 2006). The true incidence of human infections associated with *Arcobacter* is unknown, because these bacteria are not routinely investigated during human diarrheal diseases. In addition to this, a standardized protocol for their detection and characterization is not available (Collado and Figueras, 2011; Figueras *et al*., 2014). Among the several known *Arcobacter* species, *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have been recognized as those of clinical importance for animals and humans (Collado *et al*., 2011; Figueras at *al*., 2014; Hsu and Lee, 2015; Peréz-Cataluña *et al*., 2018; Ramees *et al*., 2017; Whiteduck-Léveillée *et al.*, 2015). In humans, these species have been associated with enterocolitis, peritonitis and bacteremia (Jiang *et al*., 2010; Lappi *et al*., 2013; Mottola *et al*., 2016a; Webb *et al*., 2016), while in animals they can cause gastroenteritis, mastitis, bacteremia and reproductive disorders (Arguello *et al*., 2015; Ho *et al*., 2006; Logan *et al*., 1982; Van Driessche and Houf, 2008; Whiteduck-Léveillée *et al*., 2016). Although the infectious dose has not yet been established, point-source outbreaks caused by *Arcobacter* spp. have been associated with well water ingestion, or with

the handling or consumption of contaminated raw or poorly-cooked animal food products. Also, direct contact with infected animals has been reported as a potential source of human infection (Fernandez *et al.*, 2015). In fact, the presence of *Arcobacter* has been documented worldwide from a wide range of sources and hosts with *A. butzleri* as the most prevalent species, followed by *A. cryaerophilus* and *A. skirrowii* (Collado and Figueras, 2011; Fallas-Padilla *et al*., 2014; Ramees *et al.*, 2017). *Arcobacter* spp. have also been isolated from faeces of healthy and sick humans and animals, including cattle, poultry, small ruminants, pigs and wild-living birds (Bogantes *et al*., 2015; Collado *et al*., 2009; Van Driessche *et al.*, 2003; Ottaviani *et al.*, 2017). In addition, *Arcobacter* have been detected from different foods such as fresh and ready to eat vegetables (González and Ferrús, 2011; González *et al*., 2017; Mottola *et al.*, 2016b), meat and meat products (Rivas *et al*., 2004; Rahimi, 2014, Lehmann *et al*., 2015), shellfish (Leoni *et al*., 2017; Levican *et al.*, 2014; Mottola *et al.*, 2016a), fish (Laishram *et al.*, 2016), eggs (Lee *et al.*, 2016) and drinking water (Ertas *et al*., 2010; Jalava *et al*., 2014; Jacob *et al.*, 1998). However, for better evaluating the foodborne risk linked to *Arcobacter* spp., more information on its occurrence in foods is needed (Lappi *et al.*, 2013). Regarding milk and dairy products, the detection of *Arcobacter* from these foodstuffs has been also reported (Logan *et al*., 1982; Pianta *et al*., 2007; Scullion *et al.*, 2006) but data on the occurrence of *Arcobacter* spp. in raw milk are still scarce (Shah *et al*., 2011). In Italy, 19 million tons of cow's milk are produced every year (www.agri.istat.it), mostly intended for cheese making or direct consumption as pasteurized or sterilized milk. However, in the last few years, the sale of raw milk for direct consumption via vending machines could have increased the risk of contact between humans and zoonotic agents (Haran *et al.*, 2012). Our work aims at improving the knowledge on the occurrence of *Arcobacter* spp. and its molecular characterization in bulk tank milk (BTM) samples collected in Southern Italy.

2. Matherials and methods

2.1 Sampling

The survey investigated two Italian Regions, Apulia and Basilicata, located in Southern Italy. On the whole, in these Regions are located 1.230 dairy farms with approximate 130.000 animals (www.vetinfo.it). A total of 484 BTM samples, corresponding to 39.4% of the total number of farms, were collected during 2014 to 2015. Specifically, the samples were from 396 dairy farms in Apulia and from 88 in Basilicata. The samples were aseptically collected in 500-mL sterile plastic containers, carried to laboratory in cooled containers within 24 hours after the of sampling. Samples were stored at -80 °C until analyzed.

2.2. Samples processing

BTM samples were defrosted and mixed using an agitator; then, 10 mL of milk were added to 90 mL of *Arcobacter* broth (Oxoid, Milan, Italy) supplemented with Cefoperazone, Amphotericin B and Teicoplanin (CAT selective supplement SR0174E; Oxoid) in sterile bags and homogenized using a stomacher (PBI International, Milan, Italy) at 11.000 rev min $^{-1}$ for 1 min. Then, the bags were incubated at 30 °C under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) by means of the CampyGen gas generating system (Oxoid) for 48 h.

2.3 Molecular screening

2.3.1 DNA extraction from enrichment broth

After incubation, 1 mL of enrichment broth was centrifuged at 13.000 rpm for 5 min at room temperature. The supernatant was discarded and the pellet was subjected to DNA extraction using the heat lysis and snap chilling method as described by Rasmussen et al., 2013 with some modifications. Briefly, 200 µL of sterile distilled water was added to the pellet and boiled in a water

bath at 100 °C for 15 minutes. The cell lysate was immediately transferred into ice and centrifuged at 13.000 rpm for 2 minutes. Supernatant was collected and used as DNA template for direct real-time PCR detection.

2.3.2 Real-time PCR

Genus specific real-time PCR was performed in order to screen the presence of *Arcobacter* spp. directly on the bacterial lysate. The reactions were performed in a final volume of 25 µL, using 1.25 µL EvaGreen 20X (Biotium, Hayward, USA), 0.2 nM of each dNTP, 2.5 µL of HotMaster Taq Buffer 10X (5PRIME, Hilden, Germany), 1 U of HotMaster Taq DNA Polymerase (5PRIME, Hilden, Germany), 5 pmol of each oligonucleotide primer and 2 µL of DNA template. The oligonucleotide primers used in this study (Arco-Fw and Arco-Rv), described by Gonzàlez et al., 2014. The amplification profile was carried out as follows: 95 °C for 3 min, followed by 40 cycles consisting of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. *A. butzleri* ATCC 46916T, *A. cryaerophilus* ATCC 43158T and *A. skirrowii* ATCC 51132 were used as positive controls. In order to identify nonspecific products, the melting curve was generated at the end of each run, thus exposing the final PCR product to a temperature gradient from about 60 °C to 90 °C in 20 min. The PCR reactions were processed in Applied Biosystems® 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, USA). All reactions were performed in duplicate.

2.4 Isolation and biochemical identification of Arcobater *spp.*

Ten mL of real-time PCR *Arcobacter* spp. positive enrichment broths were filtered using 0.45 µm membrane filters (Sartorius Stedim Biotech GmbH, Germany). Then, 200 µL of each filtered sample were streaked in parallel on Columbia Blood, Modified Charcoal Cefoperazone Deoxycholate (MCCD) and Karmali Agar plates (Oxoid). Plates were incubated at 30 °C under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) as described above, for 3-4 days. After

incubation, five typical *Arcobacter* spp. colonies for each sample were picked, subcultured onto Columbia Blood Agar and incubated for 48 h at 30 °C under microaerophilic conditions.

The colonies were confirmed morphologically by Gram staining and by determination of oxidase (Oxidase strips, Oxoid Microbact, Basingstoke, UK) and catalase activity (Mottola et al., 2016a; Mottola et al, 2016b). In addition, presumptive *Arcobacter* spp. colonies were further subjected to biochemical identification using API Campy® bioMerièux. The colonies identified as *Arcobacter* spp. were transferred onto *Arcobacter* broth (Oxoid, Basingstoke, UK) and incubated at 30 °C for 48 h.

2.5 Molecular identification and MLST typing of Arcobacter isolates

The extraction of DNA of isolates previously identified as *Arcobacter* spp. was performed using the GenomicPrep® kit (GE Helthcare. Illinois, USA) following the manufacturer's instructions. The identification of the *Arcobacter* isolates was determined using the *atp*A gene sequencing as described by Miller at al., 2014.

MLST was carried out on one identified isolate per positive sample using primers and conditions described by Miller et al., 2009. Specifically, the amplification and the sequencing of the seven housekeeping genes (*asp*A, *atp*A, *gln*A, *glt*A, *gly*A, *pgm* and *tkt*) included in the *Arcobacter* scheme of the PubMLST database were performed (http://pubmlst.org/arcobacter/).

The PCR products were purified using ExoSAP-IT according to supplier recommendations (GE Healthcare). Sequence reactions were carried out using BigDye 3.1 Ready reaction mix (Life Technologies) according to the manufacturer's instructions. The sequenced products were separated with a 3130 Genetic Analyzer (Life Technologies). Sequences were imported and assembled with Bionumerics 7.6 software (Applied Maths, Belgium). Any new alleles and STs were assigned by submitting the DNA sequences to the *Arcobacter* MLST database (https://pubmlst.org/arcobacter/).

3. Results

3.1 Molecular screening

The Real-Time PCR performed on enrichment broth from each BTM sample gave positive results for *Arcobacter* spp. in 64/484 (13.2 %) BTM samples. Specifically, all the positive samples were from Apulia (64/396) and none of Basilicata samples were positive for *Arcobacter* spp.

3.2 Confirmation of Real-Time PCR screening by cultural methods and identification of Arcobacter isolates

The cultural analysis carried out on the 64 Real-Time PCR positive enrichment broth showed typical *Arcobacter* colonies in 20 (31.2%) samples. On the whole, 4.1 % of BTM samples were positive for *Arcobacter* spp. Biochemical tests identified all isolates as *A. cryaerophilus*. Since the API Campy® test misidentifies all *Arcobacter* species as *A. cryaerophilus,* all the isolates identified as *A. cryaerophilus* were considered *Arcobacter* spp. The sequencing of the *atp*A gene revealed that all the isolates were *A. butzleri.*

3.3 Multi-Locus Sequence Typing

All the 20 *A. butzleri* isolates were successfully typed by MLST allowing the identification of 81 alleles of which 15 (18.5%) were previously unreported. A total of 16 STs were identified of which 14 (87.5 %) STs were previously unreported and resulted from new allele's sequences (**Table 1**).

4. Discussion

Arcobacter spp., is an important pathogen with an increasing interest for public health and food safety because of its frequent detection in different foods and its link to gastrointestinal diseases in humans (Fong *et al*., 2007). In addition, it represents a common pathogen isolated from fecal samples from people with acute enteric disease and it is responsible for traveler's diarrhea

(Collado and Figueras, 2011; Figueras *et al.*, 2014; Van den Abeele *et al.*, 2014). In order to perform proper food safety risk assessments, data on the presence of *Arcobacter* spp. and related genotypes circulating in foods are needed. Given the identification of *Arcobacter* spp. (especially *A. butzleri*) in raw milk and the evidence of *Arcobacter* spp. transmission to human that is also possible through the consumption or handling of contaminated raw milk led researchers to have more attention in this subject. Therefore, many authors focused their research on the topic through publication of data on *Arcobacter* spp. prevalences in Europe, Asia and Southern America (Ertas *et al.*, 2010; Milesi, 2010; Pianta *et al*., 2007; Revez *et al.*, 2013; Scullion *et al*., 2006; Serraino *et al*., 2013a; Shah *et al*., 2012; Yesilmen *et al.*, 2014). Surveys on BTM detected prevalence rates of 5.8%, 15% and 46% in Malaysia, Finland and Northern Ireland, respectively (Revez *et al*., 2013; Scullion *et al.*, 2006; Shah *et al*., 2012). In our survey the prevalence of *Arcobacter* spp. in BTM samples was low (4.1%) if compared to other Italian studies reporting a prevalence rate of 26% in BTM produced in Nothern Italy and of 57% as a result of an on in-line milk filters survey of dairy farms authorized to produce raw milk for direct human consumption (Milesi, 2010; Serraino *et al*., 2013a). Many factors could explain the remarkable difference between the prevalence rates reported in literature, such as, the different sampling methods, the absence of a standardized protocol for the detection of *Arcobacter*, but also the hygienic standard protocols adopted on farms, the feeding type, the climate, etc. (Collado and Figueras 2011; Hsu and Lee, 2015). In our study, all the isolates were identified as *A. butzleri* by molecular methods; these results were in agreement with other studies where *A. butzleri* was the main species isolated from raw milk and dairy plants (Ertas *et al.*, 2010; Giacometti *et al.*, 2015a; Ferreira *et al*., 2017; Milesi, 2010; Nieva-Echevarria *et al*., 2013; Pianta *et al.*, 2007; Shah *et al.* 2012; Revez *et al.*, 2013: Scuillon *et al*., 2006; Yesilmen *et al*., 2014). On the other hand, *A. butzleri* was the only species isolated probably because of the lack of standardized isolation protocols for *Arcobacter* spp. other than *A. butzleri*. In fact, our isolation procedure requires the use of an enrichment step that promotes the growth of *A. butzleri* which

297 could mask the presence of other *Arcobacter* species (Levican *et al*. 2016). This could represent a
298 procedure's drawback.
299 Furthermore, the difference in findings between the molecular screening and cultural analysis are
300 probably due to the viable but non-culturable (VNC) state of *Arcobacter* spp. in response to adverse
301 environmental conditions (Mottola *et al.*, 2016a) or to the presence of free DNA deriving from dead
302 bacterial cells. Notably, our study highlighted that a strong discrepancy between the biochemical
303 and the molecular identification of *Arcobacter* exists. In fact, all the isolates were identified as *A.*
304 *cryaerophilus* using a miniaturize biochemical identification kit and as *A. butzleri* when using the
305 molecular approach. This could be due to the difficult of identification of *Arcobacter* at species
306 level by biochemical tests; in fact the API Campy® test misidentifies all *Arcobacter* species as *A.*
307 *cryaerophilus* These findings are noteworthy because they show that the epidemiological studies
308 carried out using one or other identification method could have been affected by the chosen
309 technique.
310 The ability of *A. butzleri* to grow between 4 and 10 °C, to survive to sanitizing procedures and
311 adhere to glass, stainless steel and plastic surfaces and to form biofilm, could promote its survival,
312 colonization and persistence in farms, milking equipment and dairy plants, becoming a source of
313 contamination for milk and dairy products (Assanta *et al.,* 2002; Kjeldgaard *et al.,* 2009;
314 Rasmussen *et al*., 2013; Mottola *et al.*, 2016 a,b; Giacometti *et al*., 2014;2015 a,b; Badilla-Ramírez
315 *et al*. 2016; Serraino *et al*., 2013 a,b; Serraino and Giacometti, 2014). Contaminated raw milk and
316 dairy products represent a potential source of human infections, having significant food safety and
317 human health implications, especially for immunocompromised people for which the consumption
318 of cheese manufactured from unpasteurized milk in small processing facilities employing traditional
319 production technologies could represent a risk factor (Giacometti *et al*., 2015 b; Serraino *et al*.,
230 2013 a).
276 It is well known that *Arcobacter* spp. population show a great genetic diversity hindering the
277 epidemiologic studies, especially when the source of infection must be traced. In our study, among

20 genotyped isolates, five belonged to the already known ST66 and ST420. Both genotypes ST66 and ST420 were reported in a previous survey on dairy plants in Italy (De Cesare *et al*., 2016) . The detection of the genotypes ST66 and ST420 from our samples, supports the hypothesis that some genotypes could be associated with specific foods. Our study led to the identification of new alleles and new STs, confirming that the *A. butzleri* population has a great genetic diversity (Alonso *et al*., 2014; De Cesare *et al*., 2015; Merga *et al*., 2011; Merga *et al.,* 2013; Miller *et al.,* 2009; Perez-Cataluna *et al.,* 2017; Rasmussen *et al.,* 2013). In fact, in the present study the 87.5 % of the detected STs were unreported; the presence of new alleles among the seven analysed loci, or from new combinations of known alleles, highlights a high diversity among the strains and confirms that recombination is possible in *A. butzleri* (Alonso *et al.,* 2014). Among new alleles, the gene *gly*A was the most diverse, confirming the diversity observed by Pérez-Cataluña *et al*. (2017).

On the other hand, other authors have also reported a high heterogeneity among isolates using different genotyping techniques such as Pulsed-Field Gel Electrophoresis (PFGE), Multiple Locus Variable-Number Tandem Repeat Analysis (MLVA), Amplified Fragment Length Polymorphism (AFLP), Random Amplification of Polymorphic DNA (RAPD), and Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) (Forsythe, 2006; Douidah *et al*., 2014; Ramees *et al*., 2014).

However, in comparison with other genotyping methods, MLST is a good typing method because it gives fast and comparable results, and has been used as a routine molecular typing procedure for *Arcobacter* spp. in several studies (Ramees *et al*., 2014).

In conclusion, our study clearly shows the presence of *A. butzleri* in BTM in Southern Italy and a large genetic diversity between the isolates, contributing effectively to fill up the knowledge gap on this foodborne pathogen. The presence of *A. butzleri* in raw milk, could represent a hazard for consumers; thus, its presence should be carefully taken into account by both dairy food business operators and competent authority for reducing the foodborne risk linked to this pathogen.

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Table 1 *A. butzleri* MLST analysis results.

Isolate	Herd	*aspA*	*atpA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	ST
1	Herd 27	15	10	1	17	19	2	13	66
2	Herd 34	15	10	1	17	19	2	13	66
3	Herd 38	6	23	1	11	494	58	199	**627**
4	Herd 63	48	25	41	19	487	101	**272**	**633**
5	Herd 64	15	10	1	17	186	102	13	**628**
6	Herd 70	77	**209**	1	17	**637**	**339**	199	**634**
7	Herd 155	5	5	9	15	120	7	6	**629**
8	Herd 166	20	39	34	19	104	**340**	51	**635**
9	Herd 167	23	17	17	19	461	11	65	**630**
10	Herd 184	209	15	**186**	48	**638**	74	86	**646**
11	Herd 227	5	5	5	15	66	11	10	420
12	Herd 241	**309**	**210**	4	146	467	58	14	**636**
13	Herd 242	20	20	11	19	**639**	255	11	**647**
14	Herd 244	13	12	1	**208**	**640**	290	165	**648**
15	Herd 261	**310**	133	11	19	19	123	**271**	**637**
16	Herd 271	20	12	11	19	458	11	10	**631**
17	Herd 274	15	10	1	17	19	2	13	66
18	Herd 312	5	5	5	15	66	11	10	420
19	Herd 344	48	25	41	19	487	101	**272**	**633**
20	Herd 351	17	15	15	12	66	102	17	**632**

Highlights

Bulk tank milk produced in southern Italy is contaminated by *Arcobacter butzleri*.

Large genetic diversity of *A. butzleri* isolated from bulk tank milk in southern in Italy and identification of 14 previously unreported sequence-types.

Great discrepancy of the two technical approaches used for the identification of *Arcobacter* spp.