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Arcobacter butzleri, *Arcobacter cryaerophilus*, and *Arcobacter skirrowii* Circulation in a Dairy Farm and Sources of Milk Contamination

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Even though dairy cows are known carriers of *Arcobacter* species and raw or minimally processed foods are recognized as the main sources of human *Arcobacter* infections in industrialized countries, data on *Arcobacter* excretion patterns in cows and in milk are scant. This study aimed to identify potentially pathogenic *Arcobacter* species in a dairy herd and to investigate the routes of *Arcobacter* transmission among animals and the potential sources of cattle infection and milk contamination. A strategy of sampling the same 50 dairy animals, feed, water, and milk every month for a 10-month period, as well as the sampling of quarter milk, animal teats, the milking environment, and animals living on the farm (pigeons and cats), was used to evaluate, by pulsed-field gel electrophoresis (PFGE), the characteristic patterns in animals, their living environment, and the raw milk they produced. Of the 463 samples collected, 105 (22.6%) were positive for *Arcobacter* spp. by culture examination. All the matrices except quarter milk and pigeon gut samples were positive, with prevalences ranging from 15 to 83% depending on the sample. Only three *Arcobacter* species, *Arcobacter cryaerophilus* (54.2%), *A. butzleri* (34.2%), and *A. skirrowii* (32.3%), were detected. PFGE analysis of 370 isolates from positive samples provided strong evidence of *Arcobacter* circulation in the herd: cattle likely acquire the microorganisms by orofecal transmission, either by direct contact or from the environment, or both. Water appears to be a major source of animal infection. Raw milk produced by the farm and collected from a bulk tank was frequently contaminated (80%) by *A. butzleri*; our PFGE findings excluded primary contamination of milk, whereas teats and milking machine surfaces could be sources of *Arcobacter* milk contamination.

The genus *Arcobacter* belongs to the family *Campylobacteraceae* in the class *Epsilonproteobacteria*. It has a wide range of habitats and hosts and is ubiquitous in animals and the environment (1). Some *Arcobacter* species have recently been recognized as potential emerging enteropathogens (2).

Among the 20 recognized *Arcobacter* species (3), *Arcobacter butzleri*, *A. cryaerophilus*, and *A. skirrowii* cause infections in humans and are of prime clinical and veterinary importance, although the majority of species have never been isolated from mammals or birds. A wide range of host animals, including cattle, poultry, small ruminants, pigs, and wild-living birds, carry *Arcobacter* spp. asymptomatically and excrete them in their feces. Pets can also be asymptomatic carriers of *Arcobacter* spp. (4–6). Therefore, all these hosts can be sources of food or water contamination and consequently can pose risks for human health. In the specific case of the dairy chain, *Arcobacter* spp. have been isolated from fecal samples of dairy animals (7–13), in-line milk filters (14), cow and water buffalo milk (15–19), cheeses, and the cheese-processing environment (19–24).

Raw or minimally processed foods are usually considered the main sources of human *Arcobacter* infections in industrialized countries. The initial source in foods of animal origin seems to be fecal contamination during the various stages of production (15, 24, 25), although *Arcobacter* species can cause disease either through water-mediated food contamination or through the ingestion of contaminated water (26).

Several epidemiological studies have reported *Arcobacter* spp. in cattle or dairy farm animals used for food production (1, 7, 9, 12, 13), but few surveys have investigated the variability of excretion of *Arcobacter* species within a single herd, the carriage of these bacteria by individual cows over time, or their transmission among cows and between cows and their environment.

This longitudinal study aimed to identify potentially pathogenic *Arcobacter* species in a dairy herd, to monitor *Arcobacter* transmission among animals, and to disclose the potential sources of cattle infection and milk contamination. A strategy of sampling the same 50 dairy animals, feed, water, and milk every month for a 10-month period, as well as sampling the milking environment, was used to evaluate, by pulsed-field gel electrophoresis (PFGE), the characteristic patterns in animals, their living environment, and the raw milk they produced.

MATERIALS AND METHODS

Study farm and animals. The study was conducted on a dairy farm located in the Emilia-Romagna region, Northern Italy. The farm has a freestall barn and the usual husbandry practices and hygienic conditions. During the study, the farm housed about 170 Holstein Friesian cows, of which 80 were lactating and dry cows (>24 months old; referred to as

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TABLE 1 Samples collected during this study and *Arcobacter* species isolated from positive samples

Sample type	Total no. of samples analyzed	No. (%) of samples with:			
		Any *Arcobacter* spp.	*A. butzleri*	*A. cryaerophilus*	*A. skirrowii*
Feces	239	49 (20.5)	2 (0.8)	31 (12.9)	27 (11.2)
Water	30	24 (80)	12 (40)	19 (63.3)	4 (13.3)
Feed	20	3 (15)	ND[a]	2 (10)	2 (10)
Milk	10	8 (80)	8 (80)	ND	ND
Quarter milk	80	ND	ND	ND	ND
Teats	25	13 (52)	7 (28)	5 (20)	1 (4)
Milking system	6	5 (83.3)	5 (83.3)	ND	ND
Cat	6	3 (50)	3 (50)	ND	ND
Pigeons	47	ND	ND	ND	ND
Total	463	105 (22.67)	37 (7.99)	57 (12.3)	34 (7.3)

[a] ND, not detected.

adult animals) and 90 were calves and heifers (0 to 24 months old; referred to as young animals). The ages of the animals ranged from 0 to 5 years; each cow was identified by its unique farm identification number. Adult and young animals were housed in two different parts of the barn: adult animals were kept in cubicles and were divided into three groups depending on their milk production, whereas young animals were housed on straw in a yard with an external paddock and were grouped in five pens according to age. Calves <2 months old were not considered in this study. Lactating cows were milked twice a day, at 6 a.m. and 5 p.m., with a streamlined milking parlor system. The dairy farm performed cleaning-in-place (CIP) sanitation both for milking system equipment and for a bulk tank with a chlorinated liquid detergent (Green Forte; ICF, Cremona, Italy) and once a week with a solution of nitric acid and phosphoric acid (Green Tol; ICF, Cremona, Italy). In addition, every time the milking units were removed, they were backflushed with a chlorhexidine sanitizing solution (Sani BC3; UCL, Brescia, Italy).

The diet fed to the animals and the total number of animals in the herd remained the same throughout the study. Water was provided by a municipal supply system with several water troughs. During the study period, the farmer intermittently split, mixed, or separated and moved the herd of dairy cattle between different fields for husbandry reasons.

Sampling design and sample collection. The experiment was designed as a longitudinal study carried out for 10 months between October 2012 and July 2013. The farm was visited once a month for a total of 10 samplings. Fifty animals, 25 from the adult group and 25 from the young group, were randomly selected from the dairy herd to be repeatedly sampled throughout the study by 5 samplings at 2-month intervals. At each visit, an individual fecal sample (50 to 200 g) was collected from the rectum of each animal selected. For commercial reasons, 7 of the 25 adult cows selected were removed from the herd during the study and were not replaced; as a consequence, fewer samples were collected from the adults than from the young animals (114 versus 125, respectively), for a total of 239 fecal samples. Cross-contamination during sampling was avoided by using single-use plastic gloves.

TABLE 2 *Arcobacter* species isolated from *Arcobacter*-positive animals in the different samplings

Animal ID[a]	Sampling I	II	III	IV	V
Young animals					
289	*A. skirrowii*	*A. skirrowii*, *A. cryaerophilus*	ND[b]	ND	ND
313	ND	*A. cryaerophilus*	*A. cryaerophilus*	*A. skirrowii*	ND
319	*A. skirrowii*	*A. skirrowii*	ND	ND	ND
322	*A. skirrowii*	ND	ND	*A. skirrowii*	ND
336	ND	*A. skirrowii*, *A. cryaerophilus*	ND	*A. skirrowii*, *A. butzleri*	*A. skirrowii*
339	ND	*A. skirrowii*, *A. cryaerophilus*	ND	*A. skirrowii*	ND
340	ND	*A. cryaerophilus*	*A. cryaerophilus*	ND	ND
346	*A. skirrowii*	*A. skirrowii*	ND	ND	ND
347	ND	*A. cryaerophilus*	*A. cryaerophilus*, *A. skirrowii*	*A. cryaerophilus*	ND
348	ND	*A. cryaerophilus*, *A. skirrowii*	*A. cryaerophilus*	ND	ND
350	*A. skirrowii*	*A. skirrowii*	ND	ND	ND
352	*A. cryaerophilus*, *A. skirrowii*	*A. cryaerophilus*	*A. cryaerophilus*	ND	ND
353	ND	*A. cryaerophilus*	*A. cryaerophilus*	ND	ND
Adult animals					
243	*A. skirrowii*, *A. cryaerophilus*	ND	*A. skirrowii*, *A. cryaerophilus*	ND	ND
270	ND	*A. skirrowii*	ND	ND	*A. cryaerophilus*
274	*A. cryaerophilus*	ND	*A. cryaerophilus*	ND	ND

[a] ID, animal identification number.

[b] ND, not detected.

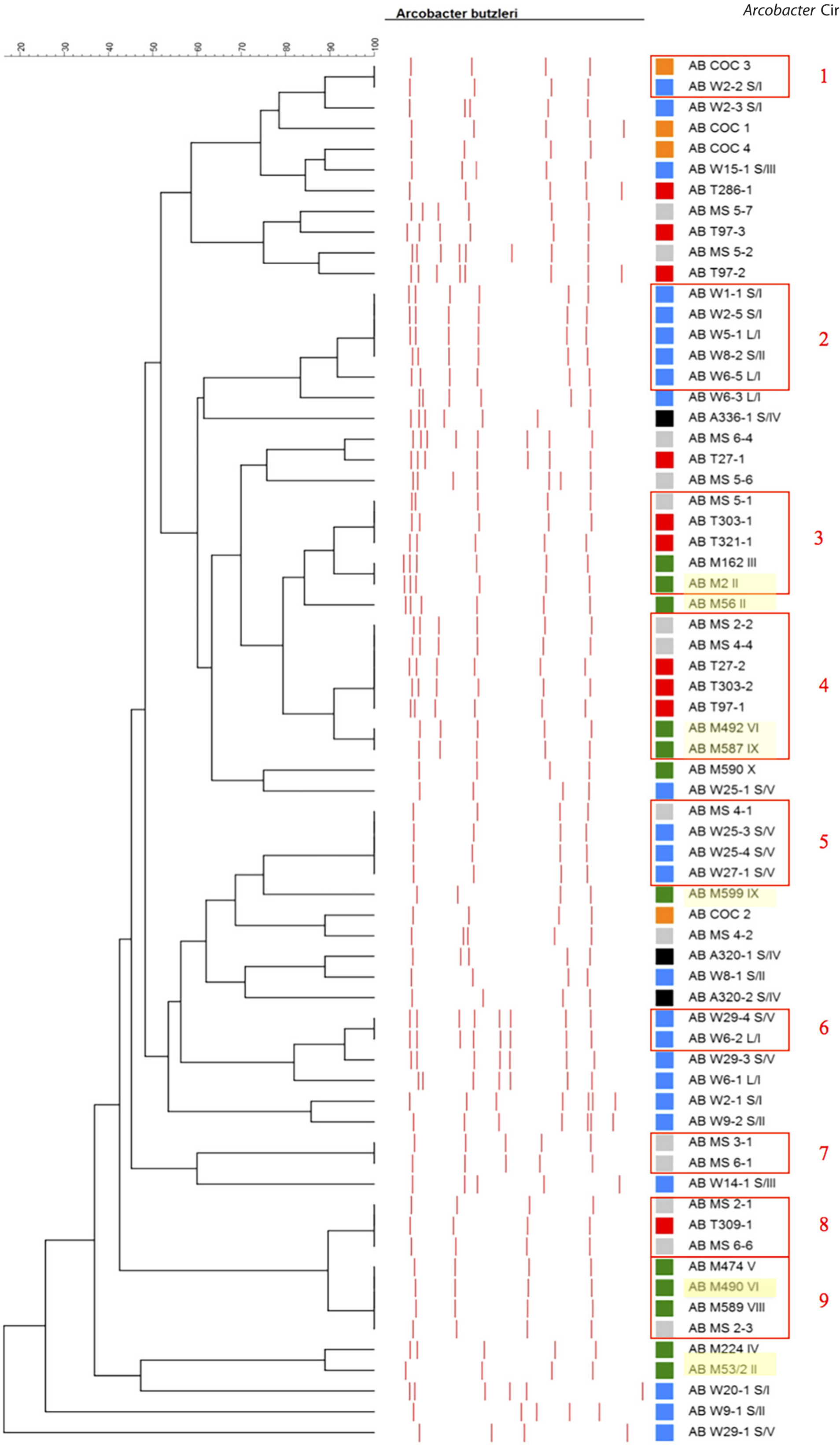
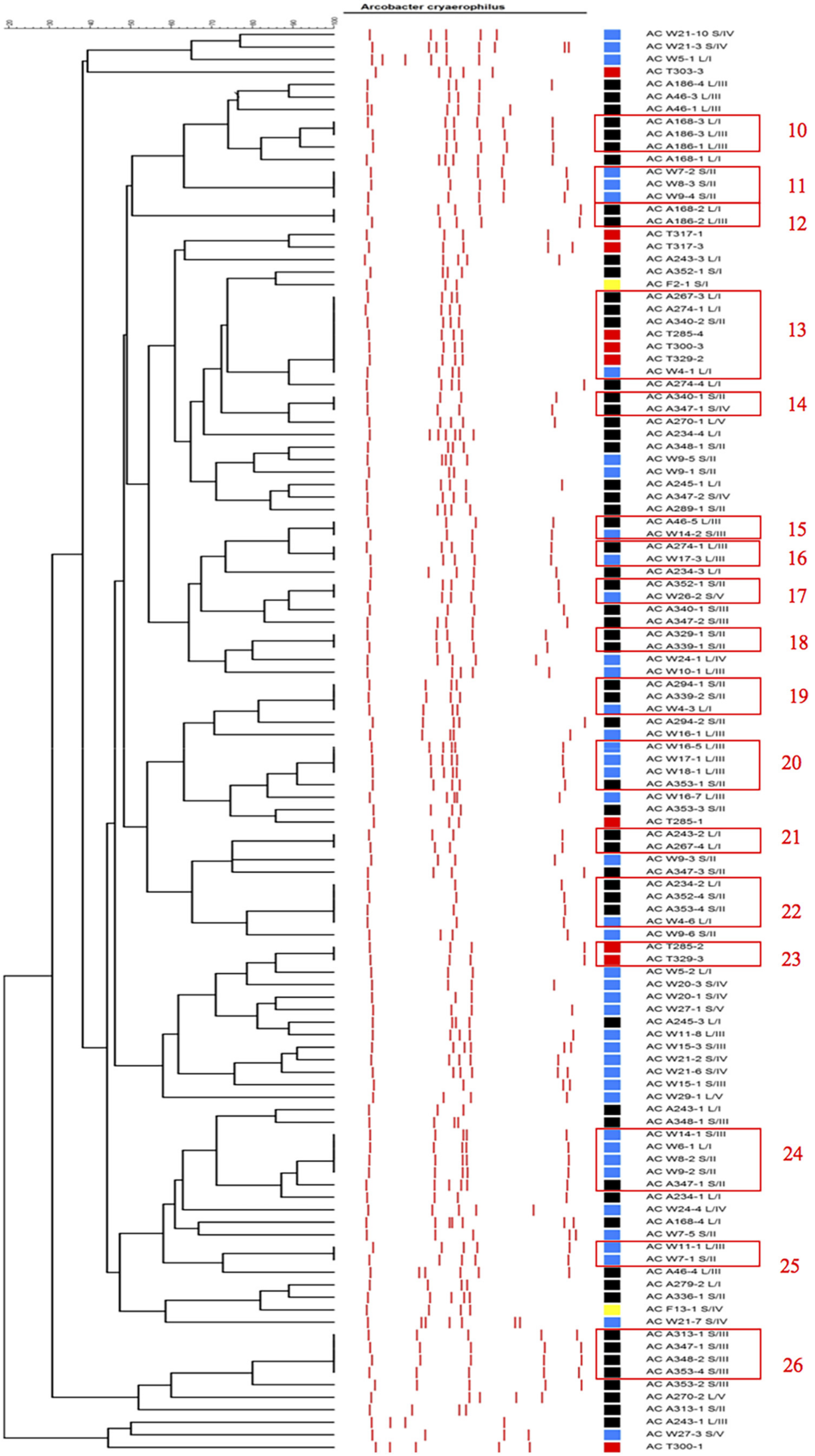


FIG 1 Distribution of SacII-digested PFGE patterns of *A. butzleri* isolates. The sources of the isolates belonging to clusters 1 to 9 are listed on the right. Sample types (with color coding indicated in parentheses) are abbreviated as follows: A, animal feces (black); COC, cat oral cavity (orange); M, milk (green); MS, milking system (gray); T, teats (red); W, water (blue). The type of sample is followed by an animal or sample identification number and the number of the isolate detected in the sample. For animal and water samples only, the type of animal (S, young; L, adult) is given. For animal, water, and milk samples, the number of the sampling (reported as I to X) is also given. Thus, for example, W2-3 S/I stands for the third *A. butzleri* isolate found in sample 2 from the water for young animals in the first sampling. Isolates with the same PFGE pattern in the same sample are reported once. The numbers above the dendrogram indicate the percentages of similarity as determined by Dice correlation coefficients and UPGMA clustering. Yellow highlighting of isolates indicates different pulsotypes isolated from the same milk sample. Isolates boxed in red are clusters.


Arcobacter cryaerophilus
20
30
40
50
60
70
80
90
100
AC W21-10 S/IV
AC W21-3 S/IV
AC W5-1 L/I
AC T303-3
AC A186-4 L/III
AC A46-3 L/III
AC A46-1 L/III
AC A168-3 L/I
AC A186-3 L/III
AC A186-1 L/III
10
AC A168-1 L/I
AC W7-2 S/II
AC W8-3 S/II
AC W9-4 S/II
11
AC A168-2 L/I
AC A186-2 L/III
12
AC T317-1
AC T317-3
AC A243-3 L/I
AC A352-1 S/I
AC F2-1 S/I
AC A267-3 L/I
AC A274-1 L/I
AC A340-2 S/II
AC T285-4
AC T300-3
AC T329-2
AC W4-1 L/I
13
AC A274-4 L/I
AC A340-1 S/II
AC A347-1 S/IV
14
AC A270-1 L/V
AC A234-4 L/I
AC A348-1 S/II
AC W9-5 S/II
AC W9-1 S/II
AC A245-1 L/I
AC A347-2 S/IV
AC A289-1 S/II
AC A46-5 L/III
AC W14-2 S/III
15
AC A274-1 L/III
AC W17-3 L/III
16
AC A234-3 L/I
AC A352-1 S/II
AC W26-2 S/V
17
AC A340-1 S/III
AC A347-2 S/III
AC A329-1 S/II
AC A339-1 S/II
18
AC W24-1 L/IV
AC W10-1 L/III
AC A294-1 S/II
AC A339-2 S/II
AC W4-3 L/I
19
AC A294-2 S/II
AC W16-1 L/III
AC W16-5 L/III
AC W17-1 L/III
AC W18-1 L/III
AC A353-1 S/II
20
AC W16-7 L/III
AC A353-3 S/II
AC T285-1
AC A243-2 L/I
AC A267-4 L/I
21
AC W9-3 S/II
AC A347-3 S/II
AC A234-2 L/I
AC A352-4 S/II
AC A353-4 S/II
AC W4-6 L/I
22
AC W9-6 S/II
AC T285-2
AC T329-3
23
AC W5-2 L/I
AC W20-3 S/IV
AC W20-1 S/IV
AC W27-1 S/V
AC A245-3 L/I
AC W11-8 L/III
AC W15-3 S/III
AC W21-2 S/IV
AC W21-6 S/IV
AC W15-1 S/III
AC W29-1 L/V
AC A243-1 L/I
AC A348-1 S/III
AC W14-1 S/III
AC W6-1 L/I
AC W8-2 S/II
AC W9-2 S/II
AC A347-1 S/II
24
AC A234-1 L/I
AC W24-4 L/IV
AC A168-4 L/I
AC W7-5 S/II
AC W11-1 L/III
AC W7-1 S/II
25
AC A46-4 L/III
AC A279-2 L/I
AC A336-1 S/II
AC F13-1 S/IV
AC W21-7 S/IV
AC A313-1 S/III
AC A347-1 S/III
AC A348-2 S/III
AC A353-4 S/III
26
AC A353-2 S/III
AC A270-2 L/V
AC A313-1 S/II
AC A243-1 L/III
AC W27-3 S/V
AC T300-1

In addition, at each farm visit, three water samples from water troughs in the stable, one trough feed sample each for the young and adult animal groups, and one bulk tank milk sample were collected, for a total of 30 water, 20 feed, and 10 milk samples. Water and feed sampling sessions were planned so as to collect one water trough sample for each group of animals and one sample for each type of feed given to animals (both a unifeed mixture and concentrated feed in pellets were fed to cows; hay and concentrated feed in pellets were fed to calves and heifers). During the study, the following one-off samplings were also performed. (i) Eighty individual quarter milk samples (milk samples from each quarter) were collected aseptically at the evening milking from 20 cows randomly selected: 10 cows were selected among those with the lowest (<200,000/ml) somatic cell count, and 10 cows were selected among those with the highest (>200,000/ml) somatic cell count. The somatic cell count was performed 1 week before the quarter milk collection within the routine control program of the Farmers' Association. (ii) Thirty-one 10- by 5-cm sterile sponges (VWR International PBI, Milan, Italy) were used to sample the internal parts of the milking systems before the beginning of milking (6 sponges) and the teats of 25 cows randomly chosen during milking (25 sponges) by using sterile gloves. The sponges were wetted with Butterfield's phosphate buffer and were placed in separate sterile bags. (iii) Six cat samples, three rectal and three oropharyngeal swabs, were collected from three cats living on the dairy farm investigated. (iv) Forty-seven guts belonging to as many pigeons (*Columba livia*) shot by the authorities during an authorized control program in the area in which the dairy farm is located were collected. Overall, 463 samples were collected and analyzed for *Arcobacter* spp.

Microbiological examination. All samples were delivered to the laboratory in a portable refrigerator (4°C ± 2°C) and were processed within 4 h of collection. *Arcobacter* was detected according to the protocol of Houf et al. (27). The samples were managed as follows: (i) 5 g of cattle feces, 25 g of feed, or the whole pigeon gut was homogenized by a stomacher in a 1:10 ratio in enrichment broth; (ii) 25 ml of water samples or 25 ml of the bulk tank milk sample was homogenized in 225 ml of enrichment broth; (iii) sponges were added to 90 ml of enrichment broth, while cat swabs were added to 9 ml of enrichment broth. The broth used for enrichment was *Arcobacter* broth (Oxoid, Basingstoke, United Kingdom) supplemented with 5% laked horse blood (Oxoid, Basingstoke, United Kingdom) and a selective supplement (cefoperazone [16 mg/liter], amphotericin B [10 mg/liter], 5-fluorouracil [100 mg/liter], novobiocin [32 mg/liter], and trimethoprim [64 mg/liter]) (27).

After 48 h of incubation at 28°C ± 1°C in plastic jars under microaerobic conditions, a 10-μl aliquot of the enrichment broth was streaked onto selective agar plates prepared by suspending 24 g of *Arcobacter* broth (Oxoid, Basingstoke, United Kingdom) and 12 g of Agar Technical (Agar No. 3) (Oxoid, Basingstoke, United Kingdom) (27) and supplementing with the selective antibiotic supplement described above. The plates were incubated microaerobically at 28°C ± 1°C for as long as 5 days. The microaerobic conditions were created by evacuating 80% of the normal atmosphere and introducing a gas mixture of 8% CO_2, 8% H_2, and 84% N_2 into the jar. If present, as many as five colonies suspected to be *Arcobacter* spp. were picked from each plate, subcultured, and subjected to presumptive identification using tests including cellular morphology by Gram staining and growth under aerobic conditions.

For identification of the presumptive *Arcobacter* colonies, two multiplex PCRs were used: DNA was extracted from the isolates using the REDExtract-N-Amp Tissue PCR kit (Sigma, St. Louis, MO, USA), and isolates were identified by the multiplex PCR described by Douidah et al. (28). Isolates identified as *A. butzleri* were confirmed by the multiplex PCR described by Houf et al. (29). Two reference strains, *A. butzleri* DSM 8739^T and *A. cryaerophilus* DSM 7289^T (Leibniz Institute DSMZ, Braunschweig, Germany), and *A. skirrowii* strain BU30CC8B1 (30), isolated from poultry, were used as controls.

Subspecies typing by PFGE. At least three single-colony isolates, when available, that were identified as *Arcobacter* spp. (*A. butzleri*, *A. cryaerophilus*, or *A. skirrowii*) were subjected to pulsed-field gel electrophoresis (PFGE) analysis by adapting the protocol reported by Ferreira et al. (31).

Agarose-embedded DNA was digested for 4 h at 37°C with 40 U of SacII (Fermentas, St. Leon-Rot, Germany). The restriction fragments were separated by PFGE (CHEF Mapper; Bio-Rad, Hercules, CA, USA) on 1.0% Certified Megabase Agarose (Bio-Rad, Hercules, CA, USA) in 0.5× Tris-borate-EDTA (TBE) buffer for 22.5 h at 14°C. The electrophoretic parameters used were as follows: two-state function; initial switch time, 5.0 s; final switch time, 40.0 s; gradient, 5 V/cm; angle, 120°; ramping factor, linear. A lambda ladder PFG marker (New England BioLabs, MA, USA) was used as a molecular size standard. One reference strain, *A. butzleri* strain DSM 8739^T, was used as a positive-control strain in each PFGE gel.

After electrophoresis, gels were stained in a GelRed (Biotium, Hayward, CA, USA) 3× solution for 60 min. Gels were visualized with a UV gel documentation system (Quantity One software, version 4.5; Bio-Rad Laboratories, Hercules, CA, USA). The fingerprinting profiles were analyzed using BioNumerics software (version 7.5; Applied Maths, Sint-Martens-Latem, Belgium) and were compared by cluster analysis using Dice coefficient analysis and the unweighted pair group method with arithmetic means (UPGMA), with a position tolerance limit of 1% and optimization of 1%. Isolates showing a PFGE pattern similarity level of 90% were grouped in the same "PFGE cluster." Isolates showing a PFGE similarity level of 100% (same pulsotype) were grouped as PFGE patterns. Isolates showing a PFGE similarity level of >90% were considered closely related and were grouped in the same PFGE cluster. PFGE clusters relative to the three recognized *Arcobacter* species were identified by progressive numbers (from 1 to 38).

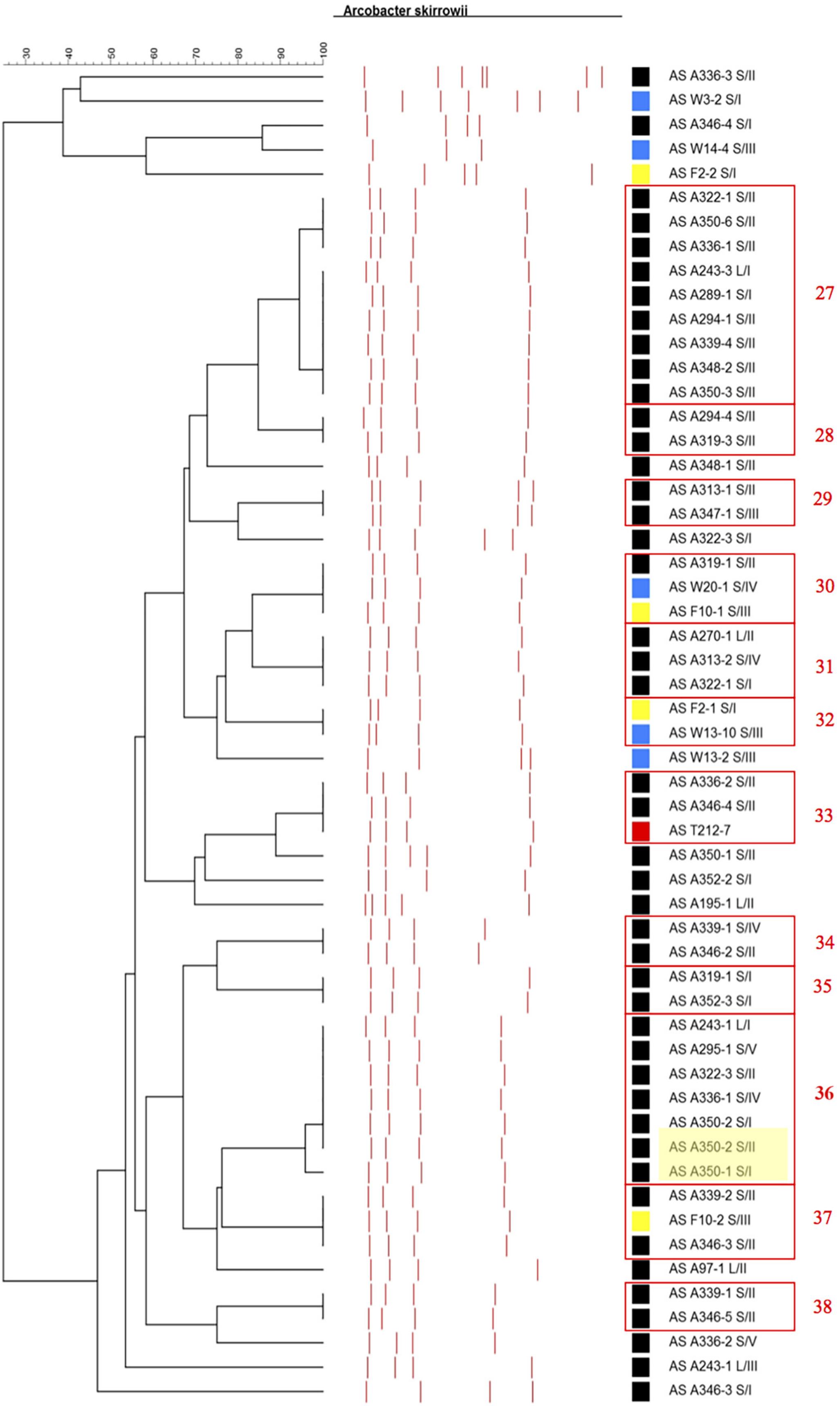
Statistical analysis. Data analysis was performed using Prism software, version 5.0. Fisher's exact test was used to compare the proportions of positive samples collected from both feces and water troughs of young and adult animals and the proportions of repetitively positive fecal samples. A chi-square test was used to compare the rates of isolation of the different *Arcobacter* species in the different types of samples analyzed. Significance was set at a P value of <0.05.

RESULTS

A total of 105 samples (22.6%) were positive for *Arcobacter* spp. by culture examination. All the quarter milk and pigeon gut samples tested negative throughout the study, whereas at least one *Arcobacter* sp. was isolated from each of the other matrices, with a prevalence of positive samples ranging from 15% to 83.3% depending on the type of sample. More details on the numbers of positive samples in different matrices are given in Table 1.

Of the 105 *Arcobacter*-positive samples, 57 (54.2%), 37 (35.2%), and 34 (32.3%) were positive for *A. cryaerophilus*, *A. butzleri*, and *A. skirrowii*, respectively. No other *Arcobacter* species were detected. *A. cryaerophilus* could be roughly evaluated as the predominant species, although on further scrutiny, it became clear that the *Arcobacter* species isolated depended on the type of sample analyzed: *A. cryaerophilus* and *A. skirrowii* were detected almost exclusively in animals and their living environments (ani-

FIG 2 Distribution of SacII-digested PFGE patterns of *A. cryaerophilus* isolates. The sources of the isolates belonging to clusters 10 to 26 are listed on the right. Sample types (with color coding indicated in parentheses) are abbreviated as follows: A, animal feces (black); F, feed (yellow); T, teats (red); W, water (blue). The type of sample is followed by an animal or sample identification number and the number of the isolate detected in the sample. For animal, feed, and water samples only, the type of animal (S, young; L, adult) is given. For animal, water, and milk samples, the number of the sampling (reported as I to X) is also given. Thus, for example, W21-10 S/IV stands for the 10th *A. cryaerophilus* isolate found in sample 21 from the water for young animals in the fourth sampling. Isolates with the same PFGE pattern in the same sample are reported once. The numbers above the dendrogram indicate the percentages of similarity as determined by Dice correlation coefficients and UPGMA clustering. Isolates boxed in red are clusters.


Arcobacter skirrowii
30
40
50
60
70
80
90
100
AS A336-3 S/II
AS W3-2 S/I
AS A346-4 S/I
AS W14-4 S/III
AS F2-2 S/I
AS A322-1 S/II
AS A350-6 S/II
AS A336-1 S/II
AS A243-3 L/I
AS A289-1 S/I
AS A294-1 S/II
AS A339-4 S/II
AS A348-2 S/II
AS A350-3 S/II
27
AS A294-4 S/II
AS A319-3 S/II
28
AS A348-1 S/II
AS A313-1 S/II
AS A347-1 S/III
29
AS A322-3 S/I
AS A319-1 S/II
AS W20-1 S/IV
AS F10-1 S/III
30
AS A270-1 L/II
AS A313-2 S/IV
AS A322-1 S/I
31
AS F2-1 S/I
AS W13-10 S/III
32
AS W13-2 S/III
AS A336-2 S/II
AS A346-4 S/II
AS T212-7
33
AS A350-1 S/II
AS A352-2 S/I
AS A195-1 L/II
AS A339-1 S/IV
AS A346-2 S/II
34
AS A319-1 S/I
AS A352-3 S/I
35
AS A243-1 L/I
AS A295-1 S/V
AS A322-3 S/II
AS A336-1 S/IV
AS A350-2 S/I
AS A350-2 S/II
AS A350-1 S/I
36
AS A339-2 S/II
AS F10-2 S/III
AS A346-3 S/II
37
AS A97-1 L/II
AS A339-1 S/II
AS A346-5 S/II
38
AS A336-2 S/V
AS A243-1 L/III
AS A346-3 S/I

mal feces and teats, as well as water and feed samples), whereas *A. butzleri* was the only species isolated from milk and the milking system, although it was also isolated frequently from water and teat samples and sporadically from feces samples. The rates of isolation of the different *Arcobacter* species from the different types of samples were significantly different ($P < 0.0001$).

In the specific case of animal feces, a significantly higher proportion of positive samples was observed for young animals (27.2% versus 13.15% for adult animals) (*P*, 0.0099), and a significantly higher proportion of repetitively positive samples was found in young animals (13 young versus 3 adult animals) (*P*, 0.0357), as shown in Table 2. The species detected in the feces were almost exclusively *A. cryaerophilus* and *A. skirrowii* (58 total isolates versus only 2 for *A. butzleri*). Cocolonization of bovine fecal pats by more than one *Arcobacter* species in the same sample occurred relatively frequently, whereas the same species was frequently isolated in different samplings for 15 of the 16 repetitively positive animals. However, PFGE analysis only once disclosed indistinguishable pulsotypes in the feces of the same animal in different samplings (see Fig. 3, cluster 36, isolates highlighted in yellow). In all other cases, different, unrelated pulsotypes were identified in different samplings of the same animal. Moreover, PFGE disclosed indistinguishable pulsotypes in more than one animal, both young and adult, and even in different areas of the barn in the same sampling or in different samplings.

A total of 370 isolates from positive samples—175 *A. cryaerophilus*-, 120 *A. butzleri*-, and 75 *A. skirrowii*-positive samples—underwent PFGE analysis. The *A. cryaerophilus* cluster analysis divided the isolates into 83 pulsotypes, 17 PFGE patterns, and 17 PFGE clusters. The *A. butzleri* cluster analysis divided the isolates into 45 pulsotypes, 11 PFGE patterns, and 9 PFGE clusters. The *A. skirrowii* cluster analysis divided the isolates into 27 pulsotypes, 13 PFGE patterns, and 12 PFGE clusters. No fragments were obtained for 13 isolates. Details on the source of isolation and sampling of the isolates belonging to the clusters are given in Fig. 1, 2, and 3.

Cluster analysis of *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* patterns revealed wide heterogeneity among these isolates, although they grouped as indistinguishable and/or closely related patterns among different matrices or herd areas and in different samplings. The following findings should be emphasized. (i) Indistinguishable and/or closely related pulsotypes in the feces of different animals (clusters 10, 12 to 14, 18, 19, 21, 22, 26 to 29, 31, 33 to 38), in water (clusters 2, 5, 6, 11, 20, 24, 25), in the feces of animals and water samples (clusters 13, 15 to 17, 19, 22, 30), in feed and water samples and/or fecal samples (clusters 30, 32, 37), in teat samples of different animals (clusters 3, 4, 13, 23), in teat and fecal samples (clusters 13 and 33), and in teat, fecal, and water samples (cluster 13) were found in the same or different samplings. (ii) For *A. butzleri* only, indistinguishable pulsotypes were found in different milking system samples (clusters 4, 7, 8), as well as indistinguishable and/or closely related pulsotypes in milking system and water samples (cluster 5) and in milking system and teat samples (clusters 3, 4, 8). (iii) Indistinguishable and/or closely related pulsotypes were found in milk, milking system, and teat samples (clusters 3 and 4) and in milk and milking system samples (cluster 9). The isolation of indistinguishable pulsotypes in different milk samples in different samplings (clusters 3, 4, 9), as well as the presence of different pulsotypes in the same milk sample (Fig. 1, isolates highlighted in yellow), should be noted.

DISCUSSION

In this study, a 20.5% prevalence of *Arcobacter* spp. in feces was found on the dairy farm investigated, and *Arcobacter* shedding was intermittent. This prevalence is similar to rates reported in other European studies, which found overall prevalences of *Arcobacter* spp. in the fecal pats of cattle ranging from 14.3% (7) to 47.7% (13). However, the sampling design for this study precludes data comparison, because this is a longitudinal study, unlike the other studies in the literature. In contrast to most of the other studies, which reported *A. butzleri* as the most frequent species isolated from healthy animals (7, 12, 24, 32, 33), in our study, *A. cryaerophilus* was the dominant species in cow fecal samples (12.9%) (in agreement with only two studies [9, 10]), immediately followed by *A. skirrowii* (11.29%) and *A. butzleri* (0.8%).

The results of our study reveal a significant difference in *Arcobacter* prevalence between young and adult animals, in line with two studies (9, 13) but in contrast with other studies (8, 12) reporting increased prevalence with increasing age of the animals. However, the different age groups examined in those studies were not housed on the same farm. Hence, it could be argued that single-point prevalence estimates are of little value unless the spatial and temporal details are reported, as already observed by Grove-White et al. (13).

Until now, it has not been clear whether dairy animals are persistently infected by *Arcobacter* and excrete the same genotypes for a long time or whether they become reinfected with new genotypes or genotypes circulating within the farm throughout their lives. Our PFGE findings provide strong evidence of *Arcobacter* circulation in the herd: the isolation of different *Arcobacter* species from the same animal in different sampling sessions, different *Arcobacter* sp. pulsotypes from the same animal in different samplings, and the same pulsotype from different animals in the same and/or different sampling sessions suggest that cattle housed in the barn throughout the year were constantly reinfected with different *Arcobacter* species and genotypes. Cattle likely acquire the microorganisms by orofecal transmission, either by direct contact or from the environment, or both (clusters 10, 12 to 22, 24, 26 to 31, 33 to 38), and it is unlikely that they could avoid reingestion of fecal material.

In this view, water appears to be a major source of infection. Water samples collected from water troughs were frequently con-

FIG 3 Distribution of SacII-digested PFGE patterns of *A. skirrowii* isolates. The sources of the isolates belonging to clusters 27 to 38 are listed on the right. Sample types (with color coding indicated in parentheses) are abbreviated as follows: A, animal feces (black); F, feed (yellow); T, teats (red); W, water (blue). The type of sample is followed by an animal or sample identification number and the number of the isolate detected in the sample. For animal, feed, and water samples only, the type of animal (S, young; L, adult) is given. For animal, water, and milk samples, the number of the sampling (reported as I to X) is also given. Thus, for example, A336-3 S/II stands for the third *A. skirrowii* isolate found in the fecal sample from animal 336 (a young animal) in the second sampling. Isolates with the same PFGE pattern in the same sample are reported once. The numbers above the dendrogram indicate the percentages of similarity as determined by Dice correlation coefficients and UPGMA clustering. Yellow highlighting of isolates indicates indistinguishable pulsotypes in the feces of the same animal in different samplings. Isolates boxed in red are clusters.

taminated (80%), and all three *Arcobacter* species, *A. cryaerophilus* (63.3%), *A. butzleri* (40%), and *A. skirrowii* (13.3%), were well represented. No significant difference in *Arcobacter* prevalence was observed between water troughs located in areas for young and adult animals, although more positive fecal samples were detected in water troughs for young animals (93.3% versus 66.6%), probably due to the higher number of positive animals shedding *Arcobacter* in the young group and the different design of water troughs for young and adult animals. The water troughs for adult animals are more modern and can be emptied completely, whereas the troughs for young animals cannot be emptied and hence are difficult to clean. The PFGE results confirmed the role of water as an important vehicle both of the transmission of *Arcobacter* species to animals (clusters 13, 15 to 17, 19, 20, 22, 24, 30) and of their spread in the farm environment; water may also be a source for the initial infection of the animals (9, 12, 17). Finally, the isolation of closely related strains in water troughs in the same area (young and/or adult animals) in sampling sessions 2 months apart (clusters 2 and 24) implies colonization of water troughs by *A. butzleri* and *A. cryaerophilus* strains. Their persistence over time could reflect the ability of some *Arcobacter* strains either to form biofilms (34), to be incorporated in a preestablished biofilm as secondary colonizers (31), or to survive in water for a temperature-dependent period with prolonged survival in the presence of organic material at low incubation temperatures (4 to 20°C) (25). Therefore, such abilities may play a role in the maintenance of the circulation of some strains.

Despite the small number of samples in this study, the total absence of *Arcobacter* spp. from pigeons living in permanent colonies within the area investigated suggests that these animals are not a primary source of *Arcobacter* infection for cattle. On the other hand, *A. butzleri* was found in the oral cavities of cats living in the farm. Based on our PFGE results, this is not relevant to cattle epidemiology but confirms that pets are carriers of *Arcobacter* spp. and that *Arcobacter* transmission from cats to humans by direct contact may be suspected in cases of close interaction between pet cats and their owners (5).

Raw milk produced on the dairy farm investigated and collected from a bulk tank was frequently contaminated (80%), but, in contrast to the fecal samples, where *A. cryaerophilus* and *A. skirrowii* were the most frequently isolated species, the only species isolated from milk was *A. butzleri*. Samplings from quarter milk, teats, and milking machines ruled out primary contamination of milk: no *Arcobacter* species were detected in quarter milk samples, but teats sampled immediately before milking and the internal surfaces of the milking system (even if sanitized) were frequently contaminated by *A. butzleri* (28% and 83%, respectively [Table 1]). The PFGE results disclosed indistinguishable or closely related pulsotypes in teat, milking machine, and milk samples (Fig. 1, clusters 3, 4, and 9), suggesting that teats and milking machine surfaces may be major sources of milk contamination. This possibility is also supported by the fact that these PFGE patterns grouped isolates from teats and/or milking machine samples with isolates from milk samples collected in several different samplings (the second sampling for cluster 3, the sixth and ninth samplings for cluster 4, and the fifth, sixth, and eighth samplings for cluster 9), indicating a common source of milk contamination.

In addition, the isolation of *A. butzleri* alone in the milking system equipment demonstrates that the microorganism is not affected (or is only partially affected) by routine sanitizing procedures, confirming once again the higher resistance of *A. butzleri* than of other potentially pathogenic *Arcobacter* species found in different environments (25, 35), including milk (36), and the ability of *A. butzleri* to persist on several food-processing surfaces (in this case, both milking systems and bulk tanks) after routine sanitizing procedures (20, 23, 31, 37–40).

All these findings led us to speculate that although different *Arcobacter* species colonized the guts of the dairy animals, the greater ability of *A. butzleri* than of other *Arcobacter* species to survive in different environments accounted for the higher isolation rate of this species in environmental samples. This became increasingly evident the farther away from the animal the samples were collected (28% of teats, 40% of water samples, and 83% of milking machine samples tested positive for *A. butzleri* [Table 1]).

In conclusion, the uninterrupted circulation of *Arcobacter* spp. observed in the barn and their circulation on the dairy farm through the transmission of indistinguishable or closely related pulsotypes among animals, water, and the environment probably represent the mechanisms for the maintenance of *Arcobacter* spp. on the farm. Nevertheless, the environment exerts selective pressure for the contamination of milk, since it is able to select the most resistant *A. butzleri* species. The recurrent and exclusive isolation of *A. butzleri* in milk and in the milking machine system confirms that the dairy environment represents a good ecological niche for *Arcobacter* spp., which behave like environmental contaminants. Therefore, fecal contamination should not be considered the only or principal source of contamination of dairy produce, at least in this context. All these considerations are important for public health, allowing the preparation and implementation of appropriate measures to control the risk of acquisition of food-borne pathogens.

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