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; Miledi, 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 (Gonzalez 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, glbA, gflA, 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).
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 allelic 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; Rasmussen 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; Serga 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

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

| Strain | Source | aspA | atpA | gltA | glyA | pgm | ttk | 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   | 196* | 48   | 638* | 74  | 86 | 646* |
| 11     | FARM 227| 5    | 5    | 5    | 15   | 66  | 11  | 10 | 420 |
| 12     | FARM 241| 308* | 210* | 4    | 146  | 467 | 58  | 14 | 656* |
| 13     | FARM 242| 20   | 20   | 11   | 19   | 638* | 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 Intergenomic Consensus) (De Cesare et al., 2015; Doudah 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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