Arcobacter spp. in bovine milk: An emerging pathogen with potential zoonotic risk

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Abstract

The aim of the present study was to assess the prevalence and genetic characteristics of *Arcobacter* spp. in bovine bulk tank milk produced in Apulia Region (Italy). Samples collected from 396 dairy farms, after enrichment in a selective broth, were subjected to an *Arcobacter* genus - specific Real Time PCR. Positive broths, previously filtered, were seeded on Karmali, MCCD and Columbia Blood Agar plates; presumptive *Arcobacter* spp. colonies were identified using an amplification and sequencing method and then characterized by Multi-Locus Sequence Typing (MLST). Prevalence of *Arcobacter* spp. in bovine milk samples was 5% (20/396); *A. butzleri* was the only isolated species, in agreement with previous studies that reported *A. butzleri* as the most commonly recovered species in milk and dairy products. MLST analysis of the 20 *A. butzleri* strains identified 81 alleles and 16 STs. Consistent with previous studies, MLST revealed a high level of heterogeneity between the *A. butzleri* isolates and confirmed the high discriminatory power of this method and its suitability for epidemiological investigations. This study confirmed the importance of raw milk as a possible source of *Arcobacter* spp. for humans.

Introduction

The *Arcobacter* genus has been linked to animal and human illness. The species *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii* have been associated with several cases of gastrointestinal disease in humans. *Arcobacter* spp. have been isolated from faeces of dairy animals and found to contaminate different foods of animal origin including milk. In fact, ingestion of *Arcobacter*-contaminated water and food is considered as the most probable route of transmission. In industrialized countries, the most important source of human *Arcobacter* infection is the consumption of raw or undercooked food (Giacometti *et al*., 2014, 2015a, 2015b; Van Driessche and Houf, 2008). Because of the lack of standardized method for the isolation of *Arcobacter* spp., the true prevalence of this potential pathogen in food is little known and probably underestimated (Collado and Figueras, 2011; Hsu and Lee, 2015; Ramees *et al*., 2017).

Currently, numerous genotyping methods are used for epidemiological molecular studies. Multi-Locus Sequence Typing (MLST) is a widely used technique for the genetic characterization of *Arcobacter* spp.; this methodology thanks to the availability of online database of all *Arcobacter* spp. isolated in the world, allows to compare strains resulting from different studies and various geographical areas (Alonso *et al*., 2014; De Cesare *et al*., 2015; Merga *et al*., 2013; Rasmussen *et al*., 2013). Few studies have investigated the presence of *Arcobacter* spp. in bovine milk and the prevalence ranged from 1 to 57% (Ertas *et al*., 2010; Milesi, 2010; Pianta and Passos, 2007; Revez *et al*., 2013; Scullion *et al*., 2006; Serraino *et al*., 2013a; Shah *et al*., 2012; Yesilmen *et al*., 2014). The aim of the present report was to establish the prevalence and characteristics of *Arcobacter* spp. in bovine bulk tank milk produced in Apulia Region (Italy).

Materials and Methods

Bovine bulk tank milk samples were collected from 396 dairy farms of Apulia Region. One milk sample per farm was aseptically collected and immediately transported under refrigeration to the laboratory where it was stored at -80°C before testing. Samples were thawed at room temperature and then 10 mL of milk were homogenized with 90 mL of Arcobacter Enrichment Broth (Oxoid, Milan, Italy) plus Cefoperazone, Amphotericin B, Teicoplanin (CAT) Selective Supplement (Oxoid) 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 (Gonzales *et al*., 2013). Ten mL of Real Time PCR *Arcobacter* spp. positive broths were filtered using 0.45 µm pore size syringe filters (Sartorius Stedim Biotech GmbH, Germany) and 0.2 mL of each filtered broth were spread in parallel onto Columbia Blood, Modified Charcoal Cefoperazone Deoxycholate (MCCD) and Karmali Agar plates (Oxoid). Plates were incubated at 30°C in microaerophilic conditions and checked daily for 3-4 days. Presumptive identification tests (Gram staining, catalase and oxidase tests) were performed on suspected colonies among those grown on Columbia Blood, MCCD and Karmali Agar plates. Colonies of Gram-negative spiral bacteria, oxidase and catalase positive were considered presumptive *Arcobacter* spp. and subjected to species identification using an *atpA* amplification and sequencing method (Miller *et al*., 2014). Colonies, all identified as *A. butzleri*, were characterized by Multi-Locus Sequence Typing (MLST) performed on seven housekeeping loci (*aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm* and *tkt*) according to the protocol published by Miller *et al.* (Miller *et al*., 2009). The different sequences were assigned as alleles and the alleles at the seven loci provided an allelic profile or ST. Allele numbers and STs were assigned using the specific MLST scheme (http://pubmlst.org/arcobacter/) (Miller *et al*., 2009).

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Results

The *Arcobacter* genus - specific PCR revealed the presence of *Arcobacter* spp. in 64 (16%) of the 396 milk samples analysed. *Arcobacter* spp. were isolated from 20 (31%) of the 64 PCR positive samples. Presumptive identification tests performed on suspected colonies always identified Gram-negative spiral bacteria, oxidase and catalase positive. Species identification of *Arcobacter* spp. presumptive colonies identified *A. butzleri* in all the 20 positive samples. Prevalence of *Arcobacter* spp. in bovine milk samples was 5% (20/396). All the 20 *A. butzleri* isolates were successfully typed by MLST and a large number of alleles and Sequence Types (STs) were recognized (Table 1).

Specifically, 81 alleles were identified across all the seven loci and 15 (19%) were previously unreported (Table 1). A total of 16 STs were identified among the 20 *A. butzleri* isolates analysed; overall 14 (87%) of the 16 STs identified were previously unreported and resulted from new alleles' sequences. Moreover, 13 (81%) of the 16 STs identified were represented by a single isolate and only ST66, ST420 and ST633 by more than one. The most common sequence type was ST66, identified in 3 (15%) of the isolates, followed by ST633 and ST420, both shared by 2 (10%) isolates.

Discussion

The aim of the present study was to assess the prevalence and characteristics of *Arcobacter* spp. in bovine bulk tank milk produced in Apulia Region. Prevalence of *Arcobacter* spp. in bovine milk samples was 5% (20/396). Studies on the presence of *Arcobacter* spp. in bovine milk have been performed in different countries and different results have been reported (Ertas *et al*., 2010; Milesi, 2010; Pianta and Passos, 2007; Revez *et al*., 2013; Scullion *et al*., 2006; Serraino *et al*., 2013a; Shah *et al*., 2012; Yesilmen *et al*., 2014). Surveys on bulk tank milk reported 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 Italy a prevalence rate of 26% was found in bulk tank milk produced in Lombardia Region and a study performed on in-line milk filters of dairy farms authorized to produce and sell raw milk for direct human consumption revealed *Arcobacter* spp. in 57% of analysed samples (Milesi, 2010; Serraino *et al*., 2013a). 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 detection of very different 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; Hsu and Lee, 2015).

A. butzleri was the only isolated species in the bulk tank milk samples analysed, in agreement with the results of previous studies that reported *A. butzleri* as the most commonly recovered species both in milk and milk products, and in dairy plants (Giacometti *et al*., 2013; Serraino *et al*., 2013a). The more frequent isolation of *A. butzleri* has been attributed to its ability to grow in several substrates and in different environmental conditions and to survive to sanitizing procedures (Giacometti *et al*., 2014; Rassmussen *et al*., 2013).

MLST analysis confirmed a considerable amount of genetic diversity between the *A. butzleri* isolates (Alonso *et al*., 2014; De Cesare *et al*., 2015; Merga *et al*., 2011, 2013; Miller *et al*., 2009; Pérez-Cataluña *et al*., 2017; Rasmussen *et al*., 2013). In fact, 13 (81%) of the 16 STs identified were represented by a single isolate and only ST66, ST420 and ST633 by more than one. Moreover, 15 (19%) of the 81 alleles and 14 (87%) of the 16 STs identified were previously unreported. A high degree of heterogeneity was demonstrated also by other authors using

Table 1. Origin and MLST typing data of *A. butzleri* strains isolated.

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

*Represent novel alleles or sequence types.

different genotyping methods such as PFGE (Pulsed-field gel electrophoresis), MLVA (Multiple Locus Variable-number Tandem repeat Analysis), AFLP (Amplified fragment length polymorphism), RAPD (Random Amplification of Polymorphic DNA), and ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus) (De Cesare *et al*., 2015; Douidah *et al*., 2014; Ramees *et al*., 2017). 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 (Alonso *et al*., 2014; De Cesare *et al*., 2015; Miller *et al*., 2009). The high variability of *A. butzleri* is believed to be due to genetic recombination (Alonso *et al*., 2014). Furthermore, the high level of heterogeneity among the *A. butzleri* isolates obtained by MLST confirms the high discriminatory power of this method and its suitability for epidemiological investigations.

Although the prevalence of *Arcobacter* spp. in bovine bulk tank milk from Apulia Region was low, the results of the present study demonstrate that raw milk could be a vehicle of this important zoonotic pathogen. Consumption of milk after heat treatment and the use of pasteurized milk in cheese production are believed to be effective in preventing potential public health risks. About 75% of raw milk produced in Apulia Region is delivered to artisanal cheese factories that transform milk in traditional dairy products, most of which, such as fresh stretched cheeses (mozzarella, burrata, scamorza etc.) are made from unpasteurized milk (Sottili, personal communication). Considering that it has been demonstrated that *Arcobacter* spp. is able to survive under the temperature conditions used for the production of many traditional dairy products such as mozzarella cheese (Serraino *et al*., 2013b), the possible public health implications due to the consumption of raw milk cheese must be seriously evaluated. Besides the risk associated to the consumption of contaminated raw milk and raw milk cheese, it must be also considered that these products could represent a potential source for the secondary contamination of ready to eat foods that are not usually cooked before consumption.

Conclusions

Although the prevalence of *Arcobacter* spp. in bovine bulk tank milk from Apulia Region was low, the results of the present study demonstrate that raw milk could be a potential source of this emerging zoonotic pathogen. Considering that most of milk produced in the investigated area is transformed in raw milk cheese, the results of the present study highlight the need for additional data in order to better assess the human health risk arising from the use of unpasteurized milk for the production of fresh and semi-hard cheese.

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