Arcobacter spp. in raw milk from vending machines in Piedmont and occurrence of virulence genes in isolates

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Abstract

Arcobacter spp. has been recognized as an emerging foodborne pathogen and a hazard to human health. In the dairy chain, it has been isolated from different sources, nevertheless data on Arcobacter occurrence in raw milk provided by vending machines are few. This study aimed to identify potentially pathogenic Arcobacter spp. in raw milk intended for human consumption sold through vending machines located in Piedmont. In an 8-month period, 37 raw milk samples were collected from 24 dairy farms: 12 (32.4%) were collected directly in the farms through automatic self-service vending machines (Table 1), were tested. Twelve (32.4%) raw milk samples were collected directly in the farms from bulk tank milk and 25 (67.6%) milk samples from vending machines (Table 1). Vending machines and farms where milk samples were collected are showed in Figure 1. For the detection of Arcobacter spp. the protocol described by Giacometti et al. (2015) was used modifying the isolation step, by using in parallel selective agar plate supplemented with 10% laked horse blood and the Campylobacter Blood-Free Selective Agar Base (CCDA – Oxoid). Suspected colonies on the two selective agar, appearing as small and transparent and flat translucent respectively, were subcultured and identified using MALDI-TOF MS (Vitek MS bioMerieux). To test the limit of detection of the protocol, we contaminated raw milk samples with 10⁴ CFU/mL, 10³ CFU/mL, 10² CFU/mL and 10 CFU/mL of A. butzleri DSM 8739TM strain; the analysis were performed twice for each contamination level. The aims of the study were to investigate the presence of Arcobacter spp. in raw milk intended for human consumption sold through vending machines located in Piedmont (North-West of Italy) or from bulk tank milk in farms authorized to production of raw milk and to define the occurrence of the virulence genes in isolates.

Introduction

The Arcobacter genus belongs to the Campylobacteraceae family and includes currently twenty-six species. Bacteria belonging to this genus are ubiquitous in the environment and animals can carry Arcobacter spp. asymptomatically in the gut. Three species, Arcobacter butzleri, A. cryaerophilus and A. skirrowii are commonly associated with human disease (Ramees et al., 2017). In industrialized countries the main source of infection for humans is the consumption of raw or minimally processed foods and water (Piva et al., 2017). Recently, Arcobacter spp. has been recognized as an emerging foodborne zoonotic pathogen and a serious hazard to human health (Ramees et al., 2017). Many putative virulence genes have been identified: cadF, HeCA and cij1349 genes are responsible for adhesion, ciaB for invasion, hecB, tlyA and pldA for lysis of erythrocytes, irgA for iron acquisition and maintaining of infection and mviN for peptidoglycan biosynthesis (Doudiah et al., 2011; Ramees et al., 2017; Giribau et al., 2015).

In the dairy chain, Arcobacter spp. has been isolated from different sources such as faeces, in-line milk filters, bulk tank milk, cheeses, and processing surfaces (Giacometti et al., 2015).

Materials and Methods

From November 2017 to June 2018, a total of 37 raw milk samples, which were from 24 dairy farms, located in Piedmont (in Turin and Cuneo provinces), that sell raw milk intended for human consumption through automatic self-service vending machines (Table 1), were tested. Twelve (32.4%) raw milk samples were collected directly in the farms from bulk tank milk and 25 (67.6%) milk samples from vending machines (Table 1). Vending machines and

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**Results**

Eight (21.6%) out of the 37 raw milk samples were positive for *Arcobacter* spp.; 4 (16%) out of the 25 samples from vending machines and 4 (33.3%) out of the 12 samples from bulk tank milk were positive. Seven (29.2%) dairy farms out of the 24 were positive. Positive sampling sites, both vending machines and bulk tank milk respectively, are showed in Figure 1.

All 8 isolates resulted to be *A. butzleri* both by MALDI-TOF MS and multiplex end-point PCR (Table 2). In dairy farm ID20, *A. butzleri* was isolated from both vending machine and bulk tank milk samples.

Dealing with the limit of detection, contaminated raw milk samples resulted to be positive for *Arcobacter* spp. isolation until the $10^2$ CFU/mL level for both determinations.

The rates of isolation of *Arcobacter* spp. from vending machines and from bulk tank milk, as well as from winter and from spring resulted to be not significantly different.

The genes *ciaB*, *miniN*, *tlyA*, *pldA* and *cadF* were detected in all the isolates (100%), the gene *cj1349* was detected in 87.5% of the isolates, the gene *hecB* in 25% of the isolates, whereas the genes *hecA*, and *argA* were detected in only 12.5% of isolates respectively (Table 2). A total of four P-types were highlighted, respectively five isolates in P-type 1 and only one isolate for each of the P-types 2-3-4. The two *A. butzleri* strains isolated from dairy farm ID20 showed different P-types: P-type 1 in the strain from vending machine and P-type 3 in the strain from bulk tank milk. Details about the identified species of isolates and the occurrence of the virulence genes are reported in Table 2.

| Dairy Farm | Strain Id | Sampling Site | Species Identification | Detection of virulence genes - PCR | Vitek MS | PldA | TlyA | CiaB | ArgA | HecA | MiniN | Cj1349 | HecB | P-type |
|------------|-----------|---------------|------------------------|-----------------------------------|----------|------|------|------|------|------|-------|-------|-------|-------|-------|
| 3          | 46550/18  | BTM           | *A. butzleri*          | +                                 | +       | +    | +    | +    | -    | -    | +     | +     | +     | -     | 1     |
| 5          | 101112/17 | VM            | *A. butzleri*          | +                                 | +       | +    | +    | +    | -    | -    | +     | +     | +     | -     | 1     |
| 6          | 46555/18  | VM            | *A. butzleri*          | +                                 | +       | +    | +    | +    | +    | +    | +     | +     | +     | +     | 4     |
| 8          | 50147/18  | BTM           | *A. butzleri*          | +                                 | +       | +    | +    | +    | +    | +    | +     | +     | +     | +     | -     |
| 20         | 18760/18  | BTM           | *A. butzleri*          | +                                 | +       | +    | +    | +    | -    | -    | +     | +     | -     | -     | 3     |
| 20         | 101129/17 | BMT           | *A. butzleri*          | +                                 | +       | +    | +    | +    | -    | -    | +     | +     | +     | -     | 1     |
| 21         | 101123/17 | BMT           | *A. butzleri*          | +                                 | +       | +    | +    | +    | -    | -    | +     | +     | -     | -     | 1     |
| 23         | 106579/17 | VM            | *A. butzleri*          | +                                 | +       | +    | +    | +    | -    | -    | +     | +     | +     | +     | 2     |

VM: vending machine; BMT: bulk tank milk.

Table 2. *Arcobacter* species isolated from raw milk samples intended for human consumption and occurrence of virulence genes in isolates.

Table 1. Samples collected during this study from vending machines and from bulk tank milk per year.

| Dairy Farm Id | Sampling site 2017 | Sampling site 2018 | Total |
|---------------|---------------------|---------------------|-------|
| Vending machine | Farm | Vending machine | Farm | |
| 1*           | 1          | 0          | 2       | 1          | 4       |
| 2*           | 1          | 0          | 2       | 0          | 3       |
| 3            | 0          | 0          | 0       | 1          | 1       |
| 4            | 0          | 0          | 1       | 0          | 1       |
| 5            | 1          | 0          | 0       | 0          | 1       |
| 6            | 0          | 0          | 1       | 0          | 1       |
| 7            | 0          | 0          | 1       | 0          | 1       |
| 8            | 0          | 0          | 1       | 1          | 2       |
| 9            | 0          | 0          | 1       | 1          | 2       |
| 10           | 1          | 0          | 0       | 0          | 1       |
| 11           | 0          | 0          | 1       | 0          | 1       |
| 12           | 0          | 0          | 0       | 1          | 1       |
| 13           | 0          | 0          | 1       | 0          | 1       |
| 14           | 0          | 1          | 1       | 0          | 2       |
| 15           | 0          | 0          | 0       | 1          | 1       |
| 16           | 0          | 0          | 1       | 0          | 1       |
| 17           | 0          | 0          | 0       | 1          | 1       |
| 18           | 0          | 0          | 0       | 1          | 1       |
| 19           | 0          | 0          | 1       | 0          | 1       |
| 20           | 0          | 1          | 1       | 0          | 2       |
| 21           | 0          | 1          | 0       | 0          | 1       |
| 22^          | 1          | 0          | 1       | 0          | 2       |
| 23           | 1          | 0          | 1       | 0          | 2       |
| 24^          | 1          | 0          | 1       | 1          | 3       |
| Total        | 7          | 3          | 18      | 9          | 37      |

*Dairy farms providing more vending machines. *In dairy farm ID22 and ID24, samples were collected from the same vending machine per year.
**Discussion**

The isolation of *Arcobacter* spp. from raw cow milk has been reported by various authors with a wide range (between 3.2 and 80%), and most of them reported *A. butzleri* as the most frequently identified species (Yesilmen *et al.*, 2014; Scullion *et al.*, 2006; Ramees *et al.*, 2017; Giacometti *et al.*, 2015); our results are in accordance with these data. As reported by many authors, differences in *Arcobacter* prevalence may be due to many factors as farm management (hygienic conditions, source of water, animal diet) as well as different sampling methods, isolation protocols and also the different type of samples (bulk tank milk, individual cow milk samples, in-line milk filters) (Scullion *et al.*, 2006; Ertas *et al.*, 2012; Serraino *et al.*, 2013).

The ciaB, mviN, tlyA, cj1349, pldA and cadF genes are mainly implicated in adhesion and invasion mechanisms and are the most frequently detected genes in the present study, with an occurrence higher than reported by Piva *et al.* (2017) in samples collected from dairy chain but with different scenarios (overall prevalence of 57.5%). From different hosts and environments like human stool, food products, processing water, faecal samples, slaughterhouse processing line equipment, the prevalence values ranging from 66 to 100% (Douidah *et al.*, 2012; Karadas *et al.*, 2013; Ferreira *et al.*, 2014; Tabatabaei *et al.*, 2014; Girbau *et al.*, 2015; Zacharow *et al.*, 2015; Laishram *et al.*, 2016; Mottola *et al.*, 2016). In accordance with the studies of Piva *et al.* (2017) and Girbau *et al.* (2015), a low occurrence of hecA, hecB and irgA genes was observed in dairy isolates whereas in other food sources and food chains a high occurrence of these genes was reported (Karadas *et al.*, 2013; Ferreira *et al.*, 2014; Lehmann *et al.*, 2015; Zacharow *et al.*, 2015; Mottola *et al.*, 2016).

In one dairy farm (ID20) *A. butzleri* was detected both from vending machine and bulk tank milk, but the two isolates showed different P-types profiles (P-type 1 and P-type 3 respectively); to note that the two samples were collected from a vending machine in 2017 and from bulk tank milk in 2018 respectively. *A. butzleri* has been reported to be an environmental microorganism able to survive in adverse conditions thanks to its ability to produce biofilm or to be incorporated in pre-existing biofilms (Giacometti *et al.*, 2015). Faecal contamination should not be considered the main source of *Arcobacter* spp. in dairy farm, as reported by Giacometti *et al.* (2015) water is an important route of transmission of *Arcobacter* both for the animals and the farm environment: different species and genotypes were detected throughout a year surveillance suggesting reinfection of cattle during the observation period.

No significative differences were found between sampling sites (bulk tank milk versus vending machines) and sampling seasons (winter versus spring). Seasonal variations in the prevalence rate of *Arcobacter* were reported in a water treatment plant in Spain with 92% prevalence in spring, 83% in summer and 75% in winter but, as reported by many other authors, no significant differences were observed (Ramees *et al.*, 2017). In Italy a seasonal association has been reported for *A. butzleri* in bivalve molluscs samples with a significantly more likely presence during the winter-spring period rather than in the summer-autumn (Leoni *et al.*, 2017). Concerning the sampling sites, no data are currently available, and further samplings were needed to investigate the possible role of sampling site in maintaining *A. butzleri* in the environment.

In Italy, the sale of raw milk through vending machines is authorized by the Italian Ministry of Health and is regulated by an agreement between the state and the regions (Intesa Stato Regioni, 2007). This agreement sets the biosafety measures, the microbiological and chemical criteria for milk, the vending machine installation and management specifications which dairy farms must comply with. Since 2008 the Italian Ministry of Health with subsequent ordinances and the Ministerial Decree 12 December 2012 made it compulsory that all raw milk vending machines carry a warning that the milk should be boiled at home before consumption. Monitoring surveys have been conducted in Piedmont to check that the raw milk sold via vending machines...

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**Figure 1.** Vending machines (A) and bulk tank milk (B) where milk samples were collected: negative sampling sites (red), positive sampling sites for *A. butzleri* (blue).
The milk chain environment can be considered a good ecological niche for *A. butzleri*, a microorganism reported to be able to survive to routine sanitizing procedures and to persist on several processing surfaces. The isolation of this emerging pathogen in raw milk intended for human consumption sold through vending machines strengthens the compulsory consumption sold after boiling as required by current legislation. Moreover, the detection of *A. butzleri* in milk suggests the importance of enlarging the analytical investigations to other emerging microorganisms to date not included among the food safety criteria.

### Conclusions

The milk chain environment can be considered a good ecological niche for *A. butzleri*, a microorganism reported to be able to survive to routine sanitizing procedures and to persist on several processing surfaces. The isolation of this emerging pathogen in raw milk intended for human consumption sold through vending machines strengthens the compulsory consumption sold after boiling as required by current legislation. Moreover, the detection of *A. butzleri* in milk suggests the importance of enlarging the analytical investigations to other emerging microorganisms to date not included among the food safety criteria.

### References

Bianchi DM, Barbaro A, Gallina S, Vitale N, Chiavacci L, Caramelli M, Decastelli L, 2013. Monitoring of food-borne pathogenic bacteria in vending machine raw milk in Piedmont, Italy. Food Control 32:435-9.

Doudah L, De Zutter L, Vandamme P, Houf K, 2010. Identification of five human and mammal associated Arcobacter species by a novel multiplex-PCR assay. J Microbiol Meth 80:281-6.

Doudah L, De Zutter L, Baré J, De Vos P, Vandamme P, Vandenberg, O, Van den Abeele AM, Houf K, 2011. Occurrence of putative virulence genes in Arcobacter species isolated from humans and animals. J Clin Microbiol 50:735-41.

Ertas N, Dogruer Y, Gonalalan Z, Guner A, Ulcer I, 2010. Prevalence of Arcobacter species in drinking water, spring water, and raw milk as determined by multiplex PCR. J Food Protect 73:2099-102.

Ferreira S, Queiroz JA, Oleastro M, Domingues FC, 2014. Genotypic and phenotypic features of Arcobacter butzleri pathogenicity. Microb Pathogenesis 76:19-25.

Giacometti F, Lucchi A, Di Francesco A, Delogu M, Grilli E, Guarniero I, Stancampiano L, Manfreda G, Merialdi G, Serraino A, 2015. Arcobacter butzleri, Arcobacter cryaerophilus and Arcobacter skirrowii circulation in a dairy farm and sources of milk contamination. Appl Environmental Microbiol 81:5055-63.

Girbau C, Guerra C, Martinez-Malaxetxebarria I, Alonso R, Fernández-Astorga A, 2015. Prevalence of ten putative virulence genes in the emerging foodborne pathogen Arcobacter isolated from food products. Food Microbiol 52:146-9.

Karadas G, Sharbati S, Hänel I, Messelhäuser U, Glocker E, Alter T, Gözl G, 2013. Presence of virulence genes, adhesion and invasion of Arcobacter butzleri. J Appl Microbiol 115:583-90.

Intesa Stato Regioni e Province Autonome di Trento e Bolzano – ISR-25th-Jan-2007a.

Laihram M, Rathlavath S, Lekshmi M, Kumar S, Nayak BB, 2016. Isolation and characterization of Arcobacter spp. from fresh seafood and the aquatic environment. Int J Food Microbiol 232:87-9.

Leonì F, Chierichetti S, Santarelli S, Talevi G, Masini L, Bartolini C, Roccheggiani E, Naceur Haouet M, Ottaviani D, 2017. Occurrence of Arcobacter spp. and correlation with the bacterial indicator of faecal contamination Escherichia coli in bivalve molluscs from the Central Adriatic, Italy. Int J Food Microbiol 245:6-12.

Ministerial Decree 12 December 2012. Informazioni obbligatorie e misure a tutela del consumatore di latte crudo o crema cruda, in attuazione dell’art. 8, commi 6 e 9, del decreto-legge 13 settembre 2012, n. 158, recante “Disposizioni urgenti per promuovere lo sviluppo del Paese mediante un piu’ alto livello di tutela della salute” convertito, con modificazioni, dalla legge 8 novembre 2012, n. 189. (13A00727) (GU Serie Generale n.24 del 29-01-2013).

Mottola A, Bonera E, Bozzo G, Marchetti P, Celano GV, Colao V, Terio V, Tantillo G, Figuera MJ, Di Pinto A, 2016. Occurrence of emerging food-borne pathogenic Arcobacter spp. isolated from pre-cut (ready-to-eat) vegetables. Int J Food Microbiol 236:33-7.

Piva S, Gariano GR, Bonilauri P, Giacometti F, Decastelli L, Florio D, Massella E, Serraino A, 2017. Occurrence of putative virulence genes on Arcobacter butzleri isolated from three different environmental sites throughout the dairy chain. J Appl Microbiol 122:1071-7.

Ramees TP, Dhuam K, Karthik K, Rathore RS, Kumar A, Saminathan M, Tiwari R, Malik YA, Singh RK, 2017. Arcobacter: an emerging food-borne zoonotic pathogen, its public health concerns and advances in diagnosis and control–a comprehensive review. Vet Quart 37:136-61.

Serraino A, Florio D, Giacometti F, Piva S, Mion D, Zanoni RG, 2013. Presence of Campylobacter and Arcobacter species in in-line milk filters of farms authorized to produce and sell raw milk and of a water buffalo dairy farm in Italy. J Dairy Sci 96:2801-7.

Seulhion R, Harrington CS, Madden RH, 2006. Prevalence of Arcobacter spp. in raw milk and retail raw meats in Northern Ireland. J Food Protect 69:1986-90.

Tabatabaei M, Aski S, Shayegh H, Khoshbakht R, 2014. Occurrence of six virulence-associated genes in Arcobacter species isolated from various sources in Shiraz, Southern Iran. Microb Pathogenesis 66:1-4.

Zacharov I, Bystron J, Walecka-Zacharska E, Podkowik M, Bania J, 2015. Genetic diversity and incidence of virulence-associated genes of Arcobacter butzleri and Arcobacter cryaerophilus isolates from pork, beef, and chicken meat in Poland. BioMed Res Int 956507.

Yesilmen S, Vural A, Erkan ME, Yildirim IH, 2014. Prevalence and antimicrobial susceptibility of Arcobacter species in cow milk, water buffalo milk and fresh village cheese. Int J Food Microbiol 188:11-4.

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