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ORIGINAL ARTICLE

Multilocus sequence typing of *Arcobacter butzleri* isolates collected from dairy plants and their products, and comparison with their PFGE types

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Keywords
Arcobacter butzleri, concordance between genotyping methods, dairy products and dairy plants, multi locus sequence typing, pulsed-field gel electrophoresis.

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Abstract

Aims: The present study aimed to determine, by multilocus sequence type (MLST), the heterogeneity level of *Arcobacter butzleri* isolates and to compare MLST and pulsed-field gel electrophoresis (PFGE) in terms of discriminatory power (DI) as well as unidirectional and bi-directional concordance.
Methods and Results: *Arcobacter butzleri* isolates ($N = 133$) from dairy products and environmental samples, collected from dairy plants, were characterized by MLST and PFGE with *Sac*II and classified in 29 sequence types (STs), 47 PFGE and 62 type strains (TS). Among the 119 alleles, 19 were previously unreported and the same for all the STs but two. A significant linkage disequilibrium was detected when the complete ST data set was analysed The DIs of MLST, PFGE and their combination were 0·937, 0·953 and 0·965 respectively. The adjusted Wallace coefficients between MLST and PFGE as well as PFGE and MLST were 0·535 and 0·720 respectively; the adjusted Rand coefficient was 0·612.
Conclusions: The *A. butzleri* studied population showed recombination to some degree. PFGE showed a DI higher than MLST. Both methods presented good concordance. The TS analysis seems to show persistence of the same strain on time and possible cross-contaminations between food and environmental sites.
Significance and Impact of the Study: This study provides insights in the *A. butzleri* population found in raw milk, cheese, and dairy production plants. The data suggest that MLST and PFGE genotypes correlate reasonably well, although their combination results in optimal resolution.

Introduction

The *Arcobacter* genus has been linked to animal and human illness (Ho *et al.* 2006). In particular, the species *Arcobacter butzleri* and *Arcobacter cryaerophilus* have been associated with several cases of gastrointestinal disease, with persistent diarrhoea as the main symptom in humans (Collado and Figueras 2011). *Arcobacter spp.* have been isolated from faecal samples of dairy animals (Piva *et al.* 2013; Shah *et al.* 2013) and found to contaminate different foods of animal origin, including milk (Ertas *et al.* 2010; Shah *et al.* 2012). Giacometti *et al.* (2013a,b) isolated *A. butzleri* from environmental samples collected from an artisanal and an industrial cheese factory, and in a ready-to-eat cheese.

One of the main challenge in *Arcobacter* research is to identify the different transmission routes of this pathogen to humans. In fact, a direct connection between consumption of *Arcobacter* contaminated foods and water and human illness has not yet been established. To trace

the sources of contamination in a food processing plant during an outbreak or during extended epidemiological investigations *Arcobacter* isolates must be characterized by discriminative typing methods. However, at present, no criterion standard typing method or strategy has been proposed.

Typing of *Arcobacter* isolates using enterobacterial repetitive intergenic consensus (ERIC)-PCR, pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) analysis, has been reviewed (Forsythe 2006). Douidah *et al.* (2014) compared six human- and animal-associated *Arcobacter* species by ERIC-PCR, PFGE with *Kpn*I and AFLP but no correlation between typing patterns from strains isolated from humans, animals and foods was noticed. In 2009 Miller *et al.* developed a multilocus sequence typing (MLST) protocol for *Arcobacter* species, based on sequence information at seven housekeeping loci. Few works have used this technique to evaluate the diversity of *Arcobacter* isolates. Merga *et al.* showed great diversity among *Arcobacter* spp. isolates from cattle faecal samples (2011) and among *A. butzleri* isolates from the same origin (2013) using MLST. Rasmussen *et al.* (2013) used the same genotyping method to evaluate the heterogeneity of *A. butzleri* isolates obtained from a Danish slaughterhouse on two consecutive production days and after the sanitizing in between. The results showed high strain variability; however,, they suggested that the repeated detection of two sequence types (STs) could be related to cross contamination. Alonso *et al.* (2014) applied MLST to type 45 *A. butzleri* isolates from poultry, raw milk, mussels, clams, pork meats and minced beef and found a large amount of diversity among them. There were no links between STs and food sources and possible recombination in *A. butzleri* strains was shown.

Carriço *et al.* (2006) and Severiano *et al.* (2011) proposed the use of the adjusted Rand coefficient (AR) and the adjusted Wallace (AW) coefficient and corresponding confidence intervals (CI) as quantitative measures of the bi-directional and uni-directional congruence between typing methods, taking into account that agreement between typing methods may arise by chance alone. These coefficients show if two typing methods provide overlapping results instead of complementary information to figure out the epidemiological correlation and the genetic diversity among strains belonging to the same species.

In view of the diversity reported for *Arcobacter* populations, the aim of this study was to apply MLST to determine the level of heterogeneity among 133 *A. butzleri* isolates obtained from different dairy products, as well as food contact and not-food contact surfaces, sampled within artisanal and industrial dairy plants in two Italian regions. The MLST profiles were added in the MLST database to increase the number of new alleles and MLST patters available for comparison with human isolates. Moreover, the MLST patterns were compared with the PFGE profiles of the same isolates, some of which previously collected. Finally, the discriminatory power and concordance between the two genotyping methods were assessed.

Materials and methods

Isolates tested

A total of 133 *A. butzleri* isolates were genotyped in this study using MLST. Moreover, 19 isolates were characterized using PFGE with *Sac*II as restriction enzyme. The PFGE profiles of the remaining 114 isolates were previously obtained (Giacometti *et al.* 2013a) and analysed as part of this comparative study.

The tested isolates were collected in two Italian regions, named Emilia Romagna (ER) ($N = 114$) and Sardinia (SS) ($N = 19$), located 650 km far away. Four samplings were performed in ER between October and December 2012, on the water buffalo milk processing days. Four samplings were performed in SS between November 2013 and April 2014 on industrial sheep ricotta cheese purchased at retail and on the environment of the industrial cheese manufacturing plant. Overall, the isolates were obtained from food samples (i.e. raw cow and buffalo milk and ricotta cheese) ($N = 43$); food contact surfaces (i.e. bulk tank valve, cheese vat, drainage table, milk pump, mozzarella moulding roller and ricotta cheese processing trolley) ($N = 49$) and not-food contact surfaces (i.e. floors of cooler room, packaging area, processing area and floor drain) ($N = 41$). The isolation details, summarized in Table 1, were previously described (Giacometti *et al.* 2013a; Scarano *et al.* 2014). Briefly, samples were transferred to the laboratory in refrigerated coolers at 5 ± 3°C and processed within 1 h. Isolation was performed according to the method of Houf *et al.* (2001). At least 10 colonies, if present, suspected of being *Arcobacter* spp. were picked from each plate, subcultured, and subjected to presumptive identification using tests that included growth under aerobic conditions and cellular morphology. Moreover, the isolates were subjected to DNA extraction using a REDExtract-N-Amp tissue PCR kit (Sigma, Milan, Italy) and identified by the multiplex PCR described by Douidah *et al.* (2010). Genomic DNA from at least three colonies from each positive sample, when available, was tested by MLST and PFGE.

Table 1 Source, sampling time, sampling area, multilocus sequence typing results, pulsed-field gel electrophoresis results and type strains (TS) of the 133 *Arcobacter butzleri* isolates included in this study

Isolate	Source	Sampling	Region	*asp*A	*atp*A	*gln*A	*glt*A	*gly*A	*pgm*	*tkt*	ST	Pulsotype	TS
Food isolates													
56	Raw cow milk	II	ER	6	20	114	15	**489**	102	2	**437**	9	437-9
2	Raw cow milk	II	ER	6	20	114	15	**490**	102	2	**438**	8	438-8
53/2	Raw cow milk	II	ER	6	20	114	15	**490**	102	2	**438**	26	438-26
41	Raw cow milk	III	ER	6	20	114	15	346	102	2	**423**	8	423-8
45	Raw cow milk	III	ER	6	20	114	15	346	102	2	**423**	8	423-8
162	Raw cow milk	III	ER	6	20	114	15	346	102	2	**423**	8	423-8
40	Raw cow milk	III	ER	6	20	114	15	346	102	2	**423**	38	423-38
42	Raw cow milk	III	ER	66	12	5	19	120	211	58	**424**	32	424-32
210	Raw cow milk	IV	ER	6	20	114	15	**489**	102	2	**437**	39	437-39
224	Raw cow milk	IV	ER	6	20	114	15	**490**	102	2	**438**	38	438-38
132	Raw WB milk	I	ER	5	5	5	15	66	11	10	**420**	42	420-42
127	Raw WB milk	I	ER	5	5	5	15	66	11	10	**420**	43	420-43
126	Raw WB milk	I	ER	3	3	1	2	65	17	13	**427**	24	427-24
149	Raw WB milk	I	ER	**225**	2	2	15	11	113	40	**432**	27	432-27
68	Raw WB milk	II	ER	20	2	1	**158**	28	26	165	**425**	20	425-20
77	Raw WB milk	II	ER	20	2	1	**158**	28	26	165	**425**	20	425-20
91	Raw WB milk	II	ER	20	2	1	**158**	28	26	165	**425**	20	425-20
95	Raw WB milk	II	ER	23	4	1	19	**486**	26	165	**428**	20	428-20
37	Raw WB milk	III	ER	17	27	15	144	**485**	36	32	**422**	22	422-22
108	Raw WB milk	III	ER	11	2	11	44	56	111	40	**429**	21	429-21
35	Raw WB milk	III	ER	20	39	34	23	**488**	101	165	**435**	18	435-18
38	Raw WB milk	III	ER	226	158	26	48	484	254	188	439	24	439-24
36	Raw WB milk	III	ER	226	158	26	48	484	254	188	439	29	439-29
39	Raw WB milk	IV	ER	17	27	15	144	**485**	36	32	**422**	22	422-22
185	Raw WB milk	IV	ER	3	3	1	2	65	17	13	**427**	34	427-34
219	Raw WB milk	IV	ER	23	7	11	15	66	102	2	**436**	25	436-25
220	Raw WB milk	IV	ER	23	7	11	15	66	102	2	**436**	25	436-25
207	Raw WB milk	IV	ER	23	7	11	15	66	102	2	**436**	48	436-48
71	Ricotta cheese	II	ER	15	10	1	17	19	2	13	66	28	66-28
82	Ricotta cheese	II	ER	15	10	1	17	19	2	13	66	28	66-28
101	Ricotta cheese	II	ER	15	10	1	17	19	2	13	66	28	66-28
117	Ricotta cheese	II	ER	15	10	1	17	19	2	13	66	28	66-28
163	Ricotta cheese	II	ER	15	10	1	17	19	2	13	66	28	66-28
198	Ricotta cheese	II	ER	15	10	1	17	65	2	13	66	28	66-28
205	Ricotta cheese	II	ER	15	10	1	17	19	2	13	66	37	66-37
SS 2	Ricotta cheese R1-I	SS I	Sardinia	153	4	40	19	**491**	102	9	**440**	47	440-47
SS 3	Ricotta cheese R1-I	SS I	Sardinia	153	4	40	19	**491**	102	9	**440**	47	440-47
SS 7	Ricotta cheese R2-I	SS I	Sardinia	10	20	11	**159**	**492**	123	11	**441**	47	441-47
SS 12	Ricotta cheese R3-I	SS I	Sardinia	20	39	11	11	98	87	178	**442**	40	442-40
SS 16	Ricotta cheese R4-I	SS I	Sardinia	20	39	11	11	98	87	178	**442**	40	442-40
SS 18	Ricotta cheese R5-I	SS I	Sardinia	10	20	11	**159**	**492**	123	11	**441**	47	441-47
SS 19	Ricotta cheese R5-I	SS I	Sardinia	10	20	11	**159**	**492**	123	11	**441**	47	441-47
SS 21	Ricotta cheese R6-I	SS I	Sardinia	10	20	11	**159**	**492**	123	11	**441**	47	441-47
Isolates from food contact surfaces													
93	Bulk tank valve	I	ER	5	5	5	15	66	11	10	**420**	45	420-45
125	Bulk tank valve	II	ER	11	2	11	44	56	111	40	**429**	21	429-21
124	Bulk tank valve	II	ER	3	3	1	32	65	21	13	**430**	14	430-14
109	Bulk tank valve	III	ER	3	3	1	32	65	21	13	**430**	14	430-14
172	Bulk tank valve	III	ER	48	25	41	37	**487**	101	55	**434**	12	434-12
183	Bulk tank valve	IV	ER	3	3	1	32	65	21	13	**430**	14	430-14
190	Bulk tank valve	IV	ER	3	3	1	32	65	21	13	**430**	15	430-15
123	Cheese vat	I	ER	66	12	5	19	120	211	58	**424**	32	424-32
92	Cheese vat	I	ER	3	3	1	2	65	17	13	**427**	24	427-24

(*Continued*)

Table 1 (*Continued*)

Isolate	Source	Sampling	Region	*asp*A	*atp*A	*gln*A	*glt*A	*gly*A	*pgm*	*tkt*	ST	Pulsotype	TS
114	Cheese vat	I	ER	3	3	1	2	65	17	13	**427**	24	427-24
5/1	Cheese vat	I	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
99	CHEESE VAT	II	ER	20	12	11	19	36	102	10	**421**	3	421-3
120	Cheese vat	II	ER	20	39	11	19	36	102	10	**431**	3	431-3
22	Cheese vat	II	ER	48	25	41	37	**487**	101	55	**434**	13	434-13
121	Cheese vat	II	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
20	Cheese vat	III	ER	48	25	41	37	**487**	101	55	**434**	36	434-36
21	Cheese vat	III	ER	48	25	41	37	**487**	101	55	**434**	36	434-36
239	Cheese vat	IV	ER	3	3	1	32	65	21	13	**430**	10	430-10
197	Cheese vat	IV	ER	3	3	1	32	65	21	13	**430**	14	430-14
199	Cheese vat	IV	ER	3	3	1	32	65	21	13	**430**	14	430-14
10	Drainage table	III	ER	48	25	41	37	**487**	101	55	**434**	41	434-41
11	Drainage table	III	ER	20	39	34	23	**488**	101	165	**435**	3	435-3
7	Drainage table	III	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
201	Drainage table	IV	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
216	Drainage table	IV	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
217	Drainage table	IV	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
113	Milk pump	I	ER	20	12	11	19	36	102	10	**421**	3	421-3
159	Milk pump	I	ER	**227**	**157**	11	19	19	**255**	11	**433**	15	433-15
58	Milk pump	II	ER	13	4	40	19	413	11	58	**419**	3	419-3
3	Milk pump	II	ER	13	4	40	19	413	11	58	**419**	17	419-17
60	Milk pump	III	ER	13	4	40	19	413	11	58	**419**	2	419-2
64	Milk pump	III	ER	5	5	5	15	66	11	10	**420**	42	420-42
49	Milk pump	III	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
86	Milk pump	III	ER	6	20	114	15	**490**	102	2	**438**	6	438-6
158	Milk pump	IV	ER	3	3	1	2	65	17	13	**427**	24	427-24
187	Milk pump	IV	ER	3	3	1	2	65	17	13	**427**	24	427-24
195	Milk pump	IV	ER	3	3	1	2	65	17	13	**427**	24	427-24
196	Milk pump	IV	ER	3	3	1	2	65	17	13	**427**	24	427-24
4	Milk pump	III	ER	13	4	40	19	413	11	58	**419**	1	419-1
106	MMR	I	ER	20	12	11	19	36	102	10	**421**	5	421-5
62	MMR	II	ER	5	5	5	15	66	11	10	**420**	43	420-43
47	MMR	II	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
46	MMR	II	ER	20	39	34	23	**488**	101	165	**435**	19	435-19
12	MMR	III	ER	5	5	5	15	66	11	10	**420**	44	420-44
13	MMR	III	ER	5	5	5	15	66	11	10	**420**	44	420-44
48	MMR	III	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
152	MMR	IV	ER	**225**	2	2	15	11	113	40	**432**	27	432-27
240	MMR	IV	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
SS 45	Trolley PA	SS II	Sardinia	77	23	11	11	90	123	11	**445**	11	445-11
Isolates from nonfood contact surfaces													
111	Floor drain	I	ER	5	5	5	15	66	11	10	**420**	46	420-46
135	Floor drain	I	ER	6	20	114	15	**490**	102	2	**438**	26	438-26
107	Floor drain	II	ER	20	12	11	19	36	102	10	**421**	3	421-3
80	Floor drain	II	ER	5	12	24	15	362	8	24	**426**	7	426-7
81	Floor drain	II	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
118	Floor drain	II	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
16	Floor drain	III	ER	17	27	15	144	**485**	36	32	**422**	17	422-17
43	Floor drain	III	ER	6	20	114	15	346	102	2	**423**	8	423-8
14	Floor drain	III	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
15	Floor drain	III	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
17	Floor drain	III	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
18	Floor drain	III	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
19	Floor drain	III	ER	20	39	34	23	**488**	101	165	**435**	17	435-17

(*Continued*)

Table 1 (*Continued*)

Isolate	Source	Sampling	Region	*asp*A	*atp*A	*gln*A	*glt*A	*gly*A	*pgm*	*tkt*	ST	Pulsotype	TS
178	Floor drain	IV	ER	5	5	5	15	66	11	10	**420**	46	420-46
179	Floor drain	IV	ER	3	3	1	2	65	17	13	**427**	24	427-24
177	Floor drain	IV	ER	3	3	1	2	65	17	13	**427**	31	427-31
SS 27	Floor drain PA	SS I	Sardinia	30	5	5	15	**495**	11	58	**446**	16	446-16
SS 41	Floor drain PA	SS II	Sardinia	5	5	5	15	66	11	10	**420**	30	420-30
SS 55	Floor drain PA	SS II	Sardinia	10	20	11	**159**	**492**	123	11	**441**	4	441-4
SS 69	Floor CR	SS II	Sardinia	10	20	11	**159**	**492**	123	11	**441**	4	441-4
SS 39	Floor PK	SS I	Sardinia	77	23	4	11	**494**	58	199	**444**	33	444-33
SS 40	Floor PK	SS I	Sardinia	77	23	4	11	**494**	58	199	**444**	35	444-35
SS 79	Floor PK	SS II	Sardinia	10	20	11	**159**	**492**	123	11	**441**	4	441-4
SS 31	Floor PA	SS I	Sardinia	8	8	**137**	**160**	**493**	200	8	**443**	34	443-34
SS 32	Floor PA	SS I	Sardinia	8	8	**137**	**160**	**493**	200	8	**443**	34	443-34
SS 33	Floor PA	SS I	Sardinia	8	8	**137**	**160**	**493**	200	8	**443**	34	443-34
105	Cooler room F	II	ER	5	5	5	15	66	11	10	**420**	45	420-45
128	Cooler room F	II	ER	5	5	5	15	66	11	10	**420**	45	420-45
57	Cooler room F	II	ER	66	12	5	19	120	211	58	**424**	32	424-32
74	Cooler room F	II	ER	66	12	5	19	120	211	58	**424**	32	424-32
78	Cooler room F	II	ER	66	12	5	19	120	211	58	**424**	32	424-32
103	Cooler room F	II	ER	66	12	5	19	120	211	58	**424**	32	424-32
116	Cooler room F	II	ER	66	12	5	19	120	211	58	**424**	32	424-32
112	Cooler room F	II	ER	11	2	11	44	11	111	40	**429**	17	429-17
67	Cooler room F	II	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
115	Cooler room F	II	ER	20	39	34	23	**488**	101	165	**435**	17	435-17
31	Cooler room F	III	ER	5	5	5	15	66	11	10	**420**	45	420-45
27	Cooler room F	III	ER	5	5	5	15	66	11	10	**420**	46	420-46
32	Cooler room F	III	ER	5	5	5	15	66	11	10	**420**	46	420-46
25	Cooler room F	III	ER	20	12	11	19	36	102	10	**421**	5	421-5
30	Cooler room F	III	ER	20	39	34	23	**488**	101	165	**435**	17	435-17

WB, water buffalo; MMR, mozzarella moulding roller; PA, processing area; PK, packaging area; CR, cooling room; F, floor; ER, Emilia Romagna; ST, sequence types.
Boldface entries represent novel alleles or STs.

Multilocus sequence typing

Multilocus sequence typing was performed on all isolates on seven housekeeping loci (*asp*A, *atp*A, *gln*A, *glt*A, *gly*A, *pgm* and *tkt*) according to the protocol published by 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 *Arcobacter* specific MLST scheme (http://pubmlst.org/arcobacter/) (Miller *et al.* 2009). Tests software (S.T.A.R.T. ver. 2; http://pubmlst.org/software/analysis/start2) was used to perform recombination and selection (d_N/d_S) tests. Sawyer's tests were relied on to provide statistical evidence of recombinational exchanges of the sequences analysed (Sawyer 1989). Linkage analysis was carried out by using the index of association (IA) (Maynard Smith *et al.* 1993) and the standardized index of association (IsA) (Haubold and Hudson 2000). For both indexes, an absolute value of zero indicates that the population is freely recombinating and is not clonal, whereas a value significantly different from 0 indicates a high genetic diversity of the population in which recombination has been rare or absent.

Pulsed-field gel electrophoresis

The DNA extracted from the 19 isolates collected in Sardinia (Table 1) was characterized by PFGE with *Sac*II, as previously described (Giacometti *et al.* 2013a). The PFGE patterns of these 19 isolates and those of the 114 isolates previously obtained (Giacometti *et al.* 2013a) were analysed by BIONUMERICS 7.5 software (BIONUMERICS; Applied Maths, Keistraat, Belgium) using the Dice similarity index and a dendrogram was constructed with the UPGMA method (Unweighted Pair Group Method with Arithmetic Mean). The optimization setting was 1·0%; the band position tolerance was 0·8%. Isolates showing a PFGE similarity level ≥90% were assigned to the same pulsotype.

Calculation of discriminatory power and concordance between typing methods

The discriminatory indexes (DI) of MLST, PFGE and their combination were calculated using the Simpson's diversity index described by Hunter and Gaston (Hunter and Gaston 1988; Hunter 1990). The adjusted Wallace's coefficient (AW) and the AR coefficient, along with the respective confidence intervals (CIs), were all calculated using the Comparing Partitions Website (http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Toll).

Results

Multilocus sequence typing

All the 133 isolates were successfully typed by MLST and classified in 29 ST (Table 1). A large number of alleles and STs were recognized in this study (Table 2). Overall, 119 alleles were identified across the seven loci, ranging from 13 for *gln*A to 25 for *gly*A. A total of 19 out of the 119 alleles (15·96%) were previously unreported. All STs were new, except for ST 439 and ST 66; 8 were unique STs, identified in single isolates, whereas 21 were shared between 2 and 23 isolates. The STs identified in foods, food contact and nonfood contact surfaces were 19, 12 and 14 respectively (Table 1). The most common ST was that named as 435, identified in 17·29% of the isolates from environmental sources and in one food sample, followed by STs 420 and 427, identified in 11·28 and 7·52% of the isolates, respectively, from all the tested sources. The remaining STs showed a frequency ranging between 5·26 and 0·75% of isolates. The number of alleles for each locus, the number of silent polymorphic sites, the proportion of nucleotide alterations that changed the amino acid sequence (nonsynonymous substitution, d_N) and the silent changes (synonymous substitution, d_S) (d_N/d_S) are shown in the Table 2. The d_N/d_S ratios varied across the seven loci, ranging between 0·0000 for *glt*A to 0·1246 for *atp*A.

Table 2 Characteristics of the multilocus sequence typing loci

Locus	Alleles	Silent polymorphic sites	d_N	d_S	d_N/d_S
*asp*A	19	24	0·0013	0·0775	0·0173
*atp*A	15	22	0·0044	0·0354	0·1246
*gln*A	13	15	0·0023	0·0386	0·0608
*glt*A	14	16	0·0000	0·0446	0·0000
*gly*A	25	31	0·0064	0·0620	0·1026
pgm	18	30	0·0019	0·0844	0·0220
tkt	15	20	0·0011	0·0698	0·0160

Pulsed-field gel electrophoresis

The 19 isolates characterized using PFGE with *Sac*II were classified in 9 different pulsotypes (Table 1). The pulsotypes indicated as 4, 34, 40 and 47 were shared between 2 and 6 isolates, whereas those named 11, 16, 30, 33 and 35 were identified in single isolates. In particular, the isolates with pulsotype 4 were collected on the floor of the packing and cooling room, as well as floor drain, during the second sampling performed in Sardinia. On the contrary, those with pulsotype 34 were all collected on the floor of the processing area during the first sampling performed in the same region. The remaining shared pulsotypes (i.e. 40 and 47) were identified among isolates from ricotta cheese, collected during the first sampling in Sardinia.

The pulsotypes of the 19 isolates collected in Sardinia were analysed using BIONUMERICS along with the isolates collected in ER, previously described (Giacometti *et al.* 2013a). Overall, the 133 isolates were classified in 47 pulsotypes. A total of 23 pulsotypes were identified in single isolates and 26 were shared between 2 and 20 isolates. The most common pulsotype was that indicated as 17, associated with environmental isolates, followed by pulsotypes 24 and 32, identified in isolates from all the tested sources and food as well as food contact surfaces respectively. The only pulsotype identified in both ER and Sardinia was the 34, associated with one isolate collected from raw water buffalo milk in ER during the fourth sampling and three environmental isolates collected on the floor of the processing area during the first sampling in Sardinia. However, the three environmental isolates shared also the same sequence type (i.e. ST 443), whereas the food isolate showed ST 427.

Type strains

The tested isolates were classified in 62 different type strains (TS) according to their MLST and PFGE profiles. Overall, 37 TSs were identified in single isolates and 25 were shared between 2 and 20 isolates. In particular, the TS 441-4, 425-20, 436-25, 66-28, 443-34, 434-36, 442-40, 420-44, 440-47 and 441-47 were associated with isolates collected from the same type of source during the same sampling (Table 3); the TS 430-14 and 420-46 to isolates from food contact and not food contact surfaces, respectively, obtained during different sampling times; the TS 423-8 to one isolate from floor drain and three isolates from raw cow milk obtained during the same sampling performed in ER; the remaining TS to isolates collected from different types of sources at different sampling times.

During each sampling, the number of TSs identified among the isolates from each source ranged between one

Table 3 Sampling source, sampling area and sampling time of the isolates belonging to the same type strain

				Sampling area and time					
Type strain	Source (n. isolates)	Source type		Emilia Romagna				Sardinia	
421-3	Cheese vat (1)	FC			2nd				
421-3	Floor drain (1)		NFC		2nd				
421-3	Milk pump (1)	FC		1st					
441-4	Floor drain (1)		NFC						2nd
441-4	Floor, cooling room (1)		NFC						2nd
441-4	Floor, packing area (1)		NFC						2nd
421-5	Cooler room floor (1)		NFC			3rd			
421-5	Mozzarella moulding roller (1)	FC		1st					
423-8	Floor drain (1)		NFC			3rd			
423-8	Raw cow milk (3)	F				3rd			
430-14	Bulk tank valve (1)	FC			2nd				
430-14	Bulk tank valve (1)	FC				3rd			
430-14	Bulk tank valve (1)	FC					4th		
430-14	Cheese vat (2)	FC					4th		
435-17	Cheese vat (1)	FC		1st					
435-17	Cheese vat (1)	FC			2nd				
435-17	Cooler room floor (2)		NFC		2nd				
435-17	Cooler room floor (1)		NFC			3rd			
435-17	Drainage table (1)	FC				3rd			
435-17	Drainage table (3)	FC					4th		
435-17	Floor drain (2)		NFC		2nd				
435-17	Floor drain (5)		NFC			3rd			
435-17	Milk pump (1)	FC				3rd			
435-17	Mozzarella moulding roller (1)	FC			2nd				
435-17	Mozzarella moulding roller (1)	FC				3rd			
435-17	Mozzarella moulding roller (1)	FC					4th		
425-20	Raw WB milk (3)	F			2nd				
429-21	Bulk tank valve (1)	FC			2nd				
429-21	Raw WB milk (1)	F				3rd			
422-22	Raw WB milk (1)	F				3rd			
422-22	Raw WB milk (1)	F					4th		
427-24	Cheese vat (2)	FC		1st					
427-24	Floor drain (1)		NFC						
427-24	Milk pump (4)	FC					4th		
427-24	Raw WB milk (1)	F		1st					
436-25	Raw WB milk (2)	F					4th		
438-26	Floor drain (1)		NFC	1st					
438-26	Raw cow milk (1)	F			2nd				
432-27	Mozzarella moulding roller (1)	FC					4th		
432-27	Raw WB milk (1)	F		1st					
66-28	Ricotta cheese (6)	F			2nd				
424-32	Cooler room floor (5)		NFC		2nd				
424-32	Raw cow milk (1)	F				3rd			
443-34	Floor, processing area (2)		NFC					1st	
434-36	Cheese vat (2)	FC				3rd			
442-40	Ricotta cheese (2)	F						1st	
420-42	Milk pump (1)	FC				3rd			
420-42	Raw WB milk (1)	F		1st					
420-43	Mozzarella moulding roller (1)	FC			2nd				
420-43	Raw WB milk (1)	F		1st					
420-44	Mozzarella moulding roller (2)	FC				3rd			
420-45	Bulk tank valve (1)	FC		1st					
420-45	Cooler room floor (2)		NFC		2nd				

(*Continued*)

Table 3 (*Continued*)

Type strain	Source (n. isolates)	Source type	Sampling area and time			
			Emilia Romagna			Sardinia
420-45	Cooler room floor (1)	NFC		3rd		
420-46	Cooler room floor (2)	NFC		3rd		
420-46	Floor drain (1)	NFC	1st			
420-46	Floor drain (1)	NFC			4th	
440-47	Ricotta cheese (2)	F				1st
441-47	Ricotta cheese (4)	F				1st

F, food; FC, food contact surface; NFC, nonfood contact surface; WB, water buffalo.

and four (Table 1). Among the 25 TSs shared between different isolates, 40% were detected among isolates collected from the same source at the same sampling time; 8% among isolates detected in the same type of source at different sampling times; 4% among isolates collected in different sources at the same sampling time and 48% between isolates collected from different sources during different sampling times (Table 3). The detection of isolates with the same TS in the same source at different sampling time could show the persistence of contamination on time, whereas the detection of isolates belonging to the same TS during the same sampling in different types of samples might suggest cross-contaminations between sources of the same or different type (Table 3).

Discriminatory power and concordance between typing methods

The DI of the applied typing methods ranged between 0·937 for MLST and 0·953 for PFGE. Combining the results collected using both typing methods the DI increased at 0·965 (Table 4). The DI calculated for MLST was significantly lower than that obtained for PFGE ($P = 0·043$) and the DI calculated for the combination of MLST and PFGE was significantly higher in comparison to that obtained using MLST ($P < 0·001$) and PFGE ($P = 0·006$) alone (Table 4).

The AW coefficient concerning the unidirectional concordance between PFGE and MLST (AW 0·720) was significantly higher ($P = 0·049$) than that between MLST and PFGE (AW 0·532) However, both methods showed a good concordance (i.e. AW > 0·50) (Table 5). This result means that the classification, in terms of different or identical genotype, obtained using PFGE can predict that found using MLST and the same for MLST *vs* PFGE, even if at a lower extent. Concerning the bi-directional concordance between the two methods, the AR value was 0·612 (0·469–0·767) confirming good correlation. The use of AW and respective CIs avoids the overestimation of unidirectional concordance between typing methods, which might due to chance alone, similar to AR and respective CIs for bidirectional concordance.

Table 4 Discriminatory power (DI) of the tested methods applied alone or in combination

Method	*N* types	*N* unique isolates	*N* clustered isolates	Cluster size	DI (95%)
MLST	29	8	125	2-23	0·937 (0·918–0·956)
PFGE	47	23	110	2-20	0·953 (0·933–0·972)
MLST/ PFGE	62	37	96	2-20	0·965 (0·949–0·982)

MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis.

Discussion

This study aimed to investigate the STs of 133 isolates of *A. butzleri* collected from dairy products, food contact and not-food contact surfaces, sampled within artisanal and industrial dairy plants in two Italian regions, named ER and Sardinia. The alleles and STs generated in this study were deposited in the PubMLST database and are available online. Overall, 19 alleles and 27 STs were previously unreported. According to Alonso *et al.* (2014), the high number of new alleles and STs generated could

Table 5 Uni-directional concordance between the applied typing methods

Typing method A	Typing method B	$AW_{A \rightarrow B}$ (95% CI)
MLST	PFGE	0·532 (0·410–0·654)
PFGE	MLST	0·720 (0·562–0·878)

AW, adjusted Wallace; MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis
P value 0·049.

reflect the heterogeneity of food and environmental *A. butzleri* isolates but also the relatively limited number of isolates currently available in the database, implemented with the new alleles and STs identified in this study. A further explanation might be the *Arcobacter* genome exposure to rapid genomic changes. In the 133 isolates tested in this study, the d_N/d_S ratios varied across the seven loci between 0·0000 for *glt*A to 0·1246 for *atp*A. These ratios are in agreement with those previously reported by Miller *et al.* 2009. Sawyer's tests revealed statistical evidence of recombinational exchanges for *asp*A (SSCF *P* value 0·001), *gly*A (SSCF *P* value 0·0049), *pgm* (SSCF *P* value 0·015) and *tkt* (SSCF *P* value 0·014). A significant linkage disequilibrium (i.e. $I_A = 3{\cdot}6717$ and IsA = 0·6119) was detected when the complete ST data set was analysed, indicating that the *A. butzleri* population is recombining to some degree.

The STs collected in this study were compared with the patterns collected by PFGE using *Sac*II as restriction enzymes. Furthermore, the discriminatory index, as well as unidirectional and bi-directional concordance between MLST and PFGE were assessed. Comparative studies on MLST and PFGE for *Arcobacter* typing were not previously published. Both MLST and PFGE exhibited a DI higher than 0·90, showing that they can discriminate *A. butzleri* isolates different from a genetic point of view with a probability higher than 90%. The discriminatory index of MLST was significantly lower than that calculated using PFGE. However, in comparison to PFGE, MLST has a high throughput, is much less labour intensive and the data generated are easier to analyse, interpret and share between laboratories. Combining both methods resulted in a probability to differentiate the isolates significantly higher than that obtained by MLST or PFGE alone. However, to apply both methods increases costs and execution time. Besides, the most promising routine epidemiological typing tool for foodborne pathogens seems to be represented by whole-genome sequencing (WGS).

The good correspondence between PFGE and MLST (AW 0·720) as well as MLST and PFGE (AW 0·532) means that the classification, in terms of different or identical genotype, obtained using PFGE can predict that found using MLST, and vice versa. However, the results on ST and pulsotype (Table 1) show isolates with the same ST displaying different pulsotypes, as well as isolates with the same pulsotype characterized by different STs. Therefore, there is concordance between those methods but they both provide a piece of information contributing to establish possible clonal relationships among the tested isolates.

The TS identified among the isolates from each source ranged between 1 and 4 (Table 1). This result underlines the importance to type a representative number of isolates from each source during each sampling when typing is performed for epidemiological investigations. Even if no TS was identified in both the regions sampled, the TS results seem to demonstrate persistence of contamination on time and cross-contaminations between environment sites as well as food and both food contact and not-food contact surfaces.

Conflict of Interest

All authors disclose any potential sources of conflict of interest that Editors may consider relevant to the present manuscript.

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