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Scuola di Dottorato in Scienze Veterinarie
per la Salute Animale e la Sicurezza Alimentare

Università degli Studi di Milano

GRADUATE SCHOOL OF VETERINARY SCIENCES
FOR ANIMAL HEALTH AND FOOD SAFETY
Director: Prof. Vittorio Dell'Orto

Doctoral Program in Animal Nutrition and Food Safety

Academic Year: 2009-2010

Emerging pathogen *Arcobacter* spp. in food of animal origin

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Index

1. Foreword 5

1.1 The Genus **Arcobacter** 7
1.2 **Arcobacter** in Human 9
1.3 Virulence Factors 10
1.4 Antimicrobial Susceptibility 12
1.5 **Arcobacter** in Animals 13
1.5.1 Arcobacter **in cattle** 13
1.5.2 Pigs as an important Arcobacter **Reservoir** 15
1.5.3 Arcobacter **in Poultry** 17
1.5.4 Arcobacter **in Other Animals** 17
1.6 **Arcobacter** in Food of Animal Origin 18
1.7 Identification and Characterization of **Arcobacter** 20
1.7.1 Molecular Identification 21
1.7.2 Molecular-Based Characterization 24
1.8 References 24

2. Objectives 39

2.1 Trial 1: Prevalence and Distribution of **Arcobacter** spp. In Veal Calves in Northern Italy 41
2.2 Trial 2: Isolation and characterization of **Arcobacter** spp. in bulk tank milk 42
2.3 Trial 3: Antimicrobial Susceptibility of Arcobacter spp. Isolated from Food of Animal Origin 42

3. Prevalence and Distribution of *Arcobacter* spp. in Veal Calves in Northern Italy 43

3.1 Abstract 45
3.2 Introduction 45
3.3 Materials and Methods 47
*3.3.*1 **Animal Selection** 47
3.3.2 Sample Collection at the Abattoir 47
3.3.3 Microbiological Analyses 48
3.3.4 Detection an Identification of Arcobacter **spp. with multiplex PCR** 48
3.4 Results 50
3.5 Discussion 56

3.6 References 58

4. Isolation and Characterization of *Arcobacter* spp. in Bulk Tank Milk 63
4.1 Abstract 65
4.2 Introduction 65
4.3 Materials and Methods 67
4.3.1 Bulk Tank Milk 67
4.3.2 Fecal Sampling 67
4.3.3 Microbiological Analyses 67
4.3.4 Detection an Identification of Arcobacter spp. with multiplex PCR 68
4.4 Results 69
4.5 Discussion 71
4.6 References 73

5. Antimicrobial Susceptibility of Arcobacter spp. Isolated from Food of Animal Origin 77
5.1 Abstract 79
5.2 Introduction 79
5.3 Materials and Methods 80
5.3.1 Bacterial Strains 80
5.3.2 Determination of Antimicrobial Susceptiility 80
5.4 Results and Discussion 81
5.5 References 83

6. General discussion 87

7. Summary 91
7.1 Foreword 93
7.2 Objectives 93
7.3 Trial 1: Prevalence and Distribution of Arcobacter spp. In Veal Calves in Northern Italy 93
7.4 Trial 2: Isolation and characterization of Arcobacter spp. in bulk tank milk 94
7.5 Trial 3: Antimicrobial Susceptibility of Arcobacter spp. Isolated from Food of Animal Origin 95
7.6 Conclusions 95

8. Acknowledgements 97

CHAPTER 1

Foreword

1. Foreword

Bacteria, now known as campylobacters, were first isolated at the beginning of the twentieth century. In 1913, McFadyean and Stockman isolated **Vibrio**-like organisms from aborted ovine fetuses (1). Five years later, Smith described the isolation of **Vibrio/Spirillum**-like organisms from aborted bovine fetuses, which he considered the same species as described by McFadyean and Stockman (2). Although the cell shape of those organisms was characteristic of the family of **Vibrionaceae**, they failed to ferment carbohyrates, ad were therefore transferred into a new genus, **Campylobacter** (3). Seventy years later, Ellis et al. (4,5) and Higgins and Degre (6) reported the isolation of aerotolerant, spiral shaped **Campylobacter**-like organisms from the organs of porcine and bovine fetuses. Examination of all **Campylobacter** and **Campylobacter**-like isolates revealed two distinct biochemical groups: the group 1 isolates were identified as **C. fetus**, whereas the group 2 isolates were considered as "aerotolerant campylobacters" (7,8).
In a comprehensive study in 1985, Neill et al. (9) provided a thoroughly documented description for a new species, **C. cryaerophila** to include these "aerotolerant-campylobacters" but emphasized also their phenotypic heterogeneity. In 1988, Thompson et al. (10) showed by partial 16S rRNA sequence analysis that **C. cryaerophila** and **C. nitrofigilis**, an organism isolated from the roots of Spartina alteniflora, exhibited more homology with each other than with other campylobacters. Suggesting that classification of both **C. cryaerophila** an **C. nitrofigilis** in another genus would be appropriate. In the early 1990s, Kiehlbauch et al. (11) found two subgroups among **C. cryaerophila** strains by phenotypic characterization and DNA-DNA hybridization. Catalase positive strains that were able to grow under aerobic conditions at 30°C but not in a medium with glycine or on McConkey agar, were considered as **C. cryaerophila**. The name **C. butzleri** was proposed for aerotolerant isolates that were negative to weakly catalase positive, and for which growth was observed in glycine minimal medium and on McConkey agar. In 1991 after a polyphasic taxonomic study, Vandamme et al. (12) transferred **C. cryaerophila** an **C. nitrofigilis** into a new genus, **Arcobacter**. Subsequently, **C. butzleri** was reclassified as **A. butzleri** and **A. skirrowii** was proposed for yet another group of animal associated **Arcobacter** strains (13).

1.1 The Genus *Arcobacter*

Due to the close phenotypic and genotypic affiliation, the genus **Arcobacter** was classified together with the genus **Campylobacter** into the family **Campylobacteriaceae** (14).

Together with the genera **Helicobacter**, **Wolinella** and **Sulfurospirillum** they constitute the most important representatives of a distinct group referred to as the rRNA superfamily VI, or as the ε-division of the class **Proteobacteria** (12).
Arcobacter are Gram-negative, nonspore-forming, motile, sphiral-shaped rods (0.2-0.8 x 0.5-5 μm), that are able to grow microaerobically or aerobically (12). They have the ability to grow fro 15 to 37°C, with an optimal growth in microaerobical conditions at 30°C. The growth at 15°C and the aerotolerance are the features that differentiate **Arcobacter** fro **Campylobacter** species (12,15). In 2007 the complete genome sequence analysis of **A. butzleri** revealed however that the majority of its proteome is most similar to those of **Sulfuromonas denitrificans Wolinella succinogenes**, and to those of the deep-sea vent Epsilonproteobacteria **Sulfurovum** and **Nitratiruptor** (16). The presence of pathways and loci associated with virulence, suggest that **A. butzleri** is a free-living, waterborne organism (16).
To date six species have been described: **A. butzleri**, **A. cryaerophilus**, **A. skirrowii**, **A. cibarius**, **A. nitrofigilis** and **A. halophilus** (12, 17-19). Predominantly **A. butzleri** has been associated with human infection as it has been isolated from stool of patients with diarrhea (20,21), but not from healthy humans (22). Furthermore the presence of virulence genes (16) and its cytopathogenic affect on in vitro cell lines (23,24) resulted in the classification of this species as an emerging pathogen (25). Besides humans, **A. butzleri** has been isolated from healthy and ill livestock (26), poultry (27,28), nonhuman primates (29-31), and diverse environmental matrices such as water and sludge (32).
A. cryaerophilus is a genotypically heterogeneous species which was originally divided into two subgroups by fatty acid analysis (13) or by restriction fragment length polymorphism (RFLP)-DNA analysis (33). As for **A. butzleri**, **A. cryaerophilus** strains have been isolated from a whole range of different matrices, including from stool and healthy humans (22) as well as from patients with enteritis (20). **A. skirrowii** has first been isolated from feces of lambs with diarrhea (13), but later on also from preputial fluids of bulls, organs of bovine, porcine and ovine aborted fetuses, animal feces, food and water. Association of **A. skirrowii** with human disease has only rarely been reported (34-35).In 2005, **A. cibarius** was described as a new **Arcobacter** species with **A. cryaerophilus** as the closest phylogenetic neighbor(18). The first representatives were isolated from broiler carcasses (18), but association with pigs has been suggested as well (36). Another potential new species has been isolated from the organs of aborted piglets and from duck feces (37,38), called **A. thereius** .
Besides the animal and human related species different free-living environmental **Arcobacter** species have been described. **A. nitrofigilis** is a nitrogen-fixing bacterium isolated from the roots of the salt marsh plant **Spartina alterniflora** (17). The strains prefer microaerobic growth conditions but aerobic growth is possible in adapted culture media (39). An obligate microaerophile marine sulfide-oxidizing

autrophic bacterium has been described which produced hydrophilic filamentous sulfur as a novel metabolic end product for which the name **Candidatus Arcobacter sulfidicus** sp. nov. has been proposed (40). A phylogenetic affiliation of a Gram-negative bacterium isolated from water collected at a hyper-saline lagoon on Hawaiian Island to the genus **Arcobacter** was confirmed for which the name A. **halophilus** was introduced (19). Cells are slightly curved, obligate halophile and growth is observed in culture media containing 2-4% salt or 0.1 potassium nitrate under aerobic and microaerobic conditions at room temperature and at 37°C, and under anaerobic conditions at 37°C. Finally, DNA from a number of **Arcobacter**-like organisms has been detected in diverse sources as salt-water lakes, coastal seawater, oil wells, biocatalytic calcification reactor, sediments in the Black Sea, sludge, and production waters (41-52).

1.2 *Arcobacter* in Humans

Interest for **Arcobacter** in veterinary and human public health enhanced since the first report of the isolation of **Arcobacter** from food of animal origin (53). Since then, studies worldwide have reported the occurrence of **Arcobacter** on food and have highlighted the possible transmission to the human population.
However, since the clarification of the taxonomic position of **Arcobacter**, only a few surveys have dealt with the clinical course of **Arcobacter** infections in humans. In 2000 Engberg et al (54) isolated one A. **butzleri** and one A. **cryaerophilus** from a total of 3267 clinical stool specimens. In the same year Lastovica et al (55) reported an A. **butzleri** prevalence of 0.39% in a study realized on 19,935 diarrheal stool of pediatric patients. During an 8-year study period Vandeberg et al (20) reported A. **butzleri** as the fourth most common **Campylobacter**-like organism isolated from 67,599 stool specimens from patients with diarrhea. In the study A. **butzleri** was more associated with a persistent and watery, and less with bloody diarrhea compared with C. **jejuni** infections and the organism has been recovered from patients of different ages (<1-90 years old). Similar results have been reported from a surveillance network in France in 2006 (21).
Most clinical **Arcobacter** infections are single cases with the source of infection rarely identified. The first association of **Arcobacter** with human infection was reported in 1987. From the feces of a 35 year-old man with acute diarrhea and abdominal pain, A. **cryaerophilus** was isolated without the detection of other pathogens (56). Since then A. **cryaerophilus** and A. **butzleri** infections were reported several times from stool samples of patients with acute diarrhea (11,35, 54, 57-60).

In 2004 the first human isolation of **A. skirrowii** was reported by Wibo et al. (34) from a 73 year-old patient with chronic diarrhea. More recently **A. skirrowii** has been detected in diarrheal stool samples in South Africa.
Besides the reports of the single cases of Arcobacter-associated enteritis, two **Arcobacter** outbreaks have been described. The first outbreak occurred in an Italian nursery and primary school where children suffered from recurred abdominal cramps without diarrhea. The isolates were identified as **A. butzleri** and the identical phenotypic characteristics and genotypic profiles of the isolates suggested an epidemiological relation for all cases (60). Person-to-person transmission was assumed as the ongoing cause of infection. A second outbreak appeared during a scout camp where 94 girls suffered from nausea, vomiting, abdominal cramps and diarrhea (61). The outbreak was assumed to be correlated with the breakdown of the automated water chlorinating system. No clear cause of infection could be identified by the examination of the stool samples, but **A. butzleri** was the only pathogen detected in the drinking water.
Besides the correlation with gastro-enteritis, **Arcobacter** has also been implicated in extra-intestinal invasive diseases. **Arcobacter butzleri** was isolated from the blood of a neonate and the clinical data indicated an **in utero** sepsis (62). Yan et al. (63) reported an **A. butzleri** isolation from two blood cultures of a man with liver cirrhosis displaying high fever and esophageal variceal bleeding, and in another bacteriemia report, mentioned by **Escherichia coli** and **Streptococcus milleri**, also the isolation of **A. butzleri** from a patient with an acute gangrenous appendicitis. Bacteriemia due to **A. cryaerophilus** has been reported in a patient with hematogenous pneumonia (64) and in a traffic accident victim (65).
The significance of **Arcobacter** as a cause of human diarrhea is still largely unknown. Since clinical samples are not routinely tested for **Arcobacter** species. The symptoms are similar to those of **Campylobacter** infections, and **Arcobacter** infections often have a spontaneous recovery (66). This make prevalence determination difficult and mostly incorrect (20, 67).

1.3 Virulence Factors

To assess the pathogenicity of **Arcobacter** for humans and animals, evaluation of potential virulence factors is required. However, up to now, a little is known about the mechanism of pathogenicity. A necessary state in the successful colonization, establishment, and ultimately production of disease by microbial pathogens is the ability to adhere to host surfaces such as mucous membranes, gastric and intestinal epithelial or endothelial tissue. Therefore it is a common trait of microbial pathogens to express adherence factors responsible for

recognizing and binding to specific receptor moieties of cells, thus enabling the bacteria to resist host strategies that would impede colonization.

Since campylobacterioses and *A. butzleri*-related illnesses have similar clinical features, it might be expected that some *C. jejuni* virulence factors would be present in *Arcobacter*. In the genomic sequence analysis of *A. butzleri* strain LMG 10828^{T}, homologs of the fibronectin binding protein CadF and Cj1349, the invasin protein CiaB, the virulence factor MviN, the phospholipase PldA and the TlyA hemolysin were detected (16). Several other *Campylobacter* virulence-associated genes however were not present with most notably the genes encoding the cytolethal distending toxin (CDT), which correlated with the study by Johnson and Murano who were unable to detect CDT-genes in *Arcobacter* species by PCR (68).

Furthermore, three additional putative virulence determinants have been identified: irgA, which encodes an iron-regulated outer membrane protein, hecA, a member of the filamentous hemagglutinin family, and hecB, a related hemolysin activation protein (16). The role and the functionality of these virulence determinants has not been determined yet.

The first *in vivo* study with *Arcobacter* strains intravenous or intraperitoneal inoculated in rodents was unsuccessful (6). No clinical symptoms were present and lesions were not observed by autopsy. However, the invasion and colonization capacity has later been demonstrated by Wesley et al. (69) as *A. butzleri* was isolated from feces and different organs up to seven days after experimental inoculation of piglets. Also the invasive capacity of *A. cryaerophilus* has been studied in an experimentally infected rainbow trout with death and clinical abnormalities reported (70). Agglutination of *A. butzleri* strains with human, rabbit, and sheep erythrocytes revealed the presence of adhesion molecules in *Arcobacter* (71). No fimbriae or pili were observed by electron microscopic examination, but an immunogenic hemagglutinin of 20 kDa was characterized. The hemagglutinin consisted of a lectin-like molecule, sensitive to heat treatment and enzymatic proteolysis, which could interact with a D-galactose-containing erythrocyte receptor.

Several *in vitro* studies on the adhesion, invasion, interleukine-8- and toxin-production capacity by *Arcobacter* strains of different origin on Vero-, Hep-2, INT407, Caco-2, IPI-2I, and HeLa-cells have been performed (22-24,72,73). A strong cytopathogenic effect was observed in Vero-cells comparable to the effect of VT-toxin of *E. coli* O157 (72, 74), whereas Hep-2- and HeLa-cells showed weak cytopathogenic effect. Cell rounding and nuclear pyknosis was observed with *Arcobacter* isolates on HeLa and Intestine 407 cells and cell elongation on CHO-cells (24). The presence of a vacuole-forming toxin, different from *Campylobacter* CDT, in *Arcobacter* strains has been demonstrated in a Vero cell culture set-up (75).

Fernandez et al. (73) and Carter (76) performed **in situ** studies about the existence of toxigenic and invasive capacities of **Arcobacter.** In those studies, the toxigenic capacity of **Arcobacter** from animal origin was determined in the rat and rabbit and pig ileal loop tests, respectively. In both studies, distention of the ileal loops with fluid accumulation and enhanced electrolyte concentrations was observed.
The information based on molecular and **in vitro** studies, presently available suggest that adhesion, invasion and toxin production could be mechanisms by which **Arcobacter** species may cause disease. However, whereas colonization of the intestine and production of cytotoxins by bacteria is generally associated with bloody diarrhea, this symptom is rarely described in the human cases reported to date. Further studies are certainly necessary to elucidate the pathobiology of **Arcobacter** species.

1.4 Antimicrobial Susceptibility

Specific standardized procedures for susceptibility testing of **Campylobacteraceae** and resistance breakpoints have not been established. Consequently, a number of different testing methods, such as agar and broth (micro)dilution (77-79), disc diffusion (80-82), and the E-test (63, 83) have been used for Arcobacter susceptibility testing in clinical, veterinary, and food microbiology. Furthermore, due to the fastidious nature and the microaerobic growth requirements of those microorganisms, the quality control limits given for nonfastidious organisms in aerobic atmosphere are not adequate (84). It is known that an increased level of carbon dioxide does decrease the effect of certain antimicrobials such as macrolides and fluoroquinolones. This will certainly occur in the microaerobic atmosphere required for the growth of **Campylobacteraceae**, and should be taken into account when interpreting susceptibility patterns (84). Comparison of the broth microdilution, the E-test and the agar dilution method showed overall comparable results for **Campylobacter** susceptibility testing when performed under the same microaerobic conditions and incubation temperature. Especially the minimum inhibitory concentration (MIC) obtained by the three methods of ciprofloxacin and erythromycin were in accordance with each other (85).
In 1992, Kiehlbauch et al. (79) applied the broth microdilution technique under aerobic atmosphere for susceptibility testing of the same panel of antimicrobials for A. **butzleri** and A. **cryaerophilus** isolates. The MICs for ciprofloxacin, erythromycin, doxycycline, and nalidixic acid differ by no more than one dilution from those obtained in the study of Houf et al. (86). However, with gentamicin, MICs for A. **butzleri** and A. **cryaerophilus** ranged from ≤0.12 to 0.5 μg/ml, whereas in the study of Houf et al. (86) the MICs ranged from 0.5 to 4 and 0.25 to 2 μg/ml, respectively. It is unknown whether the higher carbon dioxide

concentration may decrease the activity of aminoglycosides as well. In the susceptibility study by Fera et al. (77) even higher MIC ranges for both ciprofloxacin and gentamicin were obtained, although the same incubation atmosphere and temperature were applied. It is not clear if the different origin of the strains in the studies, water versus human stools and poultry carcasses, is the cause of those MIC shifts.
In contrast to thermophilic **Campylobacter** species, of which some strains demonstrate resistance to quinolones and cross-resistance between nalidixic acid and quinolones, most of the **Arcobacter** strains seem to be susceptible to both antimicrobial agents. Remarkable is however the lowered susceptibility and even resistance to ciprofloxacin of A. **butzleri** strains isolated from poultry (86). The latter finding is also demonstrated by the concentrations required to inhibit growth of 50% of the strains (MIC_{50}): the MIC_{50} for A. **butzleri** isolated from poultry is 0.12 whereas the MIC_{50} for human strains is 0.03 (86). The use of fluoroquinolones for treatment of poultry may be the basis for this decreased susceptibility.
Since particular poultry products are incriminated in the transmission of **Arcobacter** to humans, the presence of antimicrobial-resistant **Arcobacter** species in fresh poultry products can have public health implications. Today, data indicate that some **Arcobacter** isolates from poultry products are resistant and that multidrug resistance occurs. Especially the resistance to erythromycin and the decreased susceptibility to ciprofloxacin may have human health implications, as the two antimicrobials are generally prescribed as first-line drugs for the treatment of infections with **Campylobacteraceae**.

1.5 Arcobacter in Animals

Apart from A. **nitrofigilis** and A. **halophilus**, Arcobacter species have been incriminated with various animal diseases including abortion, septicemia, mastitis, gastritis, and enteritis (11, 29, 87-91). However, several studies reported the occurrence of **Arcobacter** in healthy livestock and poultry, detected by different isolation methods and molecular techniques (26, 92-99). Beside the clinical relevance, the occurrence of **Arcobacter** in healthy animals may act as significant reservoir and infection source to humans.

1.5.1 Arcobacter in Cattle

In cattle, **Arcobacter** have been associated with pathologies such as mastitis and reproduction disorders, but have more commonly been isolated from feces of clinically healthy animals (93, 95). In 1977, **Arcobacter** were isolated for the first

time from the placenta and the internal organs of aborted bovine fetuses (4). Although several studies have confirmed these observations (6, 100, 101), no studies have clearly identified **Arcobacter** as a causative agent of disease in cattle. Moreover, **Arcobacter** have been isolated from preputial sheath washing samples of bulls with no association of breeding problems in the herd (102) and detected in vaginal swabs without reproduction problems in the cows (98). Besides the association with reproduction abnormalities, there are also reports about **Arcobacter**-associated mastitis. **Arcobacter** has been isolated from raw milk samples of freshly calved dairy cows during a mastitis outbreak, characterized by the presence of fine granular clots and very high cell counts in the milk (90). The isolate was used for an experimental inoculation of the udder of young dairy cows, which developed an acute clinical mastitis in the inoculated quarters. The challenge organism was only recovered from one of the cows, in a milk sample taken 4 h after inoculation. Beside the association with disease, **Arcobacter** have been detected in the feces of healthy cows, and the occurrence in the gut of healthy cows involves a potential risk of contamination of the environment and the human food chain, with carcass contamination caused by fecal contamination during the slaughter process. The occurrence of **Arcobacter** in cattle at different stage of production has been studied by several researchers as shown in Table 1.1. The prevalence reported ranges from 2% to 39.2%, depending on the trial design (number of samples, sampling technique), country, season, age, and type of animals (calf or adult; dairy or fattening), isolation methods, and on-farm risk factors.

In the studies, A. **butzleri**, A. **cryaerophilus** and A. **skirrowii** have been recovered with A. **butzleri** as the most frequently isolated species (95, 97-99), except in the study of Van Driessche et al. (93), where A. **cryaerophilus** was the dominant species. Differences in physiological condition, housing, and feeding between calves, dairy cows and fattening cattle have an influence on the presence of **Arcobacter**, since different prevalence were observed between different animal groups (93, 97). Of the calves, only 2% of the animals excreted **Arcobacter** whereas for fattening and dairy cattle a prevalence up to 39% has been reported.

Table 1.1. Prevalence of *Arcobacter* spp. in cattle

Country	Sampling place	Animal Type	N. of Animals	Prevalence	Ref.
USA	Farm	Dairy cows	1682	14.3	95
USA	Farm	Fattening cattle	50	14.0	97
		Dairy cows	50	18.0	
		Calves	100	2.0	
Japan	Slaughterhouse	Cattle	332	3.6	98
Turkey	Slaughterhouse	Fattening cattle	200	9.5	99
Belgium	Farm	Cattle	276	11.0	26, 93
	Slaughterhouse	Fattening cattle	51	39.2	

1.5.2 Pigs as an Important Arcobacter Reservoir

As in cattle, the first reports of **Arcobacter** in pigs were associated with reproduction disorders. In several studies, **Arcobacter** have been isolated from the placenta and the internal organs of aborted fetuses observed on the farms with more late terms abortions, repeat breeding and a higher than usual rate of still-births (5, 6, 88). Antibiotic treatment or auto-vaccinations had none or only little improvement. At present, no routine screening for **Arcobacter** in pig farms has been established as the occurrence of abortions, whether caused by **Arcobacter** or not, seldom exceeds the reproduction parameters. However, results of a Danish survey suggest that the role of **Arcobacter** as etiological agent of abortion in pigs should not be ignored (88). In response to the **Arcobacter** associated abortus, some studies have dealt with the occurrence of **Arcobacter** in sows and boars. **Arcobacter** have been detected in oviductal tissues and uteri samples of sows with reproduction problems, and of sows with vaginal discharge (5, 88, 90). On farms with a history of **Arcobacter** associated abortions, sows with reproduction problems expressed high antibody serum titers in a microscopic agglutination test (103). However, **Arcobacter** have also been isolated from vaginal swabs of sows without reproduction disorders (98). On farms with and without reproduction problems, **Arcobacter** have been recovered from preputial swabs of boars, but not from the semen (103), though experimentally **Arcobacter** infected semen induced a decrease of the conception rates in sows (104). In one study, **Arcobacter** have been isolated from pig stomach samples, but their role in the etiology of gastric ulcers is not clear (91). **Arcobacter** do not always cause pathologies in pigs, since they have been isolated many times from the feces of clinically healthy animals (Table 1.2).

Table 1.2. Prevalence of *Arcobacters* spp. in pigs

Country	Sampling place	Animal Type	N. of Animals	Prevalence	Ref.
USA	Slaughterhouse	Market-age pigs	250	5	106
USA	Farm 1	Piglets	50	0	96
		Weaned pigs	50	6	
	Farm 2	Sows	10	20	
		Piglets	20	5	
	Farm 3	Sows	55	36.3	
		Piglets	20	0	
		Pigs	60	0	
		Sows	14	7.1	
Japan	Slaughterhouse	Market-age pigs	250	10	98
Belgium	Slaughterhouse	Market-age pigs	78	44.8	26, 92
	Farm	Pigs	294	41.1	

In Japan, **A. butzleri** was the most frequently isolated species in the feces at slaughterhouse level, followed by **A. cryaerophilus** (98). In an epidemiological study in which the occurrence of **Arcobacter** in animals of different age was followed, both **A. butzleri** and **A. cryaerophilus** were isolated, and characterization by pulsed field gel electrophoresis revealed the presence of different strains, suggesting colonization of animals by multiple parent genotypes that may undergo genomic rearrangements during repeated passages through the animals, or colonization with genotypes originating from different sources (96). In a Dutch study, in which the transmission routes in sows and their offspring on a breeding farm was followed, intra-uterine transmission was demonstrated, with **A. skirrowii** as the most prominent species. Furthermore, follow-up of the piglets suggested a postnatal infection from the sows, newcomers or from the environment, resulting in a demonstration of both vertical and horizontal transmission (105).

The pathogenicity of **Arcobacter** in pigs has been studied in vivo only once. After intraperitoneal inoculation of neonatal piglets, no lesions or clinical symptoms were observed and no **Arcobacter** were isolated from the organs **post mortem**. In a second trial, the effect of an infection **per os** in caesarean-derived and colostrum-deprived piglets was determined. **A. butzleri** were isolated from the feces for up to 10 days as well as from the lung, liver, kidney, ileum, and the brain (69). The intestinal colonization and multiplication of **A. butzleri** was demonstrated, in contrast with **A. cryaerophilus** and **A. skirrowii** for which only a short duration of

fecal shedding was recorded with no isolations from the organs. This may suggest the failure of those species to penetrate the intestinal barrier.

1.5.3 Arcobacter in Poultry

No association of **Arcobacter** with pathologies in poultry has been reported. Nevertheless, there are conflicting reports in literature whether or not **Arcobacter** s part of the poultry intestinal flora (94, 95, 107-111). In most of the studies, **Arcobacter** have not been isolated from cecal content nor from litter or the feathers (27, 83, 95, 110), though some studies reported the isolation of **Arcobacter** from cloacal swab samples (37, 99). In contrast with turkeys, infection experiments of chicks with **A. butzleri** were not successful (108, 112, 113). Since only some **Arcobacter** strains grow at 41°C, it is feasible that the high body temperature of birds inhibited or suppressed **Arcobacter** growth and colonization. The origin of the almost ubiquitous presence of **Arcobacter** on poultry carcasses is still under discussion as the transmission routes of these bacteria are still not established. In contrast to the related **Campylobacter**, for which the contamination at broiler house level is well documented and easily detected by conventional microbiological methods, **Arcobacter** seem however to display a different behavior. Several authors have suggested that **Arcobacter** are probably not normal inhabitants of the poultry intestine and, as formulated by Eifert et al. (108), Houf et al. (113), Gude et al. (109), and Van Driessche and Houf (94) that process water may be a potential source of the carcass contamination. An explanation for the contradictory reports in literature may be the sampling procedure. As in many studies, **Arcobacter** were isolated from the crates to transport the chickens to the slaughterhouse. One should take into account that those **Arcobacter** may contaminate the cloacal region, and this may explain the isolations reported by some authors (37, 98). As demonstrated in the study of Van Driessche and Houf (94) also the time and the sampling procedure are crucial and can affect the outcome of the study. Besides the reports of **Arcobacter** in chickens and turkeys, also the presence of **Arcobacter** in ducks and geese has been described (37, 114-116).

1.5.3 Arcobacter in Other Animals

The occurrence of **Arcobacter** in horses has been examined by Van Driessche et al. (26) who reported the isolation of **A. butzleri** out of two of the 15 examined animals. No information is available about the natural **Arcobacter** distribution in rodents, and a single report mentioned an **A. cryaerophilus** isolation from a naturally infected rainbow trout (**Oncorhynchus mykiss Walbaum**) (70). The presence of **Arcobacter** spp. in raccoons (**Procyon lotor**) was reported for the first time in

2004 (117). From a public health perspective, this observation may be important to note, since these animals share the immediate environment of human beings in certain countries and thus may play a part in the epidemiology of zoonotic bacterial infections.
Arcobacter have been isolated both from ill and healthy nonhuman primates. Several cases have been reported of the isolation of A. **butzleri** from healthy infant macaques (**Macaca nemestrina**) in a monkey nursery facility, and from Rhesus macaque (**Macaca mulatta**) with and without diarrhea (29-31).
The significance of these findings and whether they can serve as a model for human infection has not yet been determined.

1.6 *Arcobacter* in Food of Animal Origin

Beside contaminated water, food of animal origin is another possible route of transmission of **Arcobacter** to humans. The exact routes of infection are not clear, but probably include manipulation of raw meat, the consumption of undercooked products and cross-contamination. **Arcobacter**, like thermo-tolerant campylobacters, have been reported more frequently from poultry products than from red meat. Recent studies have indicated that also **Arcobacter** are common on broiler carcasses. **Arcobacter** have also been isolated from skin samples of commercially reared ducks and turkeys. Eggs seem not to be infected. Apart from chickens, **Arcobacter** have been isolated from geese and ducks. A survey of mechanically separated turkey samples showed that this meat-type can be heavy contaminated with **Arcobacter**.
A partial overview of the occurrence of **Arcobacter** on food of animal origin in different countries by multiple isolation protocols is shown in Table 1.3. At present no standard isolation method for **Arcobacter** has been proposed, therefore the true occurrence of **Arcobacter**; their contamination level and their genotypic heterogeneity are largely unknown and limit the ability to compare field data. Furthermore, the variations in recovery rates can also be due to differences in country, farm management, hygiene in slaughterhouses and processing companies (118).

Table 1.3. Presence of Arcobacter spp. on food of animal origin

Food Product	N. of Samples	Prevalence	Country	Ref.
Chicken carcasses	201	97	France	119
	170	81	Germany	82
	50	52.3	USA	120
	480	83	Belgium	27
	75	95	Turkey	121
	61	65.3	Czech Republic	122
	22	73	Australia	123
	41	48	Japan	124
	10	100	Thailand	124
Chicken meat	80	65	France	125
	220	24.1	The Netherlands	53
	52	65.4	Belgium	126
	15	20	USA	75
	100	23	Japan	80
	94	62	Northern Ireland	127
Eggs	57	0	Italy	128
Turkey meat	395	77	USA	119
	17	24	Denmark	37
Duck carcasses	10	80	UK	115
	10	70	Denmark	37
Rabbit meat	8	0	Czech Republic	122
Ground beef	45	28.9	USA	75
	32	22	Australia	123
	90	2.2	Japan	80
	108	34	Northern Ireland	127
Minced beef	68	1.5	The Netherlands	53
	97	5.1	Turkey	99
Ground pork	299	55.8	USA	129
	27	3.7	Italy	128
	200	32	USA	130
	21	29	Australia	123
	100	7	Japan	80
	101	35	Northern Ireland	127
	21	23.8	Belgium	131
Minced pork	194	0.5	The Netherlands	53
	26	19.2	Belgium	131
Sheep meat	13	15	Australia	123

1.7 Identification and Characterization of *Arcobacter*

Members of the genus **Arcobacter** are Gram-negative non-spore forming organisms. Cells are usually slender, curved rods, 0.2-0.9 μm wide and 0.5-3 μm long. S-shaped or helical cells are often present. Cells in old cultures may form spherical or coccoid bodies and loose spiral filaments up to 20 μm long (132). **Arcobacter** are motile with a characteristic corkscrew-like motion by means of a single polar unsheathed flagellum at one or both ends of the cell (13). Although the cell surface is critical in pathogenic processes such as the colonization of the host, resistance to host defense systems and invasion of cells. The cell surface characteristics of **Arcobacter** are still largely unknown (133). Research about the presence of filamentous appendages, or specific proteins with porin and adhesive properties, which have received detailed research because of their role in the pathogenesis in both **Campylobacter** and **Helicobacter** species, has not been performed. Neither specific proteins in the S-layer, as described for **Campylobacter fetus**, nor polysaccharide components responsible for serotype specificity, are known to date.

Optimal growth occurs at 30°C under microaerobic conditions, with a respiratory type of metabolism. Hydrogen is not required. After primary isolation in a microaerobic environment, growth is possible in aerobic or anaerobic atmosphere. Growth can occur at 15-37°C and growth at 42°C is described for some A. **butzleri** and A. **skirrowii** strains (134). Colonies of A. **butzleri** and A. **cryaerophilus** grown for 48 h under optimal conditions are respectively, 2-4 mm and 1-3 mm in diameter and are convex with an entire edge. Growth of A. **butzleri** has a whitish appearance whereas colonies of A. **cryaerophilus** have mostly a dirty yellow pigment. Colonies of A. **skirrowii** grown for 48 h under optimal conditions are 1-3 mm in diameter and have a flat irregular shape. Growth has a grayish appearance and is usually not profuse.

Identification of **Arcobacter** at species level by biochemical characteristics is difficult as members of the genus display little metabolic activity (13, 134). Classical biochemical differentiation of the genus **Arcobacter** from the related genera **Campylobacter** and **Helicobacter** is primarily achieved by the identification of the individual species. In general **Arcobacter** can be differentiated from **Campylobacter** by their lower optimal growth temperatures (25-30°C compared to 30-42°C for **Campylobacter**) and aerotolerance. However, in the identification of **Campylobacteraceae** at species level by the use of phenotypic tests, some fundamental problems can occur. First, many phenotypic tests used to differentiate other bacterial groups such as the members of the family **Enterobacteriaceae** have no discriminatory power for **Campylobacteraceae** because of their fastidious growth requirements and their relative metabolic inertness. For example, **Arcobacter** and **Campylobacter** species do not ferment or oxidize

carbohydrates. Second, there is a lack of standardization for the tests that are used. For example, the outcome of a given test may be influenced by the inoculum size, cultural age, and basal medium used. The third problem is the lack of objectivity in the schemes available. Most tables so far described are used by comparing the test results of the unknown with the phenotypic profiles of known taxa and more importance is often attached to a single test result that is considered as an essential character than to the remainder of the phenotype. In a comprehensive study in 1996, On et al. (135) documented an identification scheme for **Campylobacteraceae** and **Helicobacter** species, by which most **Campylobacteraceae** can be identified accurately and objectively with phenotypic tests when probabilistic methods of data assessment are employed.
In contrast to other organisms as **Salmonella**, **Campylobacter**, and **Listeria**, serology is not used for **Arcobacter** identification as attempts to perform genus or species identification by specific antibody agglutination were not successful (136). The high antigenic heterogeneity within the **Arcobacter** species may be on the basis of this failure. Due to the rather disappointing results, serological identification was not further extended, and is not further used to date.
Differentiation between **Arcobacter** and **Campylobacter** isolates is possible by the determination of the cellular fatty acid composition. **Arcobacter** species possess a unique combination of a tetradecenoic acid C14:1 and two isomers of C16:1 (137), later identified as C14:1ω7-cis, C16:1ω7-cis (common in most bacteria) and C16:1ω7-trans (considered unique for Arcobacter) (138, 139). Within the genus **Arcobacter**, A. **nitrofigilis**, A. **skirrowii** and the two A. **cryaerophilus** subgroups were differentiated by gas chromatography of the cellular fatty acids, but this technique was not able to differentiate A. **butzleri** from A. **cryaerophilus** subgroup 2 (13). To date, due to the rather complex analysis protocol and the availability of faster, less complex and cheaper molecular based methods, identification based on fatty acid profiles is not commonly applied, though its usefulness has been demonstrated by Jelinek et al. (140).
Identification based on whole-cell proteins profiles obtained by SDS-PAGE, has been the gold standard method since the description of the genus **Arcobacter**. By SDS-PAGE all known **Arcobacter** species can be identified including the differentiation between A. **cryaerophilus** subgroup 1 and 2. However, an enforced standardization of the protocol combined with a profile library of known and related species and genera is necessary. As this method is rather time consuming, it can hardly been applied in routine analysis.

1.7.1 Molecular Identification

Differentiating of **Arcobacter** species by using phenotypic tests might give erroneous results because of the shortage of clear-cut differentiating tests, a

phenomenon which has also been observed in the closely related genus **Campylobacter**. Therefore, several DNA-based assays were developed for the identification of **Arcobacter** at genus and species level. Recently, a microarray technique and a real-time fluorescence resonance energy transfer PCR for the detection and identification of **Arcobacter** spp. were reported, but are not routinely applied yet (141, 142).

RFLP. Using whole-cell chromosomal digests by the restriction enzyme PvuII and hybridization with probes derived from the **Escherichia coli** 16S and 23S rRNA genes, Kiehlbauch et al. (33, 143) developed a DNA-based method to differentiate the genera **Campylobacter**, **Arcobacter**, **Helicobacter**, and **Wolinella**. Although it is not able to distinguish A. **butzleri** from A. **skirrowii**, the method can be used for the differentiation of A. **cryaerophilus** from the other **Arcobacter** taxa and for the two subgroups of the latter species (l44). Sequence analysis of the conserved 16S rRNA gene of **Arcobacter** allowed Wesley et al. (l44) to design a genus-specific nucleic acid probe and a species-specific DNA probe for A. **butzleri**. Southern blot hybridization of PvuII digested DNA using the **Arcobacter** genus-specific probe or the A. **butzleri**-specific probe end-labeled with [γ^{32P}]ATP provided a reliable identification method for **Arcobacter** at genus level and for A. **butzleri**.

PCR. Analysis of the ribosomal gene sequence has proven to be a valuable tool in the determination of phylogenetic relationships between prokaryotes (10). A high (>94%) 16S rRNA gene sequences similarity was detected among the published **Arcobacter** species type strains. On the other hand, similarity to other members of the epsilon **Proteobacteria** was low (<90%) (18, 144). Based on the knowledge of the **Arcobacter** nucleic acid composition of the 16S rRNA, a genus- and species-specific DNA-probe was developed for identification of **Arcobacter** and A. **butzleri** strains (144). In recent years identification was done using rapid and specific PCR methods. The 16S and 23S rRNA of living organisms contain information that reflects the evolutionary relation of bacteria. Specific primers, derived from conserved rRNA gene sequences, can be used to amplify genus- or species-specific regions. Different Arcobacter genus- and species-specific PCR assays have been described in literature and were reliable in the identification of reference strains and field isolates.

One of the first described DNA-based identification techniques included a genus- and species-specific PCR developed by Bastyns et al. (145) with five primers targeting the 23S rRNA. One PCR amplification was necessary to identify the genus **Arcobacter**; a second PCR could differentiate A. **butzleri** from other species and a third amplification distinguished A. **cryaerophilus** from A. **skirrowii**. The disadvantage of this technique was the need of DNA amplification at different annealing temperatures. Based on the sequence of the **Arcobacter** and A. **butzleri**-specific DNA probes described by Wesley et al. (l44), two primer pairs

were developed that could be used in an **Arcobacter** genus- and **A. butzleri**-specific PCR with different annealing temperature (146). When the species-specific primers were replaced by the species-specific primers of Bastyns et al. (145), the first **Arcobacter** multiplex-PCR (m-PCR) was created: the genus **Arcobacter** (1223-bp) and the species **A. butzleri** (686-bp) were identified in one PCR amplification (147). The genus- and species-specific PCR assays described so far were not able to detect all known **Arcobacter** species in one PCR amplification. Therefore, m-PCR systems, targeting the 16S and 23S rRNA genes, have been developed for the simultaneous identification of the different human-related **Arcobacter** spp. in one PCR amplification and in one PCR tube (126). By means of five primers, a PCR product of 401-bp was generated for **A. butzleri**, 257-bp for **A. cryaerophilus** and 641-bp for **A. skirrowii**. Those three species were also identified by the PCR assay developed by Kabeya et al. (148), but **A. cryaerophilus** subgroup 1 and 2 were also differentiated from each other.

Some identification protocols used PCR as a part of the identification method. A PCR-hybridization protocol differentiated **A. butzleri** from the related **C. jejuni**, **C. coli**, **C. lari** and **C. upsaliensis** strains (149). The conserved glyA gene region of isolates was amplified during a PCR followed by a hybridization reaction of the amplicons with species-specific oligodeoxyribonucleotide probes. Another example is a culture-PCR method, used to detect **Arcobacter** on chicken meat (150). After enrichment of the meat sample, identification of **Arcobacter** in the medium was performed by a new developed genus-PCR assay that generated an amplicon of 181-bp for **Arcobacter** positive samples.

RFLP-PCR. Combined use of PCR with RFLP was first described by Cardarelli-Leite et al. (151). RFLP analysis of a PCR-amplified DNA fragment of the gene coding for 16S rRNA of **Campylobacter**, **Helicobacter**, **Arcobacter**, and **Wolinella succinogenes**, generated a 283-bp fragment from all species belonging to the examined genera. Initial restriction of the amplicon by DdeI, delivered a unique pattern for **A. butzleri**. Performing additional digestion using HpaII on the DdeI digested fragments, **A. cryaerophilus**, **A. skirrowii** and **A. nitrofigilis** can be distinguished as a single group from the **Campylobacter** and **Helicobacter** species, although further differentiation at species level is not possible. Hurtado and Owen (152) performed a comparable study in which amplicons ranging from 2.6 to 3.0 kb are generated by a PCR assay using primers in the conserved region of the 23S rRNA gene of **Campylobacter** and **Arcobacter**. Digesting these amplicons with HaeIII, CfoI, HpaII, and HinfI, species-specific patterns for **A. butzleri** and **A. nitrofigilis** and identical patterns for **A. cryaerophilus** and **A. skirrowii** can be obtained. In 1999 Marshall et al. (153) combined a PCR assay with RFLP for the identification of **Arcobacter** at the species level. By amplifying a 1004-bp fragment using primers targeting a conserved region of the 16S rRNA gene, followed by

restriction endonuclease digestion with DdeI and TaqI, species-specific RFLP patterns were obtained for **A. butzleri**, **A. cryaerophilus** and **A.skirrowii**.
AFLP. The genotyping AFLP technique may also be used for concurrent species identification of the family **Campylobacteraceae**, including the **Arcobacter** species (38, 154). The species **A. butzleri**, **A. cryaerophilus** and **A. skirrowii** form well-distinguished clusters in the dendrogram obtained after numerical analysis of yielded patterns.

1.7.2 Molecular-Based Characterization

The determination of the relation of isolates below species level has become very important for the identification of transmission routes and biological reservoirs in epidemiological studies. Phenotyping methods such as biotyping (155), serotyping (156), or comparisons of whole-cell proteins (13), are of limited use due to their low discriminatory power and the instability and low reproducibility of the phenotypic characteristics (134).
The suitability of pulsed-field gel electrophoresis (PFGE) and random amplification of polymorphic DNA (RAPD) as characterization techniques described by Liar and Wang (157) was confirmed for fecal or meat isolates in recent reports (96, 123, l25, 158, 159). The AFLP technique proved its qualities as genotyping method for all members of the **Campylobacteraceae** (15, 38, 154). Good distinguished clusters were obtained for **A. butzleri**, **A. cryaerophilus** and **A. skirrowii**, with profiles reproducing the clonal relation from the isolates within each species. The AFLP-method is a robust method with high discriminatory power, but nevertheless it is a demanding technique and requires a large reference database (66).
An enterobacterial repetitive intergenic consensus (ERIC) PCR was optimized for the characterization of **A. butzleri**, **A. cryaerophilus** and **A. skirrowii** strains in combination with a rapid DNA extraction method (159). Fingerprints generated with ERIC-PCR are complex enough to differentiate at strain and sub-strain level and have a good reproducibility.

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CHAPTER 2

Objectives

2. Objectives

The genus **Arcobacter**, previously classified as "aerotolerant campylobacter", from 1991 is classified as a new genus member of the family of **Campylobacteriaceae**, belonging to the epsilon-proteobacteria. The genus **Arcobacter** contains eight described species: **Arcobacter butzleri**, **A. cryaerophilus**, **A. nitrofigilis**; **A. skirrowii** ,**A. cibarius**, **A. thereius**, **A. halophilus** and **A. mytili** .
The bacteria are Gram negative, non-sporeforming, motile, curved, occasionally straight, rods which may appear as spiral. The most important differences between **Arcobacter** and **Campylobacter** are the ability of **Arcobacter** to grow at 15-25°C and its marked aerotolerance.
Arcobacter spp. have been considered as potential zoonotic foodborne and waterborne agents . **Arcobacter** spp. can be found in meat (veal, beef, pork and poultry), milk and water. Nevertheless the real occurrence of these potential pathogens in food is largely unknown.
The aim of this study is to evaluate prevalence and distribution of **Arcobacter** spp. in food of animal origin (raw meat and milk) in Northern Italy and to test the antimicrobial susceptibility of collected strains.

2.1 Trial 1: Prevalence and Distribution of *Arcobacter* spp. In Veal Calves in Northern Italy

Up to now there are many studies from different researchers about the presence, prevalence and distribution of **Arcobacter** spp. in meat of different origin (pork, beef and poultry). Data are almost entirely about the situation in Northern Europe, USA, Turkey and Israel.
In Italy, to our knowledge, there are no study to test the real occurrence of **Arcobacter** spp. both in carcasses and in raw meat.
The present study aimed to assess the **Arcobacter** spp. contamination on carcasses and in feces of veal calves slaughtered in Northern Italy. The isolates were further characterized in order to obtain insight in the heterogeneity of the **Arcobacter** species present.
We also try to determine the contamination route of carcasses and the potential risk for human health.

2.2 Trial 2: Isolation and characterization of *Arcobacter* spp. in bulk tank milk

In Italy, the sale of raw milk is legal and the consumption of raw bulk tank milk is related to the recent installation of raw milk distributors all over the country. Among the non-farming population, a growing number of consumers are claiming that raw milk is healthier and are choosing raw milk over pasteurized milk.

Arcobacter spp. find a ideal growth and survival environment in raw milk. For this reason **Arcobacter** spp. could be considered a milk-borne pathogen.

The present study aimed to assess the **Arcobacter** spp. contamination of bulk tank milk from dairy cow farms in Northern Italy, using conventional culture methods and multiplex PCR assay.

2.3 Trial 3: Antimicrobial susceptibility of *Arcobacter* spp. isolated from food of animal origin

Aim of this study is to evaluate antimicrobial susceptibility of 50 **Arcobacter** field strains collected in the two previous trials.

We used the same antimicrobial agents and techniques described in many international studies. We also tried to compare the susceptibility of our collected strains to that described in literature.

CHAPTER 3

Prevalence and Distribution of *Arcobacter* spp. In Veal Calves in Northern Italy

3. Prevalence and Distribution of *Arcobacter* spp. In Veal Calves in Northern Italy

3.1 Abstract

In this study the prevalence and distribution of **Arcobacter** spp. in samples taken from feces and carcasses of healthy veal calves, raised in Northern Italy, were investigated. A membrane filtration technique with a non-selective blood agar was employed after enrichment in **Arcobacter** selective broth to isolate a wide range of **Arcobacter** spp. In addition, the same samples were tested at the species level by using a multiplex-PCR assay. Samples from feces (50 during a summer sampling, 50 during a winter sampling) and carcasses (50 during a summer sampling a 50 during a winter sampling) were collected at the slaughterhouse from 50 veal calves originating from five different farms. Of the fecal samples examined, 36 (72%) were found positive for at least one species of **Arcobacter** during summer sampling and 38 (76%) were found positive during winter sampling. Of the sampled carcasses examined, 50 (100%) were found positive for at least one species of **Arcobacter** in summer sampling and 20 (40%) in winter sampling. From feces, 23 (46%), 17 (34%) and 5 (10%) samples were found positive by m-PCR for A. **cryaerophilus**, A. **skirrowii** and A. **butzleri**, respectively in summer. On the other side in winter 15 (30%), 12 (24%) and 2 (8%) samples were found positive by m-PCR for A. **cryaerophilus**, A. **skirrowii** and A. **butzleri**, respectively. From carcasses, 47 (94%), 17 (34%) and 5 (10%) samples were positive for A. **cryaerophilus**, A. **skirrowii** and A. **butzleri**, respectively in summer sampling whereas 5 (10%), 4 (8%) and 8 (16%) samples were positive for A. **cryaerophilus**, A. **skirrowii** and A. **butzleri**, respectively in winter sampling . Some sampled carcasses (34% in summer and 6% in winter) and some fecal samples (18% both in summer and in winter) resulted contaminated with multiple **Arcobacter** species. The present study indicates that veal calves can harbour a variety of **Arcobacter** spp. in the intestinal tract and that the presence of **Arcobacter** spp. may represent a source of contamination and dissemination in slaughterhouse.

3.2 Introduction

The genus **Arcobacter**, previously classified as "aerotolerant campylobacter", from 1991 is classified as a new genus member of the family of **Campylobacteriaceae**, belonging to the epsilon-proteobacteria (1). The genus **Arcobacter** contains eight described species: **Arcobacter butzleri**, A. **cryaerophilus**, A. **nitrofigilis**; A. **skirrowii** ,A.

cibarius, A. **thereius**, A. **halophilus** and A. **mytili** (2-6). There is also a candidate species "**Candidatus Arcobacter sulfidicus**" a highly motile sulphide-oxidising bacteria (6).
The bacteria are Gram negative, non-sporeforming, motile, curved, occasionally straight, rods which may appear as spiral. The most important differences between**Arcobacter** and **Campylobacter** are the ability of **Arcobacter** to grow at 15-25°C and its marked aerotolerance (1,7). Although their pathogenicity remains to be fully elucidated, **Arcobacter** spp. have been considered as potential zoonotic foodborne and waterborne agents (8,9). **Arcobacter** spp. can be found in meat (veal, beef, pork and poultry), milk and water. Nevertheless the real occurrence of these potential pathogens in food is largely unknown. Water and raw meat are considered the most important source of **Arcobacter** infection in human (10).
Arcobacter spp. are considered emerging human pathogens. A. **butzleri**, A **skirrowii** and A. **cryaerophilus** have been all associated with human enteritis and occasionally bacteriaemia (10, 11). Infection in human patients causes diarrhoea, abdominal pain and other symptoms including nausea, vomiting and fever (11).
On the other side, A. **butzleri**, A. **cryaerophilus** and A. **skirrowii** are associated with reproduction disorders, mastitis and gastric ulcers in cattle and swine, but are more frequently isolated from healthy animals (12-14). The occurrence of arcobacters in healthy animals may act as significant reservoir and direct infection source to humans (15).
In several studies, arcobacters have been isolated from the feces of healthy cattle on farm and prior to slaughter (16-19). However, the carcass contamination and the eventual presence of arcobacters in cattle have seldom been assessed (20).
Veal calf production in Italy is a very important sector of cattle production. Compared to other countries in EU, our country is today one of the first four markets, after France, Netherlands and Belgium, and veal calf represents about 12% of total bovine meat production in Italy (EUROSTAT, 2009). In Italy there are about 500 thousands head and veal calf facilities are distributed above all in the Northern area (Lombardia, Veneto and Piemonte).
The present study aimed to assess the **Arcobacter** spp. contamination on carcasses and in feces of veal calves slaughtered in Northern Italy. The isolates were further characterized in order to obtain insight in the heterogeneity of the **Arcobacter** species present. To our knowledge this is the first report about **Arcobacter** spp. prevalence and characterization on carcasses in Italy.

3.3 Materials and Methods

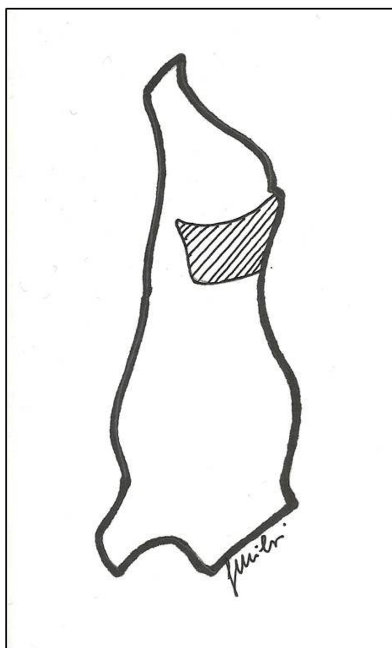
3.3.1 Animal Selection

Five veal calf farms located in Northern Italy (Lombardia and Piemonte) were selected and included in this study. Mean number of veal calves raised in each farm is about 1000 heads. Veal calves were Holstein Friesian breed and born in Italy and animals from each farm were slaughtered at the local abattoir at 8 months of age and 180-230kg of weight. Holstein-Friesian veal calves were fed with milk replacer and corn silage.
The lipid content in the milk replacer was 20% (35% coconut and 65% tallow + lard). The animals were housed in individual stalls within a single cowshed and cared for in accordance with European Union guidelines (No. 86/609/EEC) approved by the Italian Ministry of Health (L.116/92).

3.3.2 Sample Collection at the Abattoir

In the local abattoir, the activities taking place in the living area (unloading, hanging, and killing of animals) were separated from the rest of the processing activities to reduce the contamination of the evisceration room. The slaughter capacity of the plant was approximately 50 veal calves per hour. The journey and holding time prior to slaughter were usually less than a few hours.
Sampling was performed in two periods: from June to September 2009 (summer sampling) and from December 2009 to March 2010 (winter sampling).
In each period ten veal calves aging 8 months, originating from each farm, were randomly selected at the local abattoir. Sample collection was performed on different times during slaughtering: three animals at the beginning, four in the middle and three at the end of slaughtering process. In total, 50 carcasses samples and 50 fecal samples were collected in summer and 50 carcasses samples and 50 fecal samples were collected in winter.. The same sampling technique was used each time to ensure comparable results. Feces were collected directly from the rectum of each veal calf immediately after evisceration, and put into sterile-boxes (International PBI, Milan, Italy).
On corresponding carcasses, a non-destructive sampling method based on sponges moistened with 10 ml of sterile buffered peptone water (Sponge-Bag, International PBI, Milan, Italy) was used. The chosen sampling site (figure 3.1) was rump (**m. gluteus superficialis**) and the sponges were wiped over the sampling site (10 cm x 10 cm) for approximately 10 times in vertical and 10 times in horizontal directions. The sponges were placed into sterile bags, transported refrigerated to the laboratory and processed within 3 hour from collection.

Figure 3.1. Chosen sampling site on veal calf carcass.



3.3.3 Microbiological Analyses

Fecal samples (10 g) and sponges were homogenized with 90 ml of Arcobacter Enrichment broth (AEB) plus CAT (Cefoperazone, Amphotericin B, Teicoplanin) Selective Supplement (Oxoid S.p.A., Milan, Italy) in tightly sealed stomacher bags using a stomacher blender (International PBI, 123 Milan, Italy) for 1 min at normal speed.
Samples were incubated at 30°C for 4-5 days in aerobic conditions.
After enrichment culture, 0.2 ml of broth was plated on 0.45 μm pore size sterile cellulose acetate membrane filters (International PBI, Milan, Italy) placed on TSA Blood Agar (Oxoid S.p.A., Milan, Italy) to remove other enteric bacteria, as suggested by Ataby and Corry in1997(21). The filters were removed after 1 hour and plates were incubated at 30°C aerobically for at least 48 h. At least five small colourless or beige to off-white translucent colonies were selected from each plate and transferred to blood agar to obtain pure cultures.

3.3.4 Detection and identification of *Arcobacter* spp. with multiplex PCR

From each enrichment culture, after 4-5 days of incubation, DNA was extracted by the boiled lysate method. Four hundred microliters of each incubated

enrichment broth were pipetted into a micro test tube. The suspension was centrifuged at 16,500 g for 10 min.
Afterward the supernatant fluid was removed, and the remaining pellet was resuspended with 200 μl of Tris-EDTA buffer. The lysis of the bacterial cells took place during a 10 min incubation in a water bath at 100°C. Once cooled on ice, the suspension was centrifuged at 16,500 g for 10 min and 4 μl of the supernatant fluid was used for the multiplex PCR.
DNA extraction with a commercial kit was compared to the boiled lysate method on randomly selected samples to test the PCR sensitivity with both methods. Four hundred microliters of the suspension of selected enrichment broths were processed with the QIAamp DNA Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions.
The multiplex PCR using primers described by Houf et al. 2000 (33) was used for detection and identification of A. **butzleri**, A. **cryaerophilus**, and A. **skirrowii** in carcasses and fecal samples. Briefly, the DNA amplification was carried out in a C1000 Thermal Cycler (Biorad, Milan, Italy) in 25 μl solution containing 4μl of boiled lysate or purified DNA, 0.4 mM dNTP, 1.5 mM Mg2Cl, and 1 U of Taq DNA polymerase (Invitrogen, Milan, Italy). The cycling protocol was hot start at 95°C for 4 min, then 95°C for 45 sec, 61°C for 45 sec, and 72°C for 1 min for a total of 35 cycles, which was followed by 72°C for 5 min. The primers used are listed in Table 1.

Table 1. List of primers used in multiplex PCR fro detection and identification of *Arcobacter* species and eubacterial primers to check for the presence of inhibitors.

primer	sequence	sense
ARCO	5'-CGTATTCACCGTAGCATAGC-3'	forward
BUTZ	5'-CCTGGACTTGACATAGTAAGAATGA-3'	reverse
SKIR	5'-GGCGATTTACTGGAACACA-3'	reverse
CRY1	5'-TGCTGGAGCGGATAGAAGTA-3'	forward
CRY2	5'-AACAACCTACGTCCTTCGAC-3'	reverse
27f	5'-AGAGTT TGATCM TGGCTCAG-3'	forward
519r	5'- GWATTACCGCGGCKGCTG-3'	reverse

Negative controls in which DNA was replaced with sterile distilled water were included in every assay. DNA from A. **butzleri** (ATCC 49616), A. **cryaerophilus** (ATCC 43157) and A. **skirrowii** (ATCC 51132) were used as positive controls. PCR products were run on a 1.5% agarose gel in TAE buffer (Promega, Milan, Italy) at 100 V for 40 min. Resulting band sizes were 257 bp for CRY1–CRY2

(specific for *A. cryaerophilus*), 401 bp for ARCO-BUTZ (specific for *A. butzleri*), and 641 bp for ARCO-SKIR (specific for *A. skirrowii*).
Samples that were negative in the arcobacter-specific PCR were tested by PCR with eubacterial primers (27f and 519r, Table 1) to check for the presence of inhibitors (32).
For species identification of bacterial isolates from enrichment selective broths, multiplex PCR was performed on single bacterial isolates grown in nutrient Triptone-Soy Broth TSB (Oxoid, Milan, Italy) following the same protocol previously described.

3.4 Results

The results on the prevalence and distribution of the *Arcobacter* species in each season are summarized in Tables 3.2-3.5. In general, the PCR technique yielded a higher prevalence of *Arcobacter* spp. than the isolation method.

Table 3.2. Prevalence of single and multiple contamination by *Arcobacter* spp. in carcasses and feces of veal calves during summer sampling

	Carcasses		Feces	
	Isolation	PCR	Isolation	PCR
AC	11 (22%)	47 (94%)	3 (6%)	26 (52%)
AS	7 (14%)	17 (34%)	1 (2%)	17 (34%)
AB	4 (8%)	5 (10%)	4 (8%)	5 (10%)
multiple	0	17 (34%)	0	9 (18%)

AC: *A. cryaerophilus*; AS: *A. skirrowii*; AB: *A. butzleri*

Table 3.3. Prevalence of single and multiple contamination by *Arcobacter* spp. in carcasses and feces of veal calves during winter sampling

	Carcasses		Feces	
	Isolation	PCR	Isolation	PCR
AC	4 (8%)	8 (16%)	14 (28%)	24 (48%)
AS	5 (10%)	5 (10%)	8 (16%)	18 (36%)
AB	7 (14%)	9 (18%)	2 (4%)	8 (16%)
multiple	0	3 (6%)	0	10 (20%)

AC: *A. cryaerophilus*; AS: *A. skirrowii*; AB: *A. butzleri*

Table 3.4. Distribution of *Arcobacter* spp. on veal calf carcasses and feces sampled at slaughterhouse in summer (number and percentage of positive samples)

	Carcasses		Feces	
	Isolation	PCR	Isolation	PCR
AC	11 (22%)	47 (94%)	3 (6%)	26 (52%)
AS	7 (14%)	17 (34%)	1 (2%)	17 (34%)
AB	4 (8%)	5 (10%)	4 (8%)	5 (10%)
AC+AS	0	15 (30%)	0	8 (16%)
AC+AB	0	0	0	1 (2%)
AS+AB	0	0	0	0
AC+AS+AB	0	2(4%)	0	0
Total	22 (44%)	50 (100%)	8 (16%)	36 (72%)

AC: **A. cryaerophilus**; AS: **A. skirrowii**; AB: **A. butzleri**

Table 3.5. Distribution of *Arcobacter* spp. on veal calf carcasses and feces sampled at slaughterhouse in winter (number and percentage of positive samples)

	Carcasses		Feces	
	Isolation	PCR	Isolation	PCR
AC	4 (8%)	5 (10%)	14 (28%)	15 (30%)
AS	5 (10%)	4 (8%)	8 (16%)	12 (24%)
AB	7 (14%)	8 (16%)	2 (4%)	2 (4%)
AC+AS	0	1 (2%)	0	4 (8%)
AC+AB	0	2 (4%)	0	3 (6%)
AS+AB	0	0	0	0
AC+AS+AB	0	0	0	2 (4%)
Total	16 (32%)	20 (40%)	24 (48%)	38 (76%)

AC: **A. cryaerophilus**; AS: **A. skirrowii**; AB: **A. butzleri**

The isolation of **Arcobacter** species from some PCR-positive samples was unsuccessful maybe due to the overgrowth of other enteric bacteria or fungi. During summer season, with the isolation method 44% of sampled carcasses and 16% of fecal samples resulted contaminated with **Arcobacter** spp., **Arcobacter cryaerophilus** and **Arcobacter skirrowii** were the most prevalent on sampled carcasses (22% and 14% respectively), while **A. butzleri** and **A. cryaerophilus** in fecal samples

(8% and 6% respectively). In winter with isolation method 32% of sampled carcasses and 48% of fecal samples resulted contaminated with **Arcobacter** spp., **Arcobacter butzleris** and **Arcobacter skirrowii** were the most prevalent on sampled carcasses (7% and 5% respectively), while A. **cryaerophilus and** A. **skirrowii** were the most prevalent species in fecal samples (14% and 8% respectively)

It was not possible to detect multiple contamination with different **Arcobacter** spp. on both carcasses and fecal samples with the isolation method. Multiple isolates collected from the same sample were tested with multiplex PCR and confirmed to be the same species. Furthermore, **Arcobacter** spp. were not isolated from PCR negative samples.

The PCR technique detected **Arcobacter** spp. on 100% of the sampled carcasses and in 72% of fecal samples in summer (Table 3.4) and in 40% of carcasses and 76% of fecal samples in winter (Table 3.5). A. **cryaerophilus**, A. **skirrowii** and A. **butzleri** were detected on carcasses and in fecal samples with different prevalence (Table 3.2 and 3.3). During summer, the majority of the PCR positive samples from sampled carcasses and feces were identified as A. **cryaerophilus** (94% of carcasses and 52% of fecal samples). A. **skirrowii** and A. **butzleri** were detected with a lower prevalence on carcasses (34% and 10% respectively) and in fecal samples (34% and 10% respectively). During winter, the majority of the PCR positive samples from sampled carcasses was identified as A. **butzleri** (18% of carcasses) while A. **skirrowii** and A. **cryaerophilus** were detected with a lower prevalence on carcasses (10% and 16% respectively); in feces we identified A. **cryaerophilus** as prevalent species (48% of fecal samples), while A. **skirrowii** and A. **butzleri** were detected with a lower prevalence (36% and 16% respectively). Extraction method didn't influence the PCR results inasmuch as the randomly selected samples extracted with the boiled lysate method and the DNA extraction kit method gave the same PCR results (data not shown). As shown in Tables 3.4 and 3.5, the PCR method didn't detect **Arcobacter** in some enrichment fecal samples, however these samples yielded positive results for the PCR using eubacterial primers, confirming the absence of inhibiting factors.

In summer sampling, some sampled carcasses (34%) were contaminated with multiple **Arcobacter** species (A. **cryaerophilus** and A. **skirrowii** in 30% and A. **cryaerophilus**, A. **skirrowii** and A. **butzleri** in 4% of sampled carcasses). Also 18% of fecal samples harbored multiple **Arcobacter** species (A. **cryaerophilus** and A. **skirrowii** in 16% and A. **cryaerophilus** and A. **butzleri** in 2% of sampled carcasses).

Also in winter sampling some sampled carcasses (6%) were contaminated with multiple **Arcobacter** species (A. **cryaerophilus** and A. **skirrowii** in 4% and A. **cryaerophilus**, A. **skirrowii** in 2% of sampled carcasses). In fecal samples, 18% harbored multiple **Arcobacter** species (A. **cryaerophilus** and A. **skirrowii** in 8% and A. **cryaerophilus** and A. **butzleri** in 6% and A. **cryaerophilus** , A. **skirrowii** and A. **butzleri** in 4% of sampled feces).

Table 3.6 – 3.7. *Arcobacter* spp. positive samples (percentage) of the five examined farms as determined by isolation and by PCR (summer sampling)

Carcasses (%)

Farm	Method	AC	AS	AB	AC+AS	AC+AB	AC+AS+AB	Tot
1	Isolation	30	10	0	0	0	0	**40**
	PCR	90	0	0	10	0	0	**100**
2	Isolation	20	30	0	0	0	0	**50**
	PCR	60	0	0	40	0	0	**100**
3	Isolation	30	20	0	0	0	0	**50**
	PCR	50	0	0	50	0	0	**100**
4	Isolation	10	0	20	0	0	0	**30**
	PCR	40	0	20	30	0	10	**100**
5	Isolation	20	10	20	0	0	0	**50**
	PCR	60	0	10	20	0	10	**100**

AC: **A. cryaerophilus**; AS: **A. skirrowii**; AB: **A. butzleri**

Table 3.7.

Feces (%)

Farm	Method	AC	AS	AB	AC+AS	AC+AB	AC+AS+AB	Tot
1	Isolation	10	0	20	0	0	0	**30**
	PCR	30	0	30	0	0	0	**60**
2	Isolation	10	0	0	0	0	0	**10**
	PCR	20	20	0	10	0	0	**50**
3	Isolation	0	10	20	0	0	0	**30**
	PCR	50	20	0	20	10	0	**100**
4	Isolation	0	0	0	0	0	0	**0**
	PCR	10	30	0	40	0	0	**80**
5	Isolation	10	0	0	0	0	0	**10**
	PCR	30	20	10	10	0	0	**70**

AC: **A. cryaerophilus**; AS: **A. skirrowii**; AB: **A. butzleri**

Table 3.8 – 3.9. *Arcobacter* spp. positive samples (percentage) of the five examined farms as determined by isolation and by PCR (winter sampling)

Carcasses (%)

Farm	Method	AC	AS	AB	AC+AS	AC+AB	AC+AS+AB	Tot
1	Isolation	10	0	0	0	0	0	**10**
	PCR	10	0	0	0	0	0	**10**
2	Isolation	10	0	0	0	0	0	**10**
	PCR	10	0	0	0	10	0	**20**
3	Isolation	20	0	60	0	0	0	**80**
	PCR	10	0	80	0	10	0	**100**
4	Isolation	0	20	0	0	0	0	**20**
	PCR	10	20	0	0	0	0	**30**
5	Isolation	10	20	0	0	0	0	**30**
	PCR	10	20	0	10	0	0	**40**

AC: **A. cryaerophilus**; AS: **A. skirrowii**; AB: **A. butzleri**

Table 3.9.

Feces (%)

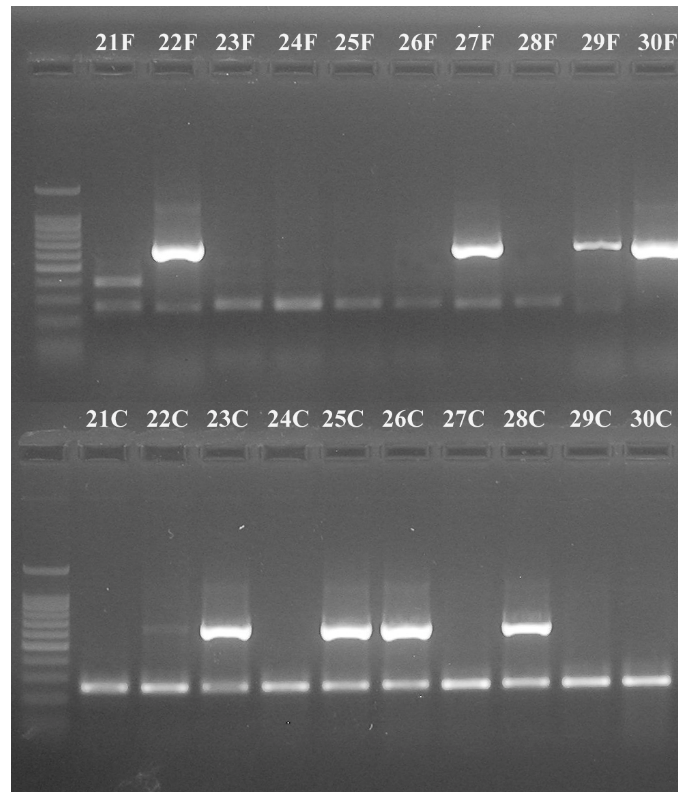
Farm	Method	AC	AS	AB	AC+AS	AC+AB	AC+AS+AB	Tot
1	Isolation	30	10	0	0	0	0	**40**
	PCR	40	10	0	0	10	0	**60**
2	Isolation	40	20	10	0	0	0	**70**
	PCR	40	20	0	0	20	10	**90**
3	Isolation	10	40	20	0	0	0	**70**
	PCR	10	50	10	0	10	0	**80**
4	Isolation	30	30	0	0	0	0	**60**
	PCR	30	30	0	20	0	0	**80**
5	Isolation	20	20	10	0	0	0	**50**
	PCR	20	10	10	20	0	10	**70**

AC: **A. cryaerophilus**; AS: **A. skirrowii**; AB: **A. butzleri**

In Tables 3.6 to 3.9 the prevalence of **Arcobacter** species in the different farms tested in each season are shown.

Depending on the isolation method, the prevalence of **Arcobacter** spp. on carcasses varied from 30% to 50% in summer and from 10% to 80% in winter. At the fecal level the prevalence varied from 0% to 30% in summer and from 40% to 70% in winter. With the PCR protocol, as shown before, 100% of carcasses were positive for **Arcobacter** spp. in summer, whereas in winter percentage varies from 10% to 100%. A. **cryaerophilus** is the most prevalent species in summer whereas in winter there isn't a dominant species. In both season there are some multiple contamination.

Figure 3.2. Example of multiplex-PCR for *Arcobacter* spp. detection in fecal (F) and corresponding carcasses samples (C) collected from veal calves of farm 3 at the slaughterhouse during summer. First lane: 100bp ladder. The ARCO-BUTZ primer pair amplified a 401-bp fragment specific for *Arcobacter butzleri*. The ARCO-SKIR primers amplified a 641-bp fragment specific for *A. skirrowii*. The CRY1-CRY2 primer pair amplified a 257-bp fragment specific for *A. cryaerophilus*.



The fecal presence of **Arcobacter** was detected in all farms, with different prevalence ranging from 50% to 100% (60%, 50%, 100%, 80% and 70% in farm 1, 2, 3, 4 and 5 respectively) in summer and from 60% to 90% (60%, 90%, 80%, 80% and 70% in farm 1, 2, 3, 4 and 5 respectively) of sampled veal calves and with a different distribution of species.
Particularly, during summer period, in herd 3 and 5, all **Arcobacter** species were detected, in herd 1 only A. **butzleri** and A. **cryaerophilus** were detected, and in herds 2 and 4 only A. **skirrowii** and A. **cryaerophilus** were detected. In winter all **Arcobacter** species were detected in herd 2, 3 and 5, in herd 1 and 4 only A. **cryaerophilus** and A. **skirrowii** were detected.
Also when 100% of carcasses at the slaughterhouse were contaminated, we did not observe a temporal distribution of **Arcobacter** species involved. Samples collected at the beginning, in the middle and at the end of slaughtering process showed a homogeneous species distribution with no accumulation of the three species in the last sampled carcasses.
Furthermore, only 16% of corresponding feces-carcass samples were characterized by the presence of the same species (single or multiple contamination) while 18% of corresponding feces-carcass samples were characterized by the presence of multiple species.

3.5 Discussion

This study confirmed that molecular methods are more sensitive than isolation for the detection of **Arcobacter** spp. With isolation methods, the prevalence of **Arcobacter** spp. could be underestimated due to difficulties in isolation of these bacteria. In particular, the intestinal flora, but also at lesser extent the contaminated carcasses, harbour a great numbers of enteric bacteria, fungi and yeasts that overgrowth tiny **Arcobacter** spp. colonies. In the present study **Arcobacter** spp. could not be isolated without filtration, but also with the use of 0.45 μm membrane filters, the prevalence was lesser than that observed by m-PCR. For example, the co-existence of more than one species in a sample was shown only by m-PCR. In general PCR gave better results for demonstration of the presence of **Arcobacter** species, which is in agreement with the experience of other research groups (22, 23).
The isolation of **Arcobacter** spp. from bovine fecal samples has been previously described. Van Driessche et al. in 2003 (14) detected the presence of **Arcobacter** at the slaughterhouse in 39.2% of bovine feces. Furthermore, Van Driessche et al. in 2005 (18) observed that in dairy farms the **Arcobacter** prevalence in bovine

faecal samples ranged from 7.5 to 15%, and particularly the prevalence for calves was determined as 27.3%.

Other studies have also reported the occurrence of arcobacters in clinically healthy cows, with a prevalence varying from 3.6 to 39.2% (14, 16, 17, 19, 24, 25). Differences in **Arcobacter** prevalence in feces from bovine animals reported in the literature can be ascribed to several causes: the sampling method, the isolation medium, the season, age of the sampled animals, the origin of the samples (such as country, farm or slaughterhouse), management (such as feeding, bedding and cleaning operation).

For example, Van Driessche et al. in 2005 (18) demonstrated that the exposure to different environmental condition and different diet between adult animals and young calves influences the prevalence of **Arcobacter** spp. The particular diet of veal calves may favor proliferation and dissemination of **Arcobacter** in the herd and between animals with consequent great prevalence. In the present study, the prevalence of **Arcobacter** contamination in veal calves feces 252 with PCR method was assessed at 72%. A. **cryaerophilus** was the most prevalent and diffuse in all selected farms, while A. **skirrowii** and A. **butzleri** somewhere were absent. Interestingly nor signs either symptoms of **Arcobacter** intestinal infection were observed in veal calves confirming previous published results where it is assumed that animals represents only a fecal carrier of this pathogen (16-18).

Arcobacters were present on 100% of sampled carcasses at the slaughterhouse. A similarly high prevalence has been reported only by Van Driessche and Houf in 2007 (26), on pork carcasses. The high prevalence may be explained by the high sensitivity of PCR method used. A. **cryaerophilus** was the most common species found on sampling sites on veal calf carcasses. The same result was observed in 2005 by Van Driessche et al. (18). On the other hand, this result contrasts with other studies where A. **butzleri** was reported as the most common species (27, 28).

As previously described by Van Driessche et al. in 2003, 2004 and 2005(4, 14, 18), on sampled carcasses we observed also co-contamination with different **Arcobacter** species (34%), according to the results of fecal contamination (18% of samples). The origin of this contamination it was not established but it is commonly assumed that enteric pathogens found on carcasses at the slaughterhouse are mainly derived from fecal origin (29).

Transfer of fecal material from the hide onto the carcasses during skinning and leaking of fecal material from the viscera during manipulation are the most probable sources.

Bacteria may also be transferred through cross-contamination between carcasses, equipment (knives) and hands of workers or particles shaken from the hide during skinnings operation (30). In the local abattoir of this study, no damage to the intestinal tract of the sampled carcasses was observed and no visible fecal

contamination was detected on selected carcasses. However the presence of aerosol or the diffusion of particles and dust from the hide or hairs during the skinning process cannot be excluded. Furthermore, this route of contamination would explain the fact that fecal samples and corresponding carcasses were often contaminated with different *Arcobacter* spp. or that positive carcasses had corresponding fecal sample negative to m-PCR. However, we hypothesized that the diffusion of *Arcobacter* by air is controlled by a ventilation and aspiration system that prevent accumulation of contaminants in the environment as demonstrated by the fact that sample collected at the end of slaughtering process had similar prevalence and distribution of the different *Arcobacter* species than samples collected at the beginning. This may underscore the fact that the slaughter environment has a major impact on contamination of carcasses by *Arcobacter*. The source of contamination on carcasses was not definitely established, but we can consider the skin and the intestinal content as the most likely sources of contamination.

In future studies, characterization of the arcobacters present in the feces and those isolated from the carcasses would help trace the contamination routes, though, as shown in previous *Arcobacter* studies in farm animals and poultry, the large heterogeneity among the isolates will complicate this investigation (4, 13, 31).

3.6 References

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CHAPTER 4

Isolation and Characterization of *Arcobacter* spp. in Bulk Tank Milk

4. Isolation and Characterization of *Arcobacter* spp. in Bulk Tank Milk

4.1 Abstract

Bulk tank milk (BTM) from 50 dairy herds in Northern Italy was examined for the presence of foodborne pathogen: **Arcobacter** spp. A membrane filtration technique with a non-selective blood agar was employed after enrichment in **Arcobacter** selective broth to isolate a wide range of **Arcobacter** spp. In addition, the same samples were tested at the species level by using a multiplex-PCR assay.
Arcobacter butzleri and A. **cryaerophilus** were detected in 40 and 8% of bulk tank milk samples, respectively. One bulk tank milk sample contained both **Arcobacter butzleri** and A. **cryaerophilus**. No BTM sample resulted positive to A. **skirrowii**. No significant association between the presence of **Arcobacter** spp. in BTM and herd management was observed. The findings of the study demonstrated the presence **Arcobacter** spp. as a potential milkborne pathogen and warrant the need for educational programs for dairy producers about the risks associated with consumption of raw milk.

4.2 Introduction

Pasteurization of commercially distributed milk has greatly reduced the risk of infection resulting from the consumption of contaminated milk (1, 2). However, a portion of the Italian population continues to consume raw milk and products made from it, namely, soft cheeses. In Italy, the sale of raw milk is legal and the consumption of raw bulk tank milk (BTM) is related to the recent installation of raw milk distributors all over the country. Among the non-farming population, a growing number of consumers are claiming that raw milk is healthier and are choosing raw milk over pasteurized milk. Certified raw milk is unpasteurized milk with a total bacterial count below a specified standard, but this is not a guarantee that the milk is free of bacterial pathogens. Although raw milk advocates claim that raw milk is healthier, research has shown no significant difference in the nutritional value of pasteurized and unpasteurized milk (3, 4).
Raw milk has been a known vehicle for pathogens for more than 100 years (1, 3, 5). In USA outbreaks associated with the consumption of raw milk occur routinely every year. In 1987 the FDA banned the interstate sale of raw milk. As of 1995, the intrastate sale of raw milk for human consumption was legal in 28 states (6). Consumption of certified raw milk has also been the source of

outbreaks (7, 8). Between 1973 and 1992, raw milk was associated with 46 outbreaks of foodborne illness in the United States. Consumption of raw milk is a high-risk behavior and will continue to cause morbidity and mortality until people stop consuming raw milk and raw milk products (9).

The risk of foodborne disease has increased over the last 20 yr (10). Outbreaks of foodborne illnesses following consumption of raw milk and products made from raw milk caused by Shiga toxin-producing **Escherichia coli** (STEC) (11, 12), **Salmonella** spp. (13, 14), **Listeria monocytogenes** (15), and **Campylobacter jejuni** (16, 17) have been reported in recent years. Gillespie et al. in 2003 (5) reported that between the years of 1992 and 2000, 52% of foodborne outbreaks in England and Wales were attributed to raw milk. Raw milk and products made from raw milk have been implicated in similar numbers of documented cases of foodborne illness in France (18).

The genus **Arcobacter** contains eight described species: **Arcobacter butzleri**, A. **cryaerophilus**, A. **nitrofigilis**; A. **skirrowii** ,A. **cibarius**, A. **thereius**, A. **halophilus** and A. **mytili** (19, 20, 21, 22).

The bacteria are Gram negative, non-sporeforming, motile, curved, occasionally straight, rods which may appear as spiral. The most important differences between **Arcobacter** and **Campylobacter** are the ability of **Arcobacter** to grow at 15-25°C and its marked aerotolerance (23, 24).

Although their pathogenicity remains to be fully elucidated, **Arcobacter** spp. have been considered as potential zoonotic foodborne and waterborne agents (25). Nevertheless the real occurrence of these potential pathogens in food is largely unknown. Water and raw meat (veal, beef, pork and poultry) are considered the most important source of Arcobacter infection in human (26).

Arcobacter spp. are considered an emerging human pathogen. A. **butzleri**, A **skirrowii** and A. **cryaerophilus** have been all associated with human enteritis and occasionally bacteriaemia (26, 27). Infection in human patients causes diarrhoea, abdominal pain and other symptoms including nausea, vomiting and fever (27).

The species A. **butzleri**, A. **cryaerophilus** and A. **skirrowii** are associated with reproduction disorders, mastitis and gastric ulcers in cattle and swine, but are more frequently isolated from healthy animals (28, 29, 30). In several studies, **Arcobacter** have been isolated from the feces of healthy cattle on farm and prior to slaughter (31, 32, 33).

The present study aimed to assess the **Arcobacter** spp. contamination of bulk tank milk (BTM) from dairy cow farms in Northern Italy, using conventional culture methods and multiplex PCR assay.

4.3 Materials and methods

4.3.1 Bulk Tank Milk

Dairy herds (n = 50) were selected randomly from the list of farms enrolled in the control of BTM quality by the Associazione Regionale Allevatori Lombardia (ARAL). Dairy herds were located in the province of Bergamo, Brescia, Cremona, Lecco, Lodi, Milano, Monza and Mantova. None of the selected herds sell raw milk by distributors. Management data (hygiene, bedding, milking routine, ecc) were collected for each herd. BTM from dairy herds was examined for the presence of milkborne **Arcobacter butzleri**, **A. skirrowii** and **A. cryaerophilus**. A single BTM sample (100 ml) was collected in sterile snap cap milk collection vial from each of the 50 dairy producers after the morning milking. Milk samples were collected between November 2009 and February 2010. The samples were shipped on ice to the laboratory and examined within 24 h of collection.

4.3.2 Fecal Sampling

Two dairy herds (one with BTM sample positive to **Arcobacter** spp. and one with BTM sample negative) were selected for fecal sample collection to detect the presence of **Arcobacter** spp. Feces were collected directly from the rectum of 20 dairy cows immediately after morning milking, and put into sterile-boxes (International PBI, Milan, Italy).

4.3.3 Microbiological Analyses

Fecal samples (10 g) and milk samples (10 ml) were homogenized with 90 ml of Arcobacter Enrichment broth (AEB) plus CAT (Cefoperazone, Amphotericin B, Teicoplanin) Selective Supplement (Oxoid S.p.A., Milan, Italy) in tightly sealed stomacher bags using a stomacher blender (International PBI, Milan, Italy) for 1 min at normal speed. Samples were incubated at 30°C for 4-5 days in aerobic conditions.
After enrichment culture, 0.2 ml of broth was plated on 0.45-µm pore size sterile cellulose acetate membrane filters (International PBI, Milan, Italy) placed on TSA Blood Agar (Oxoid S.p.A., Milan, Italy) to remove other enteric bacteria, as suggested by Ataby and Corry (34). The filters were removed after 1 hour and plates were incubated at 30°C aerobically for at least 48 h. Small colorless or beige to off-white translucent colonies were selected from each plate and transferred to blood agar to obtain pure cultures.

4.3.4 Detection and identification of Arcobacter spp. with multiplex PCR

From each enrichment culture, after 4-5 days of incubation, DNA was extracted by the boiled lysate method. Four hundred microliters of each incubated enrichment broth were pipetted into a micro test tube. The suspension was centrifuged at 16,500 g for 10 min. Afterward the supernatant fluid was removed, and the remaining pellet was resuspended with 200µl of Tris-EDTA buffer. The lysis of the bacterial cells took place during a 10 min incubation in a water bath at 100°C. Once cooled on ice, the suspension was centrifuged at 16,500 g for 10 min and 4 µl of the supernatant fluid was used for the multiplex PCR.

The multiplex PCR using primers described by Houf et al. (35) was used for detection and identification of A. **butzleri**, A. **cryaerophilus**, and A. **skirrowii** in carcasses and fecal samples. Briefly, the DNA amplification was carried out in a C1000 Thermal Cycler (Biorad, Milan, Italy) in 25µl solution containing 4µl of boiled lysate or purified DNA, 0.4 mM dNTP, 1.5 mM Mg2Cl, and 1 U of Taq DNA polymerase (Invitrogen, Milan, Italy). The cycling protocol was hot start at 95°C for 4 min, then 95°C for 45 sec, 61°C for 45 sec, and 72°C for 1 min for a total of 35 cycles, which was followed by 72°C for 5 min. The primers used are listed in Table 4.1.

Table 4.1. List of primers used in multiplex PCR fro detection and identification of *Arcobacter* species and eubacterial primers to check for the presence of inhibitors

primer	sequence	sense
ARCO	5'-CGTATTCACCGTAGCATAGC-3'	forward
BUTZ	5'-CCTGGACTTGACATAGTAAGAATGA-3'	reverse
SKIR	5'-GGCGATTTACTGGAACACA-3'	reverse
CRY1	5'-TGCTGGAGCGGATAGAAGTA-3'	forward
CRY2	5'-AACAACCTACGTCCTTCGAC-3'	reverse
27f	5'-AGAGTT TGATCM TGGCTCAG-3'	forward
519r	5'- GWATTACCGCGGCKGCTG-3'	reverse

Negative controls in which DNA was replaced with sterile distilled water were included in every assay. DNA from A. **butzleri** ATCC 49616, A. **cryaerophilus** ATCC 43157 and A. **skirrowii** ATCC 51132 were used as positive controls. PCR products were run on a 1.5% agarose gel in TAE buffer (Promega, Milan, Italy) at 100 V for 40 min. Resulting band sizes were 257 bp for CRY1–CRY2 (specific for A. **cryaerophilus**), 401 bp for ARCO-BUTZ (specific for A. **butzleri**), and 641 bp for ARCO-SKIR (specific for A. **skirrowii**).

Samples that were negative in the arcobacter-specific PCR were tested by PCR with eubacterial primers (27f and 519r, Table 1) to check for the presence of inhibitors (36).
For species identification of bacterial isolates from enrichment selective broths, multiplex PCR was performed on single bacterial isolates grown in nutrient broth (Oxoid, Milan, Italy) following the same protocol previously described.

4.4 Results

The results on the prevalence and distribution of the **Arcobacter** species in BTM are summarized in Tables 4.2. In general, the PCR technique yielded a higher prevalence of **Arcobacter** spp. than the microbiological method.
Microbiological analysis on BTM samples identified the presence of **Arcobacter** spp. in 26% of dairy herds. **Arcobacter butzleri** and A. **cryaerophilus** were detected in 24% and 2% of BTM samples, respectively, while **Arcobacter skirrowii** was never isolated. It was not possible to detect multiple contamination with different **Arcobacter** spp. on BTM samples with the isolation method. Microbiological positive samples were confirmed for the same **Arcobacter** species identification with molecular methods, even though 50% of BTM samples were positive with PCR technique. The microbiological isolation of **Arcobacter** species from some PCR-positive samples was unsuccessful maybe due to the overgrowth of other bacteria or yeast.
With the molecular method **Arcobacter butzleri** and A. **cryaerophilus** were detected in 40% and 8% of BTM samples, respectively. One bulk tank milk sample contained both **Arcobacter butzleri** and A. **cryaerophilus**. No BTM sample resulted positive to A. **skirrowii**.
As shown in Table 4.2, the PCR method didn't detect **Arcobacter** in some BTM samples, however these samples yielded positive results for the PCR using eubacterial primers, confirming the absence of inhibiting factors.

Table 4.2. Microbiological and PCR results of bulk tank milk samples collected from 50 dairy herds

	Bulk tank milk samples	
	Isolation	PCR
AC	1 (2%)	4 (8%)
AS	0	0
AB	12 (24%)	20 (40%)
AC + AS	0	0
AC + AB	0	1 (2%)
AS + AB	0	0
AC + AS + AB	0	0
negative	37 (74%)	25 (50%)
Total	50	50

To confirm the role of fecal shedding of **Arcobacter** spp. in dairy farm, two herd were selected: one with BTM positive (BTM+) for **Arcobacter butzleri** and one with BTM negative (BMT-) for any **Arcobacter** spp. Twenty lactating cows from each herd were randomly selected and feces were analyzed for the presence of **Arcobacter** spp.
The results on the prevalence and distribution of the **Arcobacter** species in BTM are summarized in Tables 4.3.

Table 4.3. Microbiological and PCR results of fecal samples (20 dairy cows) collected from a herd with a BTM sample positive to *A. butzleri* and from a herd with a negative BTM sample.

	Fecal samples			
	BTM+ herd		BTM- herd	
	isolation	PCR	isolation	PCR
AC	1/20	3/20	2/20	4/20
AS	1/20	2/20	2/20	2/20
AB	3/20	5/20	4/20	6/20
AC + AS	0/20	0/20	0/20	1/20
AC + AB	2/20	3/20	0/20	0/20
AS + AB	0/20	0/20	0/20	1/20
AC + AS + AB	0/20	0/20	0/20	0/20
negative	13/20	7/20	12/20	6/20
Total	20/20	20/20	20/20	20/20

With the molecular analysis of fecal samples, 65% of sampled cows from BTM+ herd harbored **Arcobacter** spp. in feces with **A. butzleri** as the most prevalent species (62% of positive fecal samples), while in BTM- herd, 70% of sampled cows harbored **Arcobacter** spp. in feces with **A. butzleri** as the most prevalent species (50% of positive fecal samples).
As observed for BTM samples, the microbiological isolation of **Arcobacter** species from some PCR-positive fecal samples was unsuccessful maybe due to the overgrowth of other enteric bacteria or fungi. The fecal contamination with the three species: **Arcobacter butzleri**, **A. cryaerophilus** and **A. skirrowii** was confirmed in both herd and some fecal samples resulted positive to more than one species.
As observed for BTM samples, the PCR method didn't detect **Arcobacter** in some fecal samples, however these samples yielded positive results for the PCR using eubacterial primers, confirming the absence of inhibiting factors.
After BTM sample collection, management data from each dairy herd were collected (Table 4.4). The number of lactating cows in selected dairy herds ranged from 50 to 560 with a mean of 200 lactating animals. In all dairy herds the type of bedding system is with cubicles (with mattress and/or sawdust, sand, straw, chopped straw), except for three dairy herds where the housing is loose in stalls with permanent straw bedding. During milking routine, 70% of milkers performed pre-dipping (teat disinfection prior to milking).

Table 4.4. Prevalence of contamination of BTM with *Arcobacter* spp. according to herd management

	management	prevalence	management	prevalence
pre-dipping	yes	8/15 (48,7%)	no	17/35 (53,3%)
bedding	cubicles	22/47 (46,8%)	permanent	3/3 (100%)
herd size	<200 head	13/31 (41,9%)	>200 head	12/19 (63,1%)

Even though the prevalence of contamination of BTM with **Arcobacter** spp. was greater in herd where no teat disinfection was performed before milking, in herd with a permanent bedding and in herd with more than 200 lactating animals, these differences were not significant with the other dairy herds (Fisher's Exact Test).

4.5 Discussion

The primary objective of this study was to estimate the prevalence of **Arcobacter** spp. in bulk tank milk of dairy herds by using microbiological method and PCR

assay. This is the first report of the isolation of **Arcobacter** spp from bovine milk in Italy.

This study confirmed that molecular methods are more sensitive than microbiological methods for the detection of **Arcobacter** spp. in milk, as observed in a previous study on bovine fecal and carcasses samples (chapter 3). With isolation methods, the prevalence of **Arcobacter** spp. could be underestimated due to difficulties in isolation of these bacteria. In particular, the milk flora and the contaminant of the bulk tank milk, can harbor a great number of bacteria and yeasts that overgrowth tiny **Arcobacter** spp. colonies. In fact, with microbiological methods **Arcobacter** spp. were detected in 26% of bulk tank milk samples, while with molecular methods 50% of samples were positive.

This prevalence of contamination is greater than what reported in previous surveys of raw milk where **Arcobacter** spp. prevalence rates were 3.2% (37) and 45% (38). Even though the selected dairy herds did not sell raw milk through distributors, raw milk can represents a risk for public health and raw milk samples should be examined for **Arcobacter** contamination.

The main route of contamination of BTM is represented mainly by fecal contamination of teats of mammary glands and consequently of milk during milking procedures or by fecal contamination of the tank where milk is collected and refrigerated. In support of this hypothesis, Collado et al. (40) found that **Arcobacter** spp. were most frequently isolated from samples with the highest levels of fecal contamination. In this study, microbiological or molecular analysis on milk samples collected directly from bovine mammary glands were not performed so we cannot exclude a possible role of the cow with an **Arcobacter** intramammary infection in the diffusion of this pathogen in the BTM.

A real role of **Arcobacter** spp. in bovine intramammary infection has never been fully demonstrated in dairy herds. There is only the report of Logan et al. (39) on cases of isolation of **Arcobacter** in mastitis in cows. The authors infected the animals by intramammary inoculation and each infected quarter developed an acute clinical mastitis which resolved spontaneously after 120 hours. In the present work it is not possible to confirm its possible pathogenic role on the mammary gland.

On the other side, **Arcobacter** spp. also can be found in the digestive system of healthy dairy cows that do not show any clinical signs (41, 42). Scullion et al. (43) reported that **Arcobacter** prevalence in local raw milk could readily be explained by carriage rates in dairy cattle. In the present study we confirmed that healthy and lactating dairy cows can harbor **Arcobacter** spp. at intestinal level even in dairy herd where **Arcobacter** was not isolated from BTM. Even if the BTM samples analyzed in this study resulted positive only for A. **butzleri** and A. **cryaerophilus**, all three **Arcobacter** species were identified in fecal samples, consequently also A. **skirrowii** could be a potential contaminant of BTM.

Several researchers have found that the prevalence of arcobacters in raw milk depends on the farm hygienic conditions, source of water, and animal diet (43, 44). So the second goal of this study was to attempt to identify herd management characteristics, which were likely to be associated with *Arcobacter* contamination of BTM. Because of the relatively small number of herds analyzed, some management data were not comparable. For example, three dairy herds with permanent bedding were characterized by a 100% contamination of BTM with *Arcobacter* spp. and these data were not statistically comparable with 47 dairy herds with cubicles and with 46.8% of BTM contamination. On the other side, none of the management parameters gave significant results, consequently at the herd level, *Arcobacter* prevalence could not be correlated with herd size, bedding or pre-dipping.
In conclusion, *Arcobacter* spp. were found in BTM and in feces of dairy cows. The presence of *Arcobacter* spp. in raw milk should be regarded as a hazard for human health. Therefore, raw milk should be consumed only after pasteurization. Further epidemiological studies are needed to better understand the route of diffusion of *Arcobacter* in dairy cows environment.

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CHAPTER 5

Antimicrobial Susceptibility of *Arcobacter* spp. Isolated from Food of Animal Origin

5. Antimicrobial Susceptibility of *Arcobacter* spp. Isolated from Food of Animal Origin

5.1 Abstract

Arcobacter spp. is a human pathogen and a potential animal pathogen. Thus is important to study antimicrobial susceptibility of strains collected from food of animal origin testing the most common antimicrobial agents used in human and veterinary medicine.

In the present study, we examined the antimicrobial susceptibility of 50 **Arcobacter** spp. strains collected in previous studies from veal calves carcasses and bulk tank milk (BTM), testing 8 antimicrobial agents by disk diffusion testing. Antibiotics and the concentrations of discs (μg) were kanamycin (30), streptomycin (10), gentamicin (10), tetracycline (30), cephalothin (30), ciprofloxacin (5), nalidixic acid (50) and sulfamethoxazole–trimethoprim (23.75 and 1.25, respectively). The plates were cultured at 37 °C and after 48 h of incubation, the diameter of the inhibition zones was measured with a slide caliper.

All the tested **Arcobacter** strains showed resistance to cephalotin, sulfamethoxazole+trimethoprim and nalidixic acid. A. **cryaerophilus** from veal calf carcasses showed a 100% susceptibility to tetracycline. A. **butzleri** from BTM showed 100% susceptibility to gentamycin.

5.2 Introduction

Genus **Arcobacter**, formerly known as aerotolerant **Campylobacter**, is composed of eight species: **Arcobacter butzleri**, A. **cryaerophilus**, A. **nitrofigilis**; A. **skirrowii** ,A. **cibarius**, A. **thereius**, A. **halophilus** and A. **mytili** (1-5).

A. **cryaerophilus**, A. **butzleri and** A. **skirrowii** are associated with human and animal diseases. In animals, A. **butzleri** and A. **cryaerophilus** have been isolated from diarrhea samples (6-9) and A. **skirrowii**, as well as A. **butzleri** and A. **cryaerophilus**, are known to be associated with abortion in production animals, though the pathology of the disease has not been fully understood (10-13). The organisms have also been isolated from milk samples derived from a cow with mastitis (14). In humans, A. **butzleri** and A. **cryaerophilus** have been associated with diarrhea and bacteremia (8, 15,16).

Several studies have examined the prevalence of **Arcobacter** species among domestic animals (13,17-19). **Arcobacter** can easily be found in cloacal swabs of poultry, cattle and pigs and also in carcasses or raw meat.
Thus, **Arcobacter** species seem to be highly prevalent in animal and chicken meat all over the world.
Arcobacter are human pathogens and potential animal pathogens, so the study of their antimicrobial susceptibility to evaluate their resistance to common antimicrobial agents used in veterinary and human medicine is very important.
In a review of 2006 by Snelling et al. (20) there is a description of **Arcobacter** antimicrobial susceptibility. According to this study **Arcobacter** spp. seem to be resistant to erythromycin and ciprofloxacin. On the other side, in this study they result susceptible to aminoglycosides, including kanamycin and streptomycin, tetracycline and cephalotin.
In the present study, we examined the antimicrobial susceptibility of **Arcobacter** spp. strains collected in previous studies (chapter 3 and 4), testing 8 antimicrobial agents by disk diffusion testing.

5.3 Materials and Methods

5.3.1 Bacterial Strains

A total of 50 strains of **Arcobacter** spp. (22 strains of **A. butzleri**, 16 strains of **A. cryaerophilus** and 12 of **A. skirrowii**) isolated in previous studies (chapter 3 and 4) from veal calves carcasses (11 **A. butzleri**, 15 **A. cryaerophilus** and 12 **A. skirrowii**) and from bulk tank milk (BTM) samples (11 **A. butzleri** and 1 **A. cryaerophilus**) have been tested for antimicrobial susceptibility.

5.3.2 Determination of antimicrobial susceptibility

In vitro antimicrobial susceptibility testing was conducted by the disk-diffusion testing method in accordance with the standards described in the Clinical and Laboratory Standards Institute (21). Zone diameter interpretative breakpoints for **Enterobacteriaceae** were used to determine susceptibility and resistance (table 5.1). Briefly, each isolate was added to a sterile diluent to contain approximately 10^8 CFU/ml (equivalent to 0.5 MacFarland standard) and plated on Mueller Hinton agar. **Escherichia coli** (ATCC 25922) was used as quality control organisms. A disk diffusion test was performed for the examination of antimicrobial susceptibility by using commercial disks (Oxoid, Milan, Italy). Antibiotics and the concentrations of discs (μg) were kanamycin (30), streptomycin (10), gentamicin

(10), tetracycline (30), cephalothin (30), ciprofloxacin (5), nalidixic acid (50) and sulfamethoxazole–trimethoprim (23.75 and 1.25, respectively).
The plates were cultured at 37 °C and after 48 h of incubation, the diameter of the inhibition zones was measured with a slide caliper.

Table 5.1. Zone diameter interpretative breakpoints for *Enterobacteriaceae* were used to determine susceptibility and resistance

antimicrobial	zone diameter (mm)		
	resistant	intermediate	susceptible
K	≤13	14-17	≥15
S	≤11	12-14	≥15
CN	≤12	13-14	≥15
TE	≤11	12-14	≥15
KF	≤14	15-17	≥18
CIP	≤15	16-20	≥21
NA	≤13	14-18	≥19
STX	≤10	11-15	≥16

K: kanamycin; S: streptomycin; CN: gentamicin; TE: tetracycline; KF: cephalotin; CIP: ciprofloxacin; NA: nalidixic acid; STX: sulfameth+trimeth

5.4 Results and Discussion

In this study, the antimicrobial resistance patterns of **Arcobacter** spp. isolated from veal calf carcasses in a processing plant and from bulk tank milk samples were examined and compared. To accomplish this, the CLSI criteria for **Enterobacteriaceae** were adopted to categorize the **Arcobacter** strains as susceptible or resistant because there are currently no available data that can be used for the interpretation of disk diffusion susceptibility testing for **Arcobacter** spp.
In the present study, 50 **Arcobacter** strains isolated from carcasses (11 A. **butzleri**, 15 A. **cryaerophilus** and 12 A. **skirrowii**) and BTM (11 A. **butzleri** and 1 A. **cryaerophilus**) were examined for susceptibility to 8 antimicrobial agents.
All the tested **Arcobacter** strains, from both carcasses and BTM samples, showed resistance to cephalotin, sulfamethoxazole+trimethoprim and nalidixic acid.
The results about cephalotin are in accordance to previous studies (22) and (23) where all the A. **butzleri** strains were identified as resistant to cephalothin (100% and 94.8%, respectively), even though, differently from what reported in the present study, 63.4% of A. **cryaerophilus** and 73.3% of A. **skirrowii** were revealed to be susceptible to that antimicrobial (23).

In the same study (22) all the strains of **A. butzleri** examined have been reported to be susceptible to nalidixic acid, differently from what reported in the present study where all **Arcobacter** species were resistant to that antimicrobial.
Arcobacter skirrowii strains, isolated only from carcasses, showed also resistance to streptomycin. **A. cryaerophilus** from veal calf carcasses showed relatively higher susceptibility to tetracycline (100%), gentamycin (93.3%) and ciprofloxacin (93.3%), than other **Arcobacter** species isolated from the same sources.
On the other side, **A. butzleri** from carcasses showed a greater susceptibility to streptomycin than other **Arcobacter** species from the same sources.
The incidence of drug resistance in **Arcobacters** spp. according to the source of sampling (carcasses or BTM) was comparable only for **A. butzleri**: the only difference observed was that the percentage of **A. butzleri** strains isolated from carcasses showed greater susceptibility to ciprofloxacin (55%) than strains isolated from carcasses (9%).

Table 5.2. Number of tested strains, mean and range of zone diameter interpretative results (mm) and percentage of antimicrobial susceptibility of *Arcobacter* strains from BTM samples

	A. butzleri				*A. cryaerophilus*		
antimicrobial	strain	mean	range	%	strain	mm	
K	11	16.0	11-20	**27.3**	1	14	I
S	11	17	7-22	**81.8**	1	6	R
CN	11	19.0	16-22	**100**	1	20	S
TE	11	15.9	11-19	**72.7**	1	14	I
KF	11	4.5	0-11	**0**	1	12	R
CIP	11	20.5	16-25	**54.5**	1	22	S
NA	11	2.6	0-7	**0**	1	9	R
STX	11	5.5	0-12	**0**	1	0	R

K: kanamycin; S: streptomycin; CN: gentamicin; TE: tetracycline; KF: cephalotin; CIP: ciprofloxacin; NA: nalidixic acid; STX: sulfameth+trimeth

Table 5.3 Number of tested strains, mean and range of zone diameter interpretative results (mm) and percentage of antimicrobial susceptibility of *Arcobacter* strains from veal calf carcasses.

	A. butzleri				*A. cryaerophilus*				*A. skirrowii*			
	strain	mean	range	%	strain	mean	range	%	strain	mean	range	%
K	11	17.1	10-22	**36.7**	15	11.1	0-21	**33.3**	12	15.4	12-20	**33.3**
S	11	17.3	11-22	**81.8**	15	7.9	0-17	**6.7**	12	3.3	0-11	**0**
CN	11	17.8	21	**90.0**	15	17.6	14-21	**93.3**	12	17.4	8-22	**83.3**
TE	11	16.5	8-21	**64.3**	15	18.7	14-22	**100**	12	17.9	10-24	**75**
KF	11	4.6	0-13	**0**	15	7.8	0-16	**0**	12	6.2	0-12	**0**
CIP	11	15.1	6-21	**9**	15	26.5	20-30	**93.3**	12	18.8	13-23	**33.3**
NA	11	2.7	10	**0**	15	7.0	0-12	**0**	12	8.2	0-18	**0**
STX	11	3.7	0-12	**0**	15	0	0	**0**	12	0	0	**0**

K: kanamycin; S: streptomycin; CN: gentamicin; TE: tetracycline; KF: cephalotin;
CIP: ciprofloxacin; NA: nalidixic acid; STX: sulfameth+trimeth

The differences observed between the antimicrobial resistance of this study and what previously published on Arcobacter spp. may be due to the differences of the breakpoints of the antibiotics used and the different testing methods (disk diffusion method instead of broth dilution method). However, attention should be paid to the treatment of infections with Arcobacter using nalidixic acid, cephalotin and sulfamethoxazole+trimethoprim, because the susceptibilities of Arcobacters to these antibiotics were very low among all the tested strains (mean zone diameters ranging from 0 to 8 mm).
In conclusion, Arcobacter species are broadly distributed among bovine raw food (meat and milk) in Italy. Furthermore, the strains showed resistance to a broad spectrums of antimicrobial agents.. In addition, the susceptibilities differed among species and among strains. Cephalothin should be considered for use in enrichment medium because all Arcobacter spp. were revealed to be significantly resistant.

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CHAPTER 6

General Discussion

6. General discussion

Arcobacter are commonly isolated from animals, food, and environmental samples worldwide, and cases of human **Arcobacter** infection have regularly been reported. The infection source remains however often unclear. Several major aspects actually hamper the risk assessment for these bacteria. First, besides the fact that clinical samples are not routinely tested for **Arcobacter** species as done for **Salmonella** or **Campylobacter**, most isolations are performed by methods designed for thermophilic **Campylobacter** species. The selectivity of those media is mostly achieved by incorporation of antimicrobial agents and several studies about the **Arcobacter** susceptibility show that none of those supplements allowed growth of all **Arcobacter** species or strains and at the same time they don't sufficiently suppress the accompanying flora present in biological samples.

The low recovery rate reported from human diarrheal specimens is therefore certainly an underestimation of the real prevalence. In order to determine the exposure assessment of human to **Arcobacter**, efficient robust, and reliable methods for isolation, identification and characterization of **Arcobacter** are needed.

Correct identification of **Arcobacter** is another challenge in microbiology, and too often, **Arcobacter** are misidentified as campylobacters, especially when phenotypical methods are applied. Due to the relatively metabolic inertness and the antigenic heterogeneity, biochemical or serological identification of **Arcobacter** is not recommended. Besides, they are time consuming and laborious and clearcut parameters are not always present in all strains of a certain species. For now, identification at genus level can reliable be performed by genus-specific PCR assays. As shown in this study PCR tenchnique is more sensitive than microbiological one.

Direct detection of **Arcobacter** by PCR, or even direct quantification by RT-PCR in a biological specimen is not commonly applied at this moment. In contrast to more vulnerable bacteria as **Helicobacter** species, **Arcobacter** are relatively easily to culture and there is no demand in human or veterinary medicine for a rapid analysis tool at the moment. Furthermore, as biological matrices are complex and often comprise inhibiting factors, preparation, and clean-up of the sample is expensive and hamper their use in routine laboratories. Furthermore, many studies are focused on further examination of the isolates as microbiological susceptibility and typing for which the preservation of the isolates is needed. The use of those direct methods can however have a future in large scale surveys.

The existence of considerable heterogeneity among Arcobacter in one specimen is yet another difficulty in **Arcobacter** research. This phenomenon was already reported in early ribotyping and serotyping studies. Possible explanations are multiple sources of contamination, the existence of multiple parent genotypes,

and a high degree of genomic recombination among the progeny of parent genotypes, but none of these hypotheses has been demonstrated yet.
Arcobacter should be considered an emerging pathogen. As described in this study, little is known in Italy about its presence, prevalence and distribution in cattle, both in veal calves and in dairy cows.
In Italy cattle is an important source of food for the population. We largely eat both veal and raw milk. This way **Arcobacter** could be a "new and poorly known" risk for human health.
Arcobacter spp. exist in the guts of healthy cattle and thus that they may contaminate the environment and the human food chain. Healthy cattle are an important reservoir of this microorganism.
It is important to prevent infection establishing epidemiological links between **Arcobacter** isolated from different sources, elucidating the transmission routes and studying antimicrobial susceptibility of strains collected from food of animal origin testing the most common antimicrobial agents used in human and veterinary medicine.

CHAPTER 7

Summary

7. Summary

7.1 Foreword

The genus **Arcobacter**, previously classified as "aerotolerant campylobacter", from 1991 is classified as a new genus member of the family of **Campylobacteriaceae**, belonging to the epsilon-proteobacteria. The genus **Arcobacter** contains eight described species: **Arcobacter butzleri**, A. **cryaerophilus**, A. **nitrofigilis**; A. **skirrowii** ,A. **cibarius**, A. **thereius**, A. **halophilus** and 46 A. **mytili** .
The bacteria are Gram negative, non-sporeforming, motile, curved, occasionally straight, rods which may appear as spiral. The most important differences between **Arcobacter** and **Campylobacter** are the ability of **Arcobacter** to grow at 15-25°C and its marked aerotolerance.
Arcobacter spp. have been considered as potential zoonotic foodborne and waterborne agents (. **Arcobacter** spp. can be found in meat (veal, beef, pork and poultry), milk and water. Nevertheless the real occurrence of these potential pathogens in food is largely unknown.

7.2 Objectives

The aim of this study is to evaluate prevalence and distribution of Arcobacter spp. in food of animal origin (raw meat and milk) in Northern Italy and to test the antimicrobial susceptibility of collected strains.

7.3 Trial 1: Prevalence and Distribution of *Arcobacter* spp. In Veal Calves in Northern Italy

In this study the prevalence and distribution of **Arcobacter** spp. in samples taken from feces and carcasses of healthy veal calves, raised in Northern Italy, were investigated. A membrane filtration technique with a non-selective blood agar was employed after enrichment in **Arcobacter** selective broth to isolate a wide range of **Arcobacter** spp. In addition, the same samples were tested at the species level by using a multiplex-PCR assay. Samples from feces (50 during a summer sampling, 50 during a winter sampling) and carcasses (50 during a summer sampling a 50 during a winter sampling) were collected at the slaughterhouse from 50 veal calves originating from five different farms. Of the fecal samples examined, 36 (72%) were found positive for at least one species of **Arcobacter**

during summer sampling and 38 (76%) were found positive during winter sampling. Of the sampled carcasses examined, 50 (100%) were found positive for at least one species of **Arcobacter** in summer sampling and 20 (40%) in winter sampling. From feces, 23 (46%), 17 (34%) and 5 (10%) samples were found positive by m-PCR for A. **cryaerophilus**, A. **skirrowii** and A. **butzleri**, respectively in summer. On the other side in winter 15 (30%), 12 (24%) and 2 (8%) samples were found positive by m-PCR for A. **cryaerophilus**, A. **skirrowii** and A. **butzleri**, respectively. From carcasses, 47 (94%), 17 (34%) and 5 (10%) samples were positive for A. **cryaerophilus**, A. **skirrowii** and A. **butzleri**, respectively in summer sampling whereas 5 (10%), 4 (8%) and 8 (16%) samples were positive for A. **cryaerophilus**, A. **skirrowii** and A. **butzleri**, respectively in winter sampling . Some sampled carcasses (34% in summer and 6% in winter) and some fecal samples (18% both in summer and in winter) resulted contaminated with multiple **Arcobacter** species. The present study indicates that veal calves can harbour a variety of **Arcobacter** spp. in the intestinal tract and that the presence of **Arcobacter** spp. may represent a source of contamination and dissemination in slaughterhouse.

7.4 Trial 2: Isolation and Characterization of *Arcobacter* spp. in Bulk Tank Milk

Bulk tank milk (BTM) from 50 dairy herds in Northern Italy was examined for the presence of foodborne pathogen: **Arcobacter** spp. A membrane filtration technique with a non-selective blood agar was employed after enrichment in **Arcobacter** selective broth to isolate a wide range of **Arcobacter** spp. In addition, the same samples were tested at the species level by using a multiplex-PCR assay.
Arcobacter butzleri and A. **cryaerophilus** were detected in 40 and 8% of bulk tank milk samples, respectively. One bulk tank milk sample contained both **Arcobacter butzleri** and A. **cryaerophilus**. No BTM sample resulted positive to A. **skirrowii**. No significant association between the presence of **Arcobacter** spp. in BTM and herd management was observed. The findings of the study demonstrated the presence **Arcobacter** spp. as a potential milkborne pathogen and warrant the need for educational programs for dairy producers about the risks associated with consumption of raw milk.

7.5 Trial 3: Antimicrobial Susceptibility of *Arcobacter* spp. Isolated from Food of Animal Origin

Arcobacter spp. is a human pathogen and a potential animal pathogen. Thus is important to study antimicrobial susceptibility of strains collected from food of animal origin testing the most common antimicrobial agents used in human and veterinary medicine.

In the present study, we examined the antimicrobial susceptibility of 50 **Arcobacter** spp. strains collected in previous studies from veal calves carcasses and bulk tank milk (BTM), testing 8 antimicrobial agents by disk diffusion testing. Antibiotics and the concentrations of discs (μg) were kanamycin (30), streptomycin (10), gentamicin (10), tetracycline (30), cephalothin (30), ciprofloxacin (5), nalidixic acid (50) and sulfamethoxazole–trimethoprim (23.75 and 1.25, respectively). The plates were cultured at 37 °C and after 48 h of incubation, the diameter of the inhibition zones was measured with a slide caliper.

All the tested **Arcobacter** strains showed resistance to cephalotin, sulfamethoxazole+trimethoprim and nalidixic acid. A. **cryaerophilus** from veal calf carcasses showed a 100% susceptibility to tetracycline. A. **butzleri** from BTM showed 100% susceptibility to gentamycin.

7.6 Conclusions

This study shows that **Arcobacter** spp. is largely diffused in healthy livestock and in food of animal origin, both raw meat and raw milk.

Although their pathogenicity remains to be fully elucidated, arcobacters are potential foodborne pathogens and their prevalence should be carefully investigated to guarantee public human and animal health.

CHAPTER 8

Acknowledgements

8. Acknowledgements

Ed eccomi qui, giunta alla fine di un percorso che ha impegnato tanti anni della mia vita (i migliori?).
Prima la laurea, poi le borse di studio e i co.co.co. e infine il dottorato… tra una cosa e l'altra ho passato ben *13 anni* della mia esistenza in via Celoria 10.

Mi porterò dentro molte cose: le gioie e i dolori, la paura di non farcela e le enormi soddisfazioni portate da un obiettivo raggiunto… ma anche le levatacce di ogni mattina, le lunghe code sull'A4 e in tangenziale, il caffè schifoso del bar e gli snack delle "macchinette".

E ovviamente le persone.
Quante ne ho conosciute in questi anni? Centinaia credo… e tutte hanno avuto un ruolo fondamentale.

Il primo pensiero va ovviamente a tutto il personale del Laboratorio di Ispezione degli Alimenti del Dipartimento VSA. Con tutte queste persone ho lavorato gomito a gomito ogni giorno dall'ottobre 2005 all'ottobre 2010. Cinque anni che mi hanno segnato profondamente.
Un ringraziamento particolare va alla *Prof. Soncini* che mi ha accolto, come una "povera orfanella", dopo il pensionamento del Prof. Cantoni. Grazie per i consigli, per le lunghe chiacchierate e per aver sopportato i miei sfoghi.
Ovviamente fra le persone a cui devo di più c'è anche il *Prof. Cantoni*, il mio "mentore". E' grazie a lui che ho imparato ad amare l'Ispezione degli Alimenti e nonostante sia una delle persone più confusionarie che io conosca da lui ho imparato buona parte di quello che so. E' stato lui ad indirizzarmi verso le prime esperienze lavorative, talvolta gettandomi completamente allo sbaraglio in situazioni che non ero assolutamente pronta ad affrontare da sola… e proprio grazie alla fiducia che il Professore ha sempre riposto in me ho imparato a cavarmela anche nelle situazioni più difficili e a diventare indipendente ponendo le basi della mia futura carriera.
E grazie anche a tutti gli altri: La *Prof. Cattaneo*, La *Dott.ssa Bersani*, *Simone*, *Giulia, Luciana*, *Barbara*, *"La Cri"*, *Silvia*, *Ada* e le nuove arrivate *Erica* e *Vale*.
Ognuno di loro ha dato un contributo alla mia crescita personale e professionale e mi ha insegnato qualcosa… e scusatemi se ho dimenticato qualcuno.

Ed ora passiamo alla famiglia…
Grazie ai *miei genitori* che mi hanno sempre sostenuto e rincuorato, pur non condividendo sempre appieno le mie scelte.

Grazie a *Giuliano* che mi ha aiutato in modo enorme. Ha sopportato i miei pianti di sconforto, ha condiviso con me i momenti belli e brutti da ormai quasi 14 anni. Mi ha spronato, mi ha sgridato quando doveva sgridarmi e mi ha consolato quando ero triste. E'

sempre stato il primo a riconoscere i miei pregi e i miei difetti e mi ha aiutato enormemente nella stesura di questa tesi e soprattutto nella vita. Grazie Amore Mio.

…E infine grazie a TE, il mio piccolino non ancora nato che porto dentro di me. Non ti conosco ancora, non so nemmeno se sei un maschietto o una femminuccia. Ma sono la tua mamma e sento già di amarti immensamente…

Serena