

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 farm from bulk tank milk and 25 (67,6%) from vending machines. Eight (21,6%) out of the 37 milk samples and 7 (29,2%) out of the 24 dairy farms were positive for *Arcobacter* spp. by culture examination. Four (16%) out of the 25 samples from vending machines and 4 (33,3%) out of the 12 samples from bulk tank milk were positive. All 8 isolates were identified as *A. butzleri* both by MALDI-TOF MS and multiplex end-point PCR. According to the detection of virulence genes, a total of four Patho-types were highlighted: 5 isolates in P-type 1 and only one isolate for each of the P-types 2-3-4. *A. butzleri* isolates carrying encoding virulence factors genes were iso-

lated from raw milk intended for human consumption: these findings strengthen the compulsory consumption after boiling as required by current legislation and suggest the need of enlarging the analytical investigations to other microorganisms not yet included in the food safety criteria.

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. asymptotically 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 *cj1349* 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; Girbau *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).

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.

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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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 the 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.

Arcobacter species were confirmed using the multiplex PCR end-point described by Doudiah *et al.* (2010). The presence of the nine virulence genes (*cadF*, *pldA*, *tlyA*, *ciaB*, *irgA*, *hecA*, *mviN*, *cj1349* and *hecB*) was investigated by multiplex PCR end-point according to Doudiah *et al.* (2011). DNA was extracted from

Arcobacter isolates using Instagene Matrix (Bio-Rad). *A. butzleri* DSM 8739TM and *A. cryaerophilus* DSM 7289TM were used as reference strains in both PCR. According to the presence of the virulence genes, isolates were grouped in pathotypes, later labeled as P-types. For the analysis of the results, the two-sample Wilcoxon rank-sum (Mann-Whitney) tests were performed to compare the proportions of positive samples collected from vending machines to those collected from bulk tank milk and the proportions of positive samples collected in winter to those collected in spring.

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 10² 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*, *mniN*, *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 *irgA* 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.

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.

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

Dairy Farm Id	Strain Id	Sampling Site	Species Identification		Detection of virulence genes - PCR									
			Vitek MS	PCR	<i>cadF</i>	<i>pldA</i>	<i>tlyA</i>	<i>ciaB</i>	<i>irgA</i>	<i>hecA</i>	<i>mviN</i>	<i>cj1349</i>	<i>hecB</i>	P-type
3	46550/18	BTM	<i>A. butzleri</i>	<i>A. butzleri</i>	+	+	+	+	-	-	+	+	-	1
5	101112/17	VM	<i>A. butzleri</i>	<i>A. butzleri</i>	+	+	+	+	-	-	+	+	-	1
6	46555/18	VM	<i>A. butzleri</i>	<i>A. butzleri</i>	+	+	+	+	+	+	+	+	+	4
8	50147/18	BTM	<i>A. butzleri</i>	<i>A. butzleri</i>	+	+	+	+	-	-	+	+	-	1
20	18760/18	BTM	<i>A. butzleri</i>	<i>A. butzleri</i>	+	+	+	+	-	-	+	-	-	3
20	101120/17	VM	<i>A. butzleri</i>	<i>A. butzleri</i>	+	+	+	+	-	-	+	+	-	1
21	101123/17	BTM	<i>A. butzleri</i>	<i>A. butzleri</i>	+	+	+	+	-	-	+	+	-	1
23	106579/17	VM	<i>A. butzleri</i>	<i>A. butzleri</i>	+	+	+	+	-	-	+	+	+	2

VM: vending machine; BTM: bulk tank milk.

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.*, 2010; 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% (Doudah *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

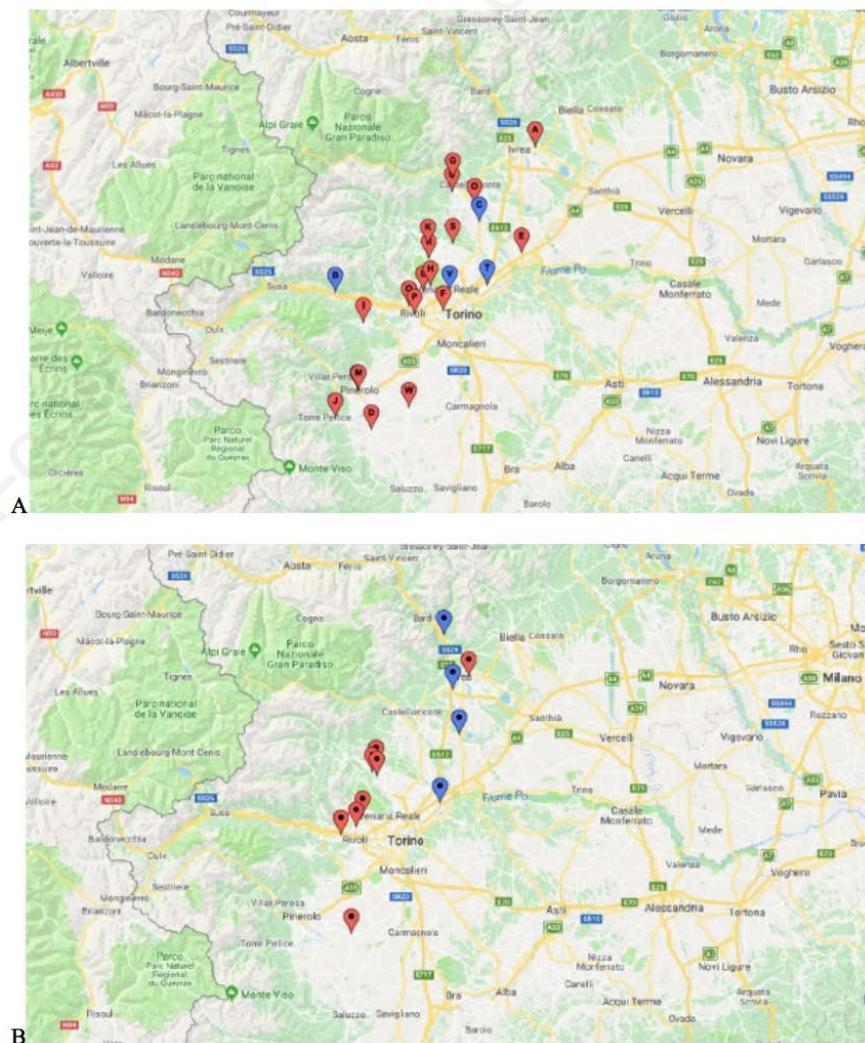


Figure 1. Vending machines (A) and bulk tank milk (B) where milk samples were collected: negative sampling sites (red), positive sampling sites for *A. butzleri* (blue).

complies with the criteria of the national regulation; Bianchi *et al.* (2013) reported the results of a three-year monitoring survey in Piedmont confirming that the unpasteurized milk could be vehicle of a variety of microorganisms and can be an important source of foodborne illness outbreaks, especially if consumers are young, elderly, or ill. The occurrence of *A. butzleri* isolates carrying encoding virulence factors genes underlines once again the importance of respecting the compulsory consumption of vending machine milk after boiling and suggests the need of widening the detection of other microorganisms not yet included in the monitoring plans.

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 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.

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