

***Arcobacter* spp., what is known and unknown about a potential foodborne zoonotic agent**

Arcobacter spp, wat is bekend en nog onbekend omtrent een
waarschijnlijk voedsel gerelateerd zoönotisch agens
(met een samenvatting in het Nederlands)

Arcobacter spp., những điều đã biết và chưa biết
về vi khuẩn gây ngộ độc thực phẩm này
(với bản tóm tắt bằng tiếng Việt)

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This is dedicated to Grandma and Mother who have lighted and guided my life.

Wherever you are I wish you would see this.

Con kính dâng lên Má, Ý thành quả này.

Người đã vun vén và dẫn dắt cuộc đời của con.

Dù Má, Ý đang ở đâu, con ước mong Người nhìn thấy con và tự hào với con.

Arcobacter spp., what is known and unknown about a potential foodborne zoonotic agent
Ph.D. Thesis

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The scope of this thesis

A new genus, the genus *Arcobacter* which is related to *Campylobacter* and also has a similar shape, was introduced in 1991 (21). Nevertheless, unlike *Campylobacter*, *Arcobacter* spp. are aerotolerant and their optimal growth temperature is at 30°C. Members of this genus inhabit very diverse environments. The type species *A. nitrofigilis* is a nitrogen fixing bacterium associated with the roots of the salt-marsh plant *Spartina alterniflora* (16) and *A. halophilus* is an obligate halophile isolated from a hypersaline lagoon (5). Three other species, *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have been found both in association with diseased as well as healthy humans and animals (1; 6; 7; 10; 11; 13; 15; 17; 20; 22-25) and are considered potential zoonotic agents. *A. cibarius* has been isolated exclusively from chicken carcasses (9).

The abundant presence of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* in food of animal origin (3; 8; 14; 19) and in soil (2) and drinking and ground water (4; 12; 18) as well as their association with illness in humans and animals caused that these bacteria were considered to play a role as foodborne or waterborne agents. Nevertheless, more than a decade after the discovery of the genus the routes of transmission and the pathogenic mechanisms of these bacteria are virtually unknown. In spite of the large number of publications on detection of *Arcobacter* spp. and, on their prevalence in various environments and foods, in animals and human, no reports on the genetic manipulation and characterisation of potential virulence factors of *Arcobacter* have appeared.

THE SCOPE OF THIS THESIS

The aim of this thesis was to investigate the routes of transmission of *Arcobacter* spp. in animals as well as some aspects of their pathogenic properties.

The status of *Arcobacter* spp. in the literature was reviewed in **Chapter 1**.

In **Chapter 2** the potential of vertical and horizontal transmission of *Arcobacter* spp. in pigs was studied. At farrowing, amniotic fluid of sows and rectal swabs of the newborn piglets were collected and tested for the presence of *Arcobacter* spp.. Rectal swabs were taken immediately after birth of the piglets and bacterial secretion by those piglets was tested during the first three weeks of their life. Pulse-field gel electrophoresis was applied to verify the vertical route of transmission for arcobacters. A Western-blot assay was used in order to find the maternal antibodies against *Arcobacter* spp. in sow colostrums.

In **Chapter 3** the vertical transmission of *Arcobacter* spp. in breeding hens to chicks was investigated. The presence of arcobacters in breeding hens and eggs was examined. At a hatchery, hatchery wastes, newly hatched chicks and unhatched eggs were sampled for *Arcobacter* spp..

Chapter 4 contains research performed in an attempt to reveal the “mystery” behind the source of *Arcobacter* contamination on carcasses in slaughterhouses. Various samples in poultry slaughterhouses including carcasses, gut contents, processing water and supply water were taken and examined for the presence of the bacteria. Eric-PCR was used to study the genotypic diversity of the isolates as well as to track down the potential source of *Arcobacter* contamination of the carcasses.

In **Chapter 5** some pathogenic properties of *Arcobacter* strains isolated from human and animals were investigated by studying their ability to adhere and invade into some human and porcine cell lines. The induction of interleukin 8- a proinflammatory cytokine was also examined.

In **Chapter 6** flagellins in *Arcobacter* species – one of the major bacterial virulence factors was characterised. The genes encoding flagellins in five *Arcobacter* species including the type species *A. nitrofigilis* were characterised. Mutants in either *fla* gene in an *A. butzleri* strain were constructed to study the role of each gene in bacterial motility. In addition, the transcription of the *fla* genes in the wild type and the mutant strains were investigated by qPCR.

Finally, in **Chapter 7** a summary and discussion of what has been achieved in this thesis and suggestions of what should be done for further understandings of *Arcobacter* spp. is presented.

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Chapter 1

***Arcobacter*, what is known and unknown about a potential foodborne zoonotic agent - A review**

Hoa T. K. Ho, Len J.A. Lipman and Wim Gaastra

Veterinary Microbiology (2006) **115**:1-13 (Review)

Abstract

Since the introduction of the genus *Arcobacter* in 1991, the association of *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii* with humans and animals has been clearly established. These bacteria have been detected world wide in products of animal origin and in healthy animals as well as in surface water. A fourth species *Arcobacter cibarius* was recently discovered on chicken carcasses. Although evidence was found for the connection of *Arcobacter* spp. with human and animal illness, *Arcobacter* spp. can be pathogens, opportunistic pathogens and commensals. Their potential as zoonotic foodborne and waterborne agents, the routes of transmission and the pathogenic mechanisms of these bacteria are largely unknown. Production of toxins or other virulence factors has not been demonstrated but adhesive and/or invasive properties were apparent. Antibiotic resistance is present in *Arcobacter* strains to significant levels. The tools to genetically access *Arcobacter*-like transformation of strains, construction of mutants are not yet available. Nor have genes (i.e. potential virulence factors) been cloned, expressed and characterized in other host organisms. Therefore those interested in the microbiology of these organisms eagerly await publication of the complete nucleotide sequence of the *Arcobacter* genome.

The abundant presence of four *Arcobacter* species in foods of animal origin and the recovery of these bacteria from surface and drinking water suggest an important role of these bacteria as foodborne or waterborne agent and possibly as zoonotic agent.

Introduction

In 1991, the genus *Arcobacter*, which together with *Campylobacter* was included in the family *Campylobacteraceae*, was proposed (65; 66). Presently the genus *Arcobacter* includes six species: *A. nitrofigilis*, a nitrogen-fixing bacterium associated with the roots of *Spartina alterniflora*- a marsh plant (42); *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* frequently associated with animals and occasionally with humans; and two recently described species, *A. cibarius*, isolated from chicken carcasses (29) and *A. halophilus*, a second environmental species isolated from a hyper saline lagoon (13). *Arcobacter* spp. are Gram-negative, non-spore-forming rods, generally 0.2–0.9 μm wide and 0.5–3 μm long. The bacteria are curved to S-shaped rods (Fig. 1). They are motile and have one unsheathed single polar flagellum at one or both ends of the cells. *Arcobacters* are able to grow under aerobic and anaerobic conditions over a wide temperature range (15–42 °C).

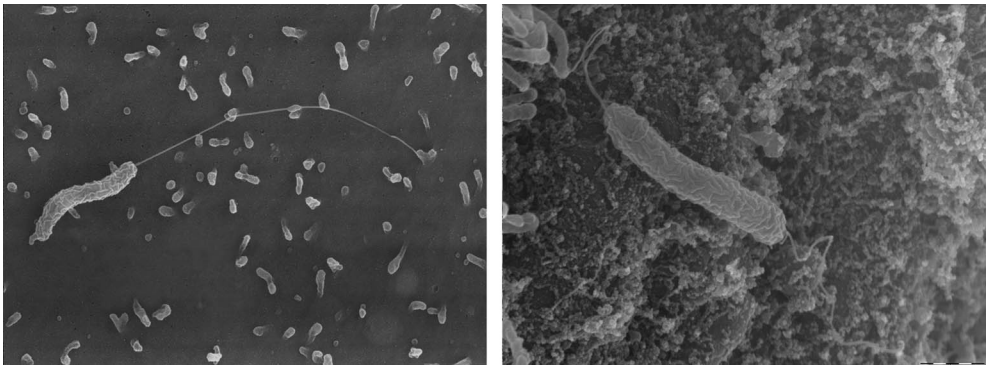


Fig. 1. Electron micrographs of *A. skirrowii* LMG 6621 on IPI-2I cells (A) and *A. cryaerophilus* LMG 7537 on Caco-2 (B).

Optimal growth occurs under microaerophilic conditions (3–10% O₂) and the bacteria do not require hydrogen (64; 66). Although their pathogenicity remains to be fully elucidated, *Arcobacter* spp. have been considered as potential zoonotic foodborne and waterborne agents (3; 28).

1. Occurrence in foods of animal origin

Arcobacter spp. are frequently isolated from products of animal origin (chickens, pork, beef and lamb). The highest prevalence is found in chickens, followed by pork and beef. In Japan 23% of chickens, 7% of pork and 2.2% of beef samples from retail shops were positive for *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* (38). An even higher prevalence was observed in Australia, where 73% of chickens, 29% of pork and 22% of beef samples were positive. *A. butzleri* was

recovered from 15% of the lamb samples (54). Among *Arcobacter* spp. isolated from meat samples, *A. butzleri* is found most, followed by *A. cryaerophilus* (9; 26; 38; 46; 51; 54). *A. skirrowii* is often not detected at all or only at low rates, which can either be explained by a low prevalence in meat or by the fact that it is more difficult to isolate than *A. butzleri* and *A. cryaerophilus* (see below). Like for *Campylobacter*, a high prevalence of *A. butzleri* and *A. cryaerophilus* is observed on chicken carcasses both from markets and abattoirs (5; 25; 38). The bacteria were detected on almost all slaughterhouse carcasses tested during a large-scale study in Belgium and up to several thousand bacteria were present per gram of neck skin prior to evisceration (25). Although a high prevalence of *Arcobacter* species on chicken meat is reported worldwide, the bacteria are rarely detected in the intestinal contents of these animals. It was therefore assumed that contamination of meat probably arises from other sources and occurs during processing (6). To detect the source of carcass contamination, Houf *et al.* (2002, 2003) sampled the surface of processing equipment (25; 26). *Arcobacter* species were recovered from 85% of swab samples taken before slaughter and after a period of a few days with no slaughter activity (25). Before the onset of slaughter, higher numbers of bacteria were detected on the last 2 days of a working week compared to the first day, which might indicate accumulation of contaminating bacteria on the processing line due to insufficient decontamination (26). The bacteria were also recovered from the water outlet of defeathering machines, the neck-skin cutter and the washer. Molecular approaches have been used to analyse the relationship between isolates from environmental sources and carcasses and to track the route of transmission, but the data was not conclusive probably due to the high genetic heterogeneity of *Arcobacter* species. Thus despite many attempts, the original source of the high contamination level of chicken carcasses and the way of spreading the contamination still remain unknown.

2. Occurrence in water

Apart from poultry and other meat, *Arcobacter* spp. (mostly *A. butzleri*) were isolated from a drinking water reservoir (33), river or surface water (12; 45; 46), ground water (53) and sewage (45; 57). There is no evidence available on the removal of arcobacters by water treatment processes, but they are probably removed to a similar extent as other bacteria. However, during an investigation in drinking water treatment plants in Germany 141 strains of *Arcobacter* spp. (of which 100 were *A. butzleri*) were isolated against only six strains of *Campylobacter jejuni* and *Campylobacter coli* (34). This may however also be a reflection of the different optimal growth temperature of these bacteria. Little is known about the effects of drinking-water treatment on *Arcobacter* spp., but *A. butzleri* was found to be sensitive to chlorine disinfection (53). In non-chlorinated drinking water the number of culturable *A. butzleri* remained at the initial level for the first 3 days and then declined to unculturable levels after 16 days, but the

number of viable bacteria was stabilised throughout the 35-day experiment (44). Thus consumption of contaminated water is a likely source of exposure to arcobacters. *A. butzleri* can easily attach to water-distribution pipe surfaces (stainless steel, copper and plastic), which makes regrowth in the water distribution system a possibility (3). This can become a significant problem in drinking water and food processing plants with respect to public health (3).

3. Association with animals

3.1. Presence in healthy animals

Arcobacter spp. are present in the digestive tract of healthy farm animals without causing clinical signs of illness (32). The presence of *Arcobacter* spp. in faecal samples from healthy cattle (37; 51; 63), pigs (32; 37; 62), chickens (37), sheep and horses (61) has been reported.

The prevalence of *Arcobacter* species in these animals varies from country to country. In general, *Arcobacter* spp. are found more frequently in swine than in cattle. Monitoring for 1 year in a slaughterhouse in Japan revealed that 3.6% of faecal samples from cattle and 10% of samples from pigs were positive for *A. butzleri*, *A. cryaerophilus* or *A. skirrowii* (37). In Belgium these percentages were 39 and 44% (61). In farm animals *Arcobacter* species are also found more frequently in pigs than in cattle provided the same isolation method is used (62; 63). An abundance of *Arcobacter* spp. is present in healthy sows. In Belgium, high numbers of gestating sows (59%) on one farm and of sows after farrowing (85%) on another farm were positive for *A. butzleri*, *A. cryaerophilus* or *A. skirrowii* (62). The prevalence in sows ranged from 7.2 to 36.4% in the US (32). On a pig farm in the Netherlands, 42% of the tested sows carried one or more *Arcobacter* species (23).

Although *Arcobacter* spp. are isolated with high frequencies from chicken meat (see above), the bacteria are not frequently detected in the intestinal tract of these birds. Culture of the contents of gizzard, small intestine, caecum and colon of chickens where arcobacters were detected on all carcasses, yielded only one isolate from a colonic sample (6). Similar data were obtained in other studies. *Arcobacter* spp. were isolated from swabs taken from the cloacae of only 1% of 8-week-old chickens (whereas 32% in the same group was positive for *Campylobacter*), 3% of 16-week-old birds in one flock and none from another flock of 28-week-old chickens (71). No *Arcobacter* was isolated from the contents of the large intestine of slaughtered chickens despite the high prevalence on chicken carcasses (higher even than that of *Campylobacter*) (25). After experimental oral infection of 3- and 5-day-old chickens and turkeys with various *A. butzleri* and *C. jejuni* strains, no *A. butzleri* was detected in either cloacal or tissue samples of the chickens (71). In the

turkeys only 2.9% of cloacal samples but 6% of tissue samples were positive for *A. butzleri*. *C. jejuni* was recovered from cloacal samples of all infected chickens and turkeys tested. Oral infection of 1-week-old chickens with high bacterial doses (10^6 and 10^9 CFUs per bird) led to excretion of *A. butzleri* by animals but not by those infected with low doses (10^3 CFUs) of the bacteria (14). Remarkably, 22% of the cloacal swabs from a group of chickens infected with 10^9 CFUs per bird were positive for *Arcobacter*, whereas in another group that received the same bacterial dose *Arcobacter* could not be detected in the contents of any part of the intestine tested (jejunum, ileum, caecum and colorectum). Statistical analysis revealed a significantly higher percentage of positive surface samples than of faecal swabs in all groups. Taken together, these data indicate the pass-through but not colonisation by the bacteria of the intestinal tract, followed by contamination of the environment (14). It was suggested that the intestinal tract of poultry, due to the high body temperature (41 °C), might not be a favourable habitat for non-thermophilic *Arcobacter* spp. Although *Arcobacter* species are aerotolerant and can survive and spread in the environment for a long period, it is paradoxical that they are not recovered from gut contents but can be isolated from swabs taken from the cloaca and almost all carcasses tested. The presence of *Arcobacter* spp. in live chickens, the mechanism by which these organisms spread and contaminate the environment are topics that clearly need further study because of the epidemiological and food safety aspects involved.

In general, *A. butzleri* is the species most frequently found in healthy livestock followed by *A. cryaerophilus* (37; 51; 61; 73). However, *A. cryaerophilus* was the dominant species identified in cattle followed by *A. skirrowii* in a study by Van Driessche *et al.* (2005) and *A. skirrowii* was most frequently detected in sow faeces on a farm in the Netherlands (23). While judging these prevalence data it should be kept in mind that the isolation technique used can influence which *Arcobacter* species is isolated (63). The difficulty in isolation of *A. cryaerophilus* and particularly *A. skirrowii* compared to *A. butzleri* will be discussed. In addition, shifts in the type of species excreted over time or intermittent excretion of *Arcobacter* species have been observed (23; 32).

Co-colonization of a single host with more than one species or more than one genetically distinct strain of *Arcobacter* is quite common. More than one *Arcobacter* species was detected in 26% of positive cattle faecal samples (63). An individual animal (pig or cow) can simultaneously harbour three species of *Arcobacter* (61-63).

Substantial genetic variation among isolates of the same *Arcobacter* species has been observed. Genotyping revealed 24 different genotypes among 46 colonies of *A. butzleri*, 25 genotypes among 33 isolates of *A. cryaerophilus* whereas 18 isolates of *A. skirrowii* had distinguishable genotypes (62). Individual pigs were found to excrete up to seven *A. butzleri* genotypes, ten *A. cryaerophilus* genotypes and six

of *A. skirrowii*. Likewise, in cattle, an individual cow was found to excrete six *A. cryaerophilus* genotypes and two *A. skirrowii* genotypes (63). Ho *et al.* (2006) detected in utero transmission of two genetically different strains of *A. cryaerophilus* from a single sow to her newborn piglets in the same litter. Like for *Campylobacter* genotypic diversity of *Arcobacter* among farm animals is explained by genomic rearrangements of parent genotypes during recurring exposure (32; 62).

Arcobacter spp. have been found frequently in the environment on farms. Various strains of *Arcobacter* were discovered in samples taken from farmers' boots and from the water supply on cattle and pig farms (62; 63). Experimental infection of chickens with *A. butzleri* led to the presence of this *Arcobacter* in more than 90% of the litter floors samples and in all feather samples (14). *A. butzleri* could still be detected on the surface of the housing several weeks after the birds were removed suggesting a high capacity for survival in the environment. Therefore, contaminated farming facilities and insufficient hygiene probably add to the transmission of the bacteria through farms and among stocks.

3.2. Factors that influence the prevalence of *Arcobacter* species in animals

A number of factors affect the determination of the prevalence of *Arcobacter* spp. or specific *Arcobacter* species in animals and animal products. The experimental design, sample size and identification/isolation method used influence the recovery rate in field studies. Direct isolation notably leads to lower recovery rates than isolation after enrichment (61-63). *Arcobacter* spp. were isolated from 10% of cattle samples by direct isolation but from 39% after enrichment. Similar results were obtained for pigs (28% versus 44%); sheep (5% versus 16%) and horses (0% versus 15%) (61). Direct isolation or isolation after enrichment using the same sample led to the recovery of different species or different strains of a particular species (62; 63). A co-infection detected by direct isolation sometimes was not observed after enrichment, probably because one strain outgrows the other. For example, *A. cryaerophilus* and *A. skirrowii* were detected in swine and cattle faeces by direct isolation but only the former was isolated after enrichment (62; 63). Furthermore, routine procedures for bacterial isolation (picking a few colonies from enrichment/selective plates for further identification) may result in missing co-existing strains or species.

Differences in the ease by which each *Arcobacter* species can be isolated should also be taken into account. For example, *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* could be detected in seawater and plankton samples by PCR (77.8, 55.5 and 8.3%, respectively) but only *A. butzleri* could be isolated albeit with a lower rate (41.7%) (18). No optimal isolation method for all *Arcobacter* species is available (27). The difficulty in isolation of *A. cryaerophilus* and *A. skirrowii* is probably due to their susceptibility to antimicrobials and other components used in

the isolation media (7; 27; 37; 38). Furthermore, colonies of *A. skirrowii* isolated from animal faeces are only visible after 72 hours (61) or after 4 days of incubation in a human case study (76). Tiny colonies of *A. skirrowii* from swine faeces were only retrieved after filtering the enrichment culture through 0.45 µm syringe filters and incubation for 4 days (23). Poor growth of *A. skirrowii* may allow overgrowth by enteric bacteria in samples that are incubated for 1 or 2 days in routine procedure. The use of membrane filters was suggested as a possibility to remove other enteric bacteria but leads to additional costs in diagnostics (6; 51).

To overcome these difficulties and to allow detection of the simultaneous presence of *Arcobacter* species, multiplex PCR (m-PCR) methods have been used for screening enrichment samples prior to isolation (54; 74). Different m-PCR reactions have been developed (21; 30; 36). However, with the introduction of *A. cibarius* (29) these m-PCR reactions developed to detect the rDNA of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* seem to have a limited application. A bias in the m-PCR should be considered since only detection of the species present in the highest number is obtained. Simultaneous detection of two or three *Arcobacter* species was only accomplished when more or less equal amounts of template were present (23).

The age of the sampled animals may influence the prevalence. Wesley and Baetz (1999) observed a low prevalence of *Arcobacter* spp. in 8- and 16-week-old chickens (1 and 3%, respectively), while the highest prevalence was observed in 56-week-old breeders (57%). In swine, the prevalence in sows was higher than in fattening pigs (62). The incidence of *Arcobacter* spp. in pigs was found to increase with age due to prolonged exposure to a contaminated environment (32). However, the influence of age on *Arcobacter* prevalence in cattle is disagreed among research groups. Higher incidence of *A. butzleri* was observed in feedlot and dairy cattle than in calves (20). The exposure to different environmental conditions and different diet (high-energy feed to feedlot and dairy cattle versus grass- and milk-based diet to calves) between adult animals and young calves was put forward as explanation. This is in contrast to the observation that the highest prevalence of *Arcobacter* spp. is found in calves, followed by young cattle and the lowest in dairy cows (63). These authors argued that the samples in the latter study were taken from animals housed in the same farm while those in the former study were collected from various farms.

Variation in prevalence on different farms is attributed to differences in farming practices: herd size (73), hygiene, water supply and feeding (20; 63; 73). According to Wesley *et al.* (2000) individual water suppliers seem to reduce the chance of *Arcobacter* uptake, probably due to the diminished spread through contaminated water. Alley flushing to remove manure may increase *Arcobacter* prevalence due to the ability of *Arcobacter* spp. to grow at low temperature and under aerobic conditions, which may help them to survive and contaminate

recycled water resource better than *Campylobacter* (73). As for other enteric bacteria the type of diet and supplement used may influence colonization of the intestinal tract by *Arcobacter* spp. due to alteration of gut homeostasis. A protective effect against *Arcobacter* infection was observed in dairy cattle fed with alfalfa, but not in those consuming cottonseed or hulls. Concentrated high-energy feed, as compared to a grass- and milk-based diet possibly favours colonization of cattle by *A. butzleri* (20). The difference in prevalence of *Arcobacter* spp. in various groups of cattle was explained by differences in rearing practice used for feedlot and dairy cattle (63).

A seasonal influence on *Arcobacter* prevalence in animals (chicken, pigs and cattle) has been suggested. A higher prevalence was reported in spring and summer than in winter (62). However, no statistical differences in summer and winter prevalence were observed in Japan (37). Similarly, *Arcobacter* spp. in sows in the Netherlands were detected at the same rate in June and December (23).

The role of each *Arcobacter* species in association with animals and humans is still unclear. Whether some *Arcobacter* species are opportunistic colonisers or even commensals for a particular host (host specific or age dependent) and others are specific pathogens (causing diarrhoea or reproductive disorders) will influence the outcome of tested samples.

3.3. Potential pathogens in animals

In the late 1970s aerotolerant *Campylobacter*-like microorganisms were isolated from aborted bovine and porcine fetuses (15; 16; 48). These organisms were later classified in the genus *Arcobacter* belonging to the family *Campylobacteraceae* (65; 66). Nowadays, strains of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have been isolated from aborted fetuses and placentas of bovine, porcine, and ovine origin (68). *A. cryaerophilus* were found most frequently in Brazil (10) and the US (56) while in Denmark *A. cryaerophilus* and *A. skirrowii* were isolated from 50% of the aborted porcine fetuses at relatively equal rates (50). No other organisms frequently associated with porcine reproductive disorders (including PPV-Porcine Parvovirus and PRRSV-Porcine Reproductive and Respiratory Syndrome Virus) were detected. However, no remarkable pathological signs were identified. According to Schroeder-Tucker (1996), the clinical signs associated with *Arcobacter* infection include infertility, chronic discharge during oestrus, chronic stillborn problems and late-term abortions. Venereal transmission of the bacteria is likely since *A. butzleri* and *A. cryaerophilus* were recovered from preputial fluid of bulls (11) and *A. skirrowii* from preputial fluid of boars and fattening pigs (68).

As mentioned, *Arcobacter* spp. were detected in clinically healthy sows and live newborn piglets (16; 23). Furthermore, different *Arcobacter* species were detected in a single aborted fetus and in different fetuses from the same litter (50). Apparently some *Arcobacter* strains play a primary role in abortions and

reproductive disorders while others merely are opportunistic pathogens (50). Whether or not this reflects differences in virulence is unknown. The roles of *Arcobacter* spp. in chronic reproductive disorders in farm animals and the economic effects thereof have not been investigated.

An aerotolerant *Campylobacter* (later identified as *A. cryaerophilus*) was also isolated from milk during a mastitis outbreak (40). Experimental infection of udders of healthy cows with this strain produced an acute clinical mastitis with fever and a remarkable reduction in milk yield (40).

Apart from reproductive disorders in farm animals, *Arcobacter* spp. are found to be associated with enteritis and diarrhoea in animals: i.e. *A. butzleri* from faeces of diarrhoeic pigs, cattle, horses, ostriches and tortoises; *A. skirrowii* from sheep and cattle with diarrhoea and hemorrhagic colitis (64; 68). Several reports on the association of *A. butzleri* with diarrhoea in non-human primates are available (2; 22). The bacteria were isolated from 14 diarrhoeic rhesus macaques of which seven were co-infected with *C. coli* and *C. jejuni*. No other potential enteric pathogens were isolated (2). Histological examination of colonic specimens showed acute and chronic inflammation, identical to *Campylobacter* colitis. Ribotyping of the strains suggested that *A. butzleri* might be endemic in this primate population (2).

4. Potential pathogens in humans

After *C. jejuni*, *C. coli* and *Campylobacter upsaliensis*, *A. butzleri* is the fourth most common *Campylobacter*-like organism isolated from the stool of human patients in Belgium and France (52; 62). Twenty percent of the samples positive for *A. butzleri* were also infected with another entero-pathogen. *A. butzleri* was found more regularly in diarrhoeic stools than in non diarrhoeic stools. *A. cryaerophilus* but not *A. skirrowii* was also detected. The association of *Arcobacter* spp. with human gastroenteric illness was also reported in other countries. *A. butzleri* was the only potential enteric pathogen isolated during an outbreak in an Italian nursery and primary school where ten infected children suffered from recurrent abdominal cramps with no diarrhoea (67) and the bacterium was isolated from diarrhoeic children in Thailand (58). *A. cryaerophilus* was detected in an Australian adult with intermittent diarrhoea and abdominal pain (59). In 2004, the first case of *A. skirrowii* isolated from an elderly person with chronic diarrhoea was reported (76). The role of *A. skirrowii* in human disease is not clear, but is believed to be limited.

Human bacteraemia caused by *A. butzleri* and *A. cryaerophilus* has been reported in a few cases. In three cases, the patients were elderly people of whom two suffered from chronic illness (31; 39; 77). Transmission of bacteria occurred via an infected arteriovenous fistula in one case (31), by the oral route in another (39) and

was unknown in the third (77). Vertical/transplacental transmission was assumed in a case of newborn bacteraemia caused by *A. butzleri* although the mother showed no signs of an infectious disease before delivery, except recurrent haemorrhages due to placenta praevia (49). A more severe case is that of a boy who fell into a mud pool, developed acute respiratory distress, disseminated intravascular coagulation, acute renal failure and died. *A. cryaerophilus* was isolated from blood and the source of infection was supposed to be coming from the mud via the respiratory system (75).

Except for the latest case of bacteraemia (75), *Arcobacter* infection described in human case reports, has been mainly detected in patients with chronic illnesses, both elderly and children. In the study by Vandenberg *et al.* (2004) (69) the age of the patients suffering from *A. butzleri* diarrhoea ranged from 30 days to 90 years. From the infected patients tested, 16% suffered from immunocompromising or other chronic diseases. Diarrhoea associated with *A. butzleri* seems more persistent, watery and asymptomatic but less acute than diarrhoea caused by *C. jejuni*.

Apart from the specific transmission in the case of bacteraemia and the potential person-to-person transmission during the outbreak at the Italian school, like for other enteric bacteria, *Arcobacter* infection of humans probably occurs by the oral route via contaminated food or water. Four *Arcobacter* species (*A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. cibarius*) have been isolated in high numbers from meat, especially from chicken carcasses. The bacteria are detected regularly in oysters (55). The presence of *Arcobacter* spp. in river and canal water, in well water that served for drinking purposes, in drinking water reservoir and drinking water treatment plants has been demonstrated. All these data indicate that the infection pressure for humans is probably high.

5. Pathogenicity

More than a decade after the genus was established and despite many reports on the presence of *Arcobacter* spp. in food and its association with human and animal illness, there is still a limited knowledge on the pathogenicity of these bacteria. A few studies on *in vitro* adhesion, invasion and cytotoxicity of *A. butzleri* and *A. cryaerophilus* but not *A. skirrowii* are available (Table 1). In a rat ileal loop model, distensions with fluid accumulation and an increase in electrolyte concentration caused by two *A. cryaerophilus* isolates from an aborted bovine foetus and from swine faeces were reported (19). However, assays on CHO, Hela and INT407 cell lines and PCR using degenerated primers based on genes coding for cytolethal distending toxin (CTD) of *E. coli* and *C. jejuni* failed to detect CDT activity and CDT genes in isolates of *A. butzleri* and *A. cryaerophilus* from environmental, animal and human samples (35; 47). The *in vitro* cytotoxicity of *Arcobacter* species reported were changes in cell morphology (elongation, vacuolisation,

rounding up, nuclear pyknosis and detachment from the well bottom) (8; 35; 47; 70). No further characterisation of this toxicity has been reported except that of a speculation of a <10 kDa toxic molecule (35). Whether or not *Arcobacter* spp. produce toxins and what the nature of these toxins is, remains to be elucidated. Haemagglutination activity with human and animal erythrocytes of two *Arcobacter* strains was detected and the haemagglutinin was characterised as a lectin-like protein of approximately 20 kDa that reacts with a D-galactose-containing receptor on the cell surface of erythrocytes (60).

Table 1. *In vitro* studies on pathogenicity of *Arcobacter* spp.

	Sample origin	Adhesion	Invasion	Cytotoxicity ^a	References
<i>A. butzleri</i>					
Hep-2	Sea water	6/17	–	–	(8)
	Human stool	12/12	4/12	3/12	(69)
Hela	Sea water	6/17	–	–	(8)
	River water	1/8	0/8	–	(47)
	River water	–	–	3/3	(35)
INT407	Animal and human	–	–	3/3	(35)
	River water	1/8	0/8	–	(47)
	River water	–	–	3/3	(35)
Vero	Animal and human	–	–	3/3	(35)
	River water	–	–	17/18	(47)
	Sea water	–	–	5/17	(8)
CHO	Meats	–	–	76/80	(70)
	River water	–	–	17/18	(47)
<i>A. cryaerophilus</i>					
Hep-2	Swine feces	–	1/1	–	(19)
	Aborted bovine fetus	–	1/1	–	(19)
Hela	River water	–	–	3/3	(35)
	Animal and human	–	–	3/3	(35)
INT407	River water	–	–	3/3	(35)
	Animal and human	–	–	3/3	(35)
Vero	Meats	–	–	2/2	(70)
<i>A. skirrowii</i>					
Vero	Meats	–	–	17/19	(70)

(–) not tested

^a Number of positive samples/number of tested samples

A possible reason for the limited knowledge of the molecular mechanisms of *Arcobacter* pathogenesis is the genomic difference with *Campylobacter* spp. Attempts to study *Arcobacter* pathogenicity were mainly based on knowledge obtained from *Campylobacter* species and other gastrointestinal pathogens like

Helicobacter. However, since the genome of *A. butzleri* RM4018 (of which a draft genome has been produced) is most similar to *Wollinella succinogenes* (43), attempts to study *Arcobacter* pathogenicity should be redirected.

There have been two animal studies on the pathogenicity of *Arcobacter* spp. In the first, experimental oral infection of caesarean-derived 1-day-old piglets was performed (72). All strains colonised and multiplied in the gut but only *A. butzleri* strains (from human faeces and swine) were able to invade into internal organs of infected animals. This was in contrast to an *A. cryaerophilus* strain (from an aborted bovine foetus) and an *A. skirrowii* strain (from a diarrheic lamb). While invasion was observed in all four piglets infected with *A. butzleri* strains in the first trial, bacteria were only detected in two out of four challenged animals in the second trial. In addition, the mortality due to the *A. butzleri* and *A. skirrowii* strains in the first trial was not observed when the experiment was repeated. The variable results were explained by different susceptibility of the piglets and their age (even though they were all 1-day-old and caesarean-derived from sows on day 115th of gestation, it was stated that those in the second experiment seemed younger than those in the previous experiment). In the second study, 3- and 5-day-old chickens and turkeys were infected orally with the human *A. butzleri* strain used in the above pig study and with chicken and turkey field isolates (71). The human strain could not colonise and invade the White Leghorn chickens and commercially outbred turkeys, but was recovered from cloacal swabs and tissues of highly inbred Beltsville White turkeys. The results showed that the invasive capacity and virulence of the *A. butzleri* strains were host-dependent with respect to species and breed. Thus it may be difficult to draw conclusions on the virulence of *Arcobacter* species in this type of experiment when the strains originate from a different host species.

Many *Arcobacter* strains isolated from humans, chicken carcasses, meat or the environment were found to be resistant to antimicrobials commonly used in human and veterinary medicine. A remarkable multi-drug resistance was observed in all studies. High resistance was found against trimethoprim, trimethoprim-sulphamethoxazole and members of the broad-spectrum beta-lactams including cephalosporins (4; 17; 38). Even to antimicrobial agents used to treat multi-drug resistant Gram-negative bacteria like aztreonam resistance was found (4). Also to erythromycin and ciprofloxacin, commonly used for the treatment of infections by campylobacters and related bacteria, increased resistance was observed (28). Recently, the *gyrA* gene was identified in *Arcobacter* species (1). DNA gyrase which is a bacterial enzyme consisting of two subunits encoded by *gyrA* and *gyrB* catalyses the negative supercoiling of DNA and is involved in DNA replication and RNA transcription. The antibacterial activity of some antibiotics including coumarins (e.g. novobiocin) and quinolones are known to be directed against DNA gyrase (41). Mutations of the genes coding for DNA gyrase may confer resistance to these antibiotics (24). A QRDR (quinolone-resistance determining region)

mutation, associated with quinolone resistance, was found in two clinical isolates of *A. butzleri* and one *A. cryaerophilus*, resistant to ciprofloxacin (1). In the study by Houf *et al.* (2001) *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* were found to be highly resistant to novobiocin (27).

Conclusion

Evidence clearly indicates an association of *Arcobacter* spp. with human and animal disease. Although there is still no direct evidence, the abundant presence of four *Arcobacter* species in foods of animal origin and the recovery of these bacteria from surface and drinking water suggests an important role of contaminated food and water in the transmission of these bacteria. Very little is known about how arcobacters cause disease, their virulence factors or their pathogenicity. The difficulties encountered in the establishment of the role of each *Arcobacter* species in causing human and animal illness, the sources and routes of infection are probably at least partly due to the fastidiousness to culture them as well as their high genetic diversity.

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Chapter 2

Potential routes of acquisition of *Arcobacter* species by piglets

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Abstract

The aim of this study was to determine the prevalence and the transmission routes of *Arcobacter* spp. in sows to their offspring on a breeding farm. Twelve *Arcobacter*-positive sows and their litters were studied for this purpose. Analysis of rectal samples showed a high prevalence of *Arcobacter* spp. among the sows (approximately 42% of the sows carried one or more *Arcobacter* species). Intermittent excretion of one particular species and shifts in excretion from one species to another were observed in individual animals over time. The detection of *Arcobacter* spp. in amniotic fluid of the sows and in rectal samples from newborn piglets (ranging from 38.5–83.3% per litter), as well as the high similarity between PFGE profiles of *Arcobacter* isolates from sows and their respective newborns indicated the existence of an intra-uterine transmission route for *Arcobacter* spp. Specific antibodies against *Arcobacter* spp. were detected in colostrum by Western blot. At 2 weeks of age, only a few piglets were positive for *Arcobacter*. The reappearance of *Arcobacter* in these piglets at Week 3 and the shift in the *Arcobacter* species detected (from a prominent presence of *A. cryaerophilus* at birth to the presence of *A. skirrowii* and *A. butzleri* at 3 weeks after birth) showed that a post-natal infection route from their mothers, newcomers or the environment to the piglets existed. Thus, in this manuscript the transmission of *Arcobacter* spp. (both vertical and horizontal) from carrying sows to their offspring is demonstrated.

Introduction

The genus *Arcobacter*, previously named “aerotolerant campylobacters”, was introduced in 1991 (25). Presently this genus is composed of six species. Two of them are environmental bacteria: *A. nitrophilus*, a nitrogen-fixing microorganism (15) and *A. halophilus*, a halophile (3). *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* are frequently found in faecal samples of animals (chicken, pigs, cattle, sheep and horses) (22). They have been isolated from meat (10; 20), oysters (21), groundwater (19), seawater and plankton (5). *A. cibarius*, a recently detected species, was isolated from chicken carcasses (6). In humans, *Arcobacter* spp. have been reported to be associated with diarrhoea and bacteraemia (12).

Among domestic animals, swine are an important host/reservoir of *Arcobacter* spp. Pork has been reported as a source of *Arcobacter* contamination (17; 26). The bacteria have been isolated from faeces of healthy pigs and sows at high prevalence (up to 85%) (8; 23). Association of *Arcobacter* spp. with porcine infertility and abortion has been reported worldwide (2). In Denmark, a high prevalence of *Arcobacter* spp. associated with pig abortions was found while no other organisms associated with porcine reproductive disorders were detected (18). It is thought that some *Arcobacter* strains play a primary role in abortion while others may be opportunistic pathogens colonising the foetus (18). In spite of the fact that *Arcobacter* spp. are present in live newborn piglets and their placenta (4), and their ability to colonise and invade 1-day-old piglets (29), infection of nursing piglets by *Arcobacter*-positive mothers has not yet been demonstrated (8).

Since pigs can be either clinical hosts of *Arcobacter* spp. and/or reservoirs shedding the bacteria, more understanding of the epidemiology among the stock is needed. The aim of this study is to observe the transmission of *Arcobacter* species from carrying sows to their piglets, both congenital and post-natal.

Materials and Methods

Sampling

The study was carried out on a breeding farm in The Netherlands that has 180 sows. Rectal swabs were collected from pregnant and non-pregnant sows in June (61 sows) and December 2004 (83 sows). Among the sows tested, nine were sampled twice.

One week before farrowing, sows were moved to individual pens. To study the transmission from mothers to piglets, rectal and vaginal swabs were taken from sows that were ready to deliver at the time of observation. The first 12 animals that were shown to be *Arcobacter*-positive by PCR (see below) were included in the

study. At parturition, swabs of the amniotic fluid were taken from inside the vagina of the sows at the moment the uterus contracted. In order to test the presence of antibodies against *Arcobacter* spp. (by Western blot, see below), colostrum of these sows was sampled by hand-milking the first four teats within 2 hours of giving birth.

Immediately after they were born, all piglets were rectally swabbed. The area around the anus was widely and carefully wiped with ethanol before sampling to prevent contamination from the amniotic fluid and environment. Piglets were ear-tagged for further observation. Rectal samples of piglets were taken again at 2 and 3 weeks of age.

Detection of Arcobacter spp. by species-specific multiplex PCR (m-PCR)

Swabs were inoculated in 10 ml *Arcobacter* broth (Oxoid) containing Cefoperazone, Amphotericin, Teicoplanin supplement (CAT, Oxoid) under microaerophilic conditions (5% O₂, 10% CO₂ and 85% N₂) at 30°C. After 48 hours of incubation, 0.5 ml culture was centrifuged at 6000 × g for 5 minutes. Pellets were resuspended in the same volume with water, boiled for 10 minutes at 95°C, and left on ice for 10 minutes before centrifugation for 10 minutes at 16000 × g to obtain DNA for PCR. The remainder of the broth culture was kept at 4°C under microaerophilic conditions for reisolation 1 day later, if needed.

Arcobacter species were detected using a multiplex PCR assay with primers based on the rDNA genes of *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* (7). Samples that reacted negative were tested for the presence of PCR inhibitors by a PCR using the eubacterial primers 27F and 519R (also based on rDNA genes) (11). All primer sequences used in the two PCR assays are shown in Table 1.

Table 1. Primers used in this study

Primers	Sequence (5' to 3')
m-PCR	
ARCO (1357–1338)	CGTATTCACCGTAGCATAGC
BUTZ (959–983)	CCTGGACTTGACATAGTAAGAATGA
SKIR (705–723)	GGCGATTACTGGAACACA
CRY1 (105–124)	TGCTGGAGCGGATAGAAGTA
CRY2 (359–340)	AACAACCTACGTCCTTCGAC
PCR for eubacteria	
27F	AGAGTTTGATCMTGGCTCAG
519R	GWATTACCGCGGCKGCTG

K = G, T; M = A, C; W = A, T.

Sensitivity of detection of multiple-species by m-PCR

In order to examine the sensitivity of the multiplex PCR for the detection of different *Arcobacter* species that are simultaneously present in one sample, three control strains, namely *A. butzleri* LMG 6620, *A. skirrowii* LMG 6621 and *A. cryaerophilus* LMG 7537 obtained from the Bacteria Collection of the Laboratory of Microbiology, Gent University, Belgium were used. Bacteria were inoculated in Brain Heart Infusion broth (BHI, Oxoid) and grown overnight at 30°C under microaerophilic conditions. In an attempt to have the same DNA concentration in all samples, each culture was adjusted to the same optical density of 0.25 at 600 nm, and 1 ml culture of each strain was used to extract DNA by the boiled lysis method as described above. DNA samples were serially diluted 10-fold. Mixtures of templates containing different DNA proportions of these strains were prepared as described in Table 2. Three microlitres of each template mixture was used in 25 µl PCR reaction. All combined DNA templates were run with the whole set of five m-PCR primers as mentioned above. Mixtures were also amplified with each species-specific primer set (ARCO and BUTZ for *A. butzleri*, ARCO and SKIR for *A. skirrowii*, and CRY1 and CRY2 for *A. cryaerophilus*). Ten microlitres of each reaction was loaded onto 0.8% agarose gel containing 0.6 µg/ml ethidium bromide for analysis by electrophoresis.

Table 2. Combination scheme of DNA of three species for testing the sensitivity of detection of simultaneous species by m-PCR (proportion of each strain in each mixture)

Mixtures ^a	butz ^b	skir ^b	cry ^b	Mixtures	butz	skir	cry
1	1	1	1	17	10 ⁻¹	1	1
2	10 ⁻¹	10 ⁻¹	1	18	10 ⁻²	1	1
3	10 ⁻²	10 ⁻²	1	19	10 ⁻³	1	1
4	10 ⁻³	10 ⁻³	1	20	10 ⁻⁴	1	1
5	10 ⁻⁴	10 ⁻⁴	1	21	10 ⁻⁵	1	1
6	10 ⁻⁵	10 ⁻⁵	1	22	1	1	10 ⁻¹
7	10 ⁻¹	1	10 ⁻¹	23	1	1	10 ⁻²
8	10 ⁻²	1	10 ⁻²	24	1	1	10 ⁻³
9	10 ⁻³	1	10 ⁻³	25	1	1	10 ⁻⁴
10	10 ⁻⁴	1	10 ⁻⁴	26	1	1	10 ⁻⁵
11	10 ⁻⁵	1	10 ⁻⁵	27	1	10 ⁻¹	1
12	1	10 ⁻¹	10 ⁻¹	28	1	10 ⁻²	1
13	1	10 ⁻²	10 ⁻²	29	1	10 ⁻³	1
14	1	10 ⁻³	10 ⁻³	30	1	10 ⁻⁴	1
15	1	10 ⁻⁴	10 ⁻⁴	31	1	10 ⁻⁵	1
16	1	10 ⁻⁵	10 ⁻⁵				

^a Thirty-one DNA mixtures were created for the test

^b butz: *A. butzleri*; skir: *A. skirrowii*; cry: *A. cryaerophilus*

Isolation of Arcobacter spp.

From each PCR-positive sample, 0.1 ml broth culture was dropped onto an *Arcobacter* agar plate (*Arcobacter* broth containing 1.5% agar) or BHI blood agar plate (plus 6% horse blood) on which a sterile cellulose nitrate filter was laid (1). Initially, filters of 0.8 μm pore size were used for isolation of *Arcobacter* spp. from the sow samples. Later, to improve the removal of other enteric bacteria in samples filters of 0.65 μm were used for isolation of *Arcobacter* species from the amniotic fluid and piglet samples. Plates were left for 1 hour at 30°C, the filters were removed and the agar plates were incubated at 30°C for 48 hours under microaerophilic conditions. Plates without growth were incubated for two more days. Three colonies from each plate were transferred onto blood agar. Isolates were examined microscopically for motility and confirmed to be *Arcobacter* species by m-PCR.

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed in order to examine the genetic relatedness of isolates of *A. cryaerophilus* obtained from infected sows and their respective newborn piglets. Digestion of the genomic DNA with *EagI* (New England Biolabs), as recommended for genotypic strain differentiation of *Arcobacter* spp. (8), was used to obtain the DNA fragments for PFGE. From each litter, four *Arcobacter* isolates were analysed, including: one isolate from the rectal swab (before parturition) and one from the amniotic fluid sample of the same sow, and two isolates each from one of its respective newborn piglets.

Preparation of the agarose plugs was based on the procedure by Hume *et al.* (2001). Bacterial cells were collected from blood agar plates in phosphate-buffered saline pH 7.2 and adjusted to obtain an absorbance of 1.7 at OD_{600 nm}. Equal volumes of prewarmed bacterial suspensions and 1% RESolve low melting agarose (Biozym, Landgraaf, The Netherlands) were mixed and loaded into CHEF disposable plug molds (Bio-Rad Laboratories), and solidified for 10 minutes at 4°C. The plugs were incubated for 72 hours at 50°C in 1.5 ml of lysis buffer (1% *N*-lauroylsarcosine; 0.5 M EDTA, pH 9–9.3; and 0.2 mg/ml proteinase K) and the buffer was replaced twice a day. The treated plugs were washed twice for 30 minutes each in cold (4°C) TE buffer (10 mM Tris, pH 8.0 and 1 mM EDTA), then three times each for 1 hour in cold TE containing 1.5 mM phenylmethylsulphonylfluoride (PMSF), and finally three times each for 1 hour in cold TE buffer. Subsequently, the plugs were preincubated in the required restriction buffer for 30 minutes at room temperature and digested for 16 hours at 37°C in 200 μl of fresh restriction buffer containing 20 Units of the restriction endonuclease *EagI*. The plugs with digested DNA and marker Lambda Ladder (New England Biolabs) were included in a 1% SeaKem agarose gel (Biozym, Landgraaf, The Netherlands) and DNA fragments were separated by a contour-

clamped homogeneous electric field (CHEF) DR-III apparatus (Bio-Rad, Hercules, USA) in 0.5 x TBE buffer (89 mM Tris, 89 mM Boric acid and 2 mM EDTA, pH 8.3). Run conditions were: initial switch time, 0.1 s; final switch time, 90 s; included angle, 120°; 6 V/cm; buffer temperature, 14°C; and running time, 20 hours. The gels were stained in 1 x TBE buffer containing 1 mg/ml ethidium bromide for 20 minutes and the bands were visualised using an UV transilluminator (Syngene, Westburg).

Western blot assay for detection of colostrum antibodies against Arcobacter spp.

Ten of the 12 colostrum samples were tested for the presence of antibodies against *Arcobacter* spp. using a Western blot assay. An isolate of *A. cryaerophilus* from the amniotic fluid of one sow and the three reference *Arcobacter* strains (mentioned above) were included in the experiment. Cells were pelleted, washed with saline and resuspended in water, and whole cell lysates were prepared by heating at 95°C for 10 minutes in Laemmli buffer. The lysates were loaded and proteins were separated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). Whole cell protein was blotted onto nitrocellulose membranes (Schleicher and Schuell, BioScience) (using Tank Transfer Systems, Bio-Rad; blotting overnight at 20 V, 4°C). Blotted membranes were soaked in blocking buffer (3% gelatine in Tris-buffered saline, TBS pH 7.5, 0.05 M Tris-HCl and 0.15 M NaCl) for 1 hour at room temperature. The membranes were incubated with diluted colostrum in blocking buffer (1:100, 1:200, 1:500, 1:1000, and 1:5000) for 2 hours at room temperature. The blots were then washed three times (each including three brief washes with water, followed by a 5 minutes wash with TBS - 0.05% Tween 20) before incubation with anti-pig IgG (whole molecule), peroxidase antibody (Sigma) for 1 hour at room temperature. Subsequently, the washing step was repeated three times as described above. Colour was developed by incubation of the blots for 20 minutes in 4-chloro-1-naphthol solution (0.06 g 4-chloro-1-naphthol (Sigma), in 100 ml TBS and 20 ml methanol; 60 µl H₂O₂ 30% was added just before use). The reaction was stopped by washing the membranes with water. All incubation and washing steps were performed with gentle agitation.

Results

Presence of Arcobacter spp. in sows detected by m-PCR

Samples taken in June and December yielded similar prevalence rates (Table 3). However, the frequency with which each of the *Arcobacter* species was detected changed over time. In June, *A. skirrowii* was detected in 23 out of 26 PCR-positive samples. Six months later this species still was present in most positive samples, but more *A. cryaerophilus* and *A. butzleri* positive samples were observed. This

was especially striking for the former that was found in one sample (1/26) in June but in 13 (13/35) in December.

Table 3. Prevalence of *Arcobacter* spp. in sows on the farm by m-PCR of enrichment both

	Period of sampling	
	June 2004 (n = 61) ^a	December 2004 (n = 83) ^a
Numbers positive sows	26 (42.6%)	35 (42.2%)
<i>A. butzleri</i>	6	10
<i>A. cryaerophilus</i>	1	13
<i>A. skirrowii</i>	23	15
Multiple-strain detection		
<i>A. butzleri</i> and <i>A. skirrowii</i>	3	–
<i>A. cryaerophilus</i> and <i>A. skirrowii</i>	1	3

^a Rectal swabs from nine sows were taken at both samplings.

There were also shifts in the excretion of the various species by individual sows Table 4. *A. butzleri* was detected from sows 52 and 112 in June, but *A. skirrowii* was demonstrated in the former and *A. cryaerophilus* in the latter in December. A similar phenomenon was observed with sows 141 and 151 with a shift from *A. skirrowii* in June to *A. cryaerophilus* in December.

Table 4. Shift in species ^a excretion of nine sows over the two sampling times (based on m-PCR)

Sows	June	December	Sows	June	December
52	butz	skir	151	skir	cry
56	ND ^b	butz	167	ND	ND
105	skir	skir	1013	skir	skir
112	butz	cry	1033	ND	ND
141	skir	cry			

^a butz: *A. butzleri*; skir: *A. skirrowii*; cry: *A. cryaerophilus*.

^b ND: not detected.

Isolation of Arcobacter species

Initially *Arcobacter* agar supplemented with CAT and filters of 0.8 µm pore size were used for the isolation of bacteria from PCR-positive enrichments. Bacteria were isolated from eight samples in June and 16 samples in December. The co-excretion of two species that was observed by m-PCR could not be detected by

isolation. Overgrowth by enteric microflora was observed. When enrichment samples were passed through 0.45 μm syringe filters, two samples yielded very tiny colonies after 4-days of incubation. These colonies were confirmed to be *A. skirrowii* by m-PCR. The isolation of *Arcobacter* species from the amniotic fluid samples and piglets was improved by using 0.65 μm membranes on top of blood agar plates (without CAT supplement).

Sensitivity of multiple-species detection by m-PCR

In the reaction of mixed template 1 where undiluted DNA of the three strains was combined in equal amounts, all bands representing the three species were obtained (Fig. 1).

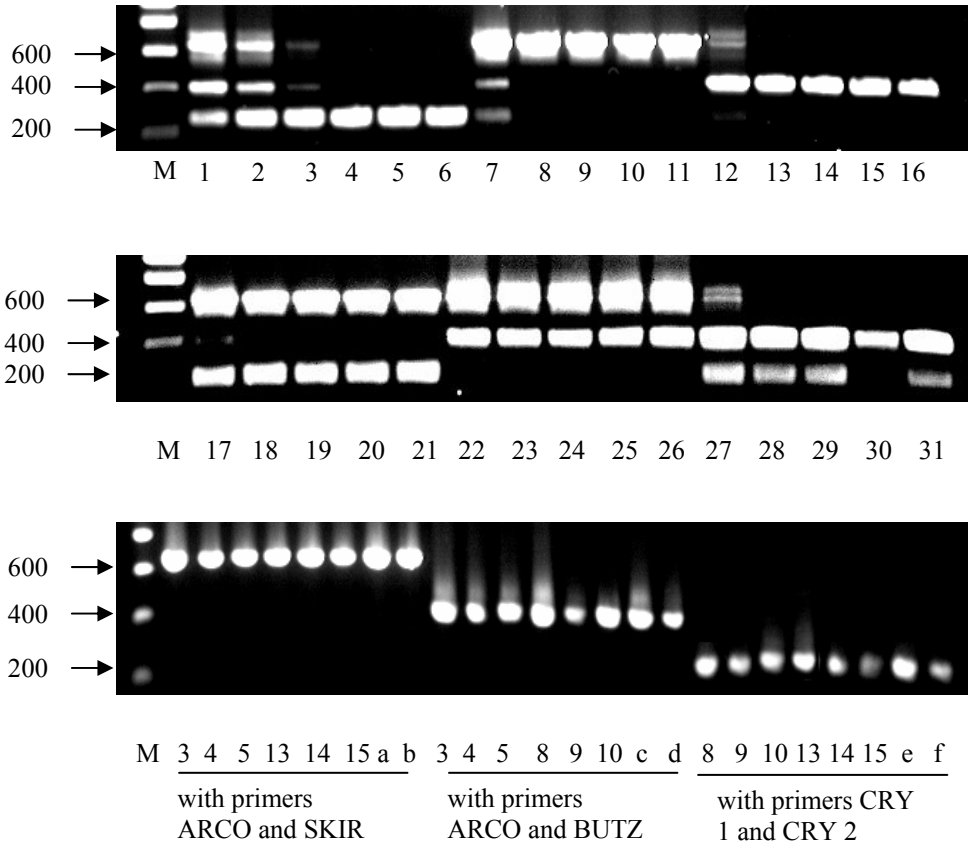


Fig. 1. Assay for the sensitivity of multiple-species detection by m-PCR. Lanes M were loaded with a molecular weight marker. The numbered lanes correspond to DNA mixed templates of three strains (*A. butzleri*, *A. skirrowii*, and *A. cryaerophilus*) (described in Table 2). Lane a and b contained DNA template of *A. skirrowii*; c and d, of *A. butzleri*; e and f, of *A. cryaerophilus* at dilutions of 10^{-2} (a, c, d) and 10^{-3} (b, d, e), for positive controls. In (A) and (B) samples were run with five primers of the m-PCR. In (C) samples were run with one-species-specific primer set as indicated.

All three bands were not obtained from pooled DNA templates with different DNA ratios in the mixtures. For example, when performing a PCR with the whole set of five primers and DNA ratios of *A. butzleri* and *A. skirrowii* to *A. cryaerophilus* in combinations four and five of respectively 10^{-3} and 10^{-4} to 1, only the band for *A. cryaerophilus* was obtained (Fig. 1A). However, a PCR of the same DNA mixtures using one-species primer sets – ARCO and BUTZ, or ARCO and SKIR – resulted in thick bands corresponding to *A. butzleri* or *A. skirrowii* (Fig. 1C). Similar results were obtained with combinations 8, 9, 10 and 13, 14, 15.

Transmission of Arcobacter species from carrying sows to their piglets

Vaginal swabs taken from the sows a few days before delivery were examined for the presence of *Arcobacter* spp. and all were PCR-negative. Congenital infection of the newborn piglets ranged from 38.5 to 83.3% (Table 5). Although the other two species were also identified or co-detected from rectal swabs of sows 1 week before farrowing, only *A. cryaerophilus* was recovered from the amniotic fluid of the sows and detected in all positive newborns by m-PCR and culture. Only one or two newborn piglets of five litters showed co-infection with *A. butzleri* or *A. skirrowii*. Interestingly, *A. cryaerophilus* was identified by m-PCR from amniotic fluid of sow 3, but *A. skirrowii* was isolated, and one of her piglets showed infection with both species. Two litters (8 and 9) were not further sampled since unrelated health problems occurred in the sows and their piglets were removed and nursed by other sows.

Whereas *A. cryaerophilus* was detected in all positive piglets at birth, 2 weeks later this species was only found in one piglet from each of four litters and in four piglets of a fifth litter. The prevalence *A. skirrowii* and *A. butzleri* increased, and the former became the most dominant species detected. Some piglets that were congenitally infected did not excrete *Arcobacter* spp. at 2 weeks of age. No positive PCR results were obtained in two litters (3 and 6).

Three weeks after birth more piglets in each litter were found positive and *A. skirrowii* still remained the most prominent *Arcobacter* species present followed by *A. butzleri*. At this stage, three more litters (1, 2 and 4) were not sampled because most of the piglets from these litters were redistributed with those from other litters.

Pulsed-field gel electrophoresis (PFGE)

The analysis was performed on five litters from which the whole set of four isolates per litter were obtained. However, DNA of some isolates did not react with the enzyme used for digestion. Consequently, PFGE fragment patterns of *EagI*-digested DNA of four isolates obtained from each of two litters and two isolates from amniotic fluid and rectal swabs of the sow in litter three are shown in Fig. 2.

Table 5. Detection of *Arcobacter* spp. from newborn, 2-week- and 3-week-old piglets

Litters	No. positive newborn/ litter	Species ^a (numbers of piglets) detected by m-PCR from				
		Mothers (rectum)	Amniotic fluid	Newborns	2-wk-old piglets ^b	3-wk-old piglets ^c
1	7/17 (41.2%)	skir	cry	cry (7)	cry (1); skir (2); ND ^d (6); M/D ^d (8)	NT ^d
2	6/10 (60.0%)	cry	cry	cry (5) cry & butz ^e (1)	cry (4); ND (3); M/D (3)	NT
3	6/12 (50.0%)	cry & skim	cry	cry (5) cry & skim ^e (1)	ND (8); M/D (4)	skir (3) ND (2); M/D (3)
4	9/16 (56.3%)	cry	cry	cry (9)	cry (1); ND (10); M/D (5)	NT
5	6/10 (60.0%)	cry	cry	cry (5); cry & skim (1)	skir (3); cry & skim (1); ND (3); M/D (3)	skir (4) cry & skim (2) ND (1)
6	8/10 (80.0%)	cry	cry	cry (8)	ND (10)	cry (1) ND (7); M/D (2)
7	8/12 (66.7%)	butz	cry	cry (8)	skir (4); butz & skim ^e (1); ND (4); M/D (3)	skir (7) ND (1)
8	8/10 (80.0%)	cry	cry	cry (8)	NT	NT
9	2/4 (50.0%)	cry	cry	cry (2)	NT	NT
10	5/13 (38.5%)	cry	cry	cry (4) butz (1)	skir (5); ND (3); M/D (5)	skir (6) ND (2)
11	5/6 (83.3%)	cry	cry	cry (5)	butz (1); butz & skim (5)	butz & skim (6)
12	10/14 (71.4%)	cry & skim	cry	cry (8) cry & skim (2)	butz & cry (1); butz & skim (4); butz (6); M/D (3)	butz & cry (1); butz & skim (4); butz (6)

^a butz: *A. butzleri*; skim: *A. skirrowii*; cry: *A. cryaerophilus*.

^b Total numbers of piglets per litter counted at week two are the same as the numbers at birth.

^c Total numbers of piglets per litter counted at week three exclude those that were removed or dead (M/D) at week two.

^d NT: not tested; ND: not detected; M/D: moved to other litters or dead.

^e Removed to another litter.

For the first two litters identical profiles were observed for the isolates from rectal and amniotic fluid samples from the sow and from the rectal sample from one piglet while the isolate from the other piglet showed a different pattern. For the sow of litter 3, the PFGE pattern of isolates from rectal and amniotic fluid samples

were also identical. Isolates from different litters showed dissimilar fragment patterns

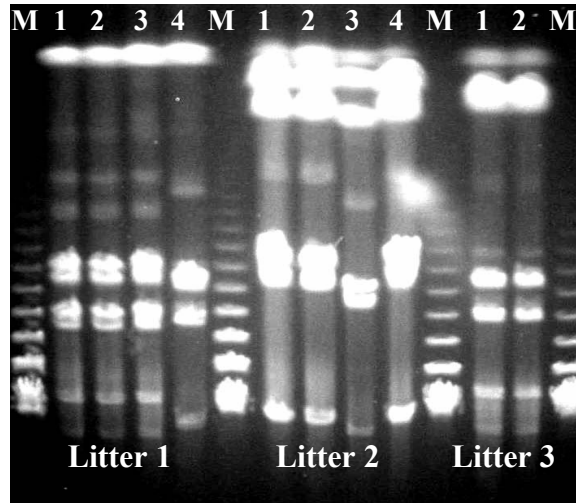


Fig. 2. PFGE profiles (*Eag* I) of *A. cryaerophilus* isolates from three litters. For each litter: lane 1, isolate from rectal sample of pregnant sow (before birth); lane 2, from amniotic fluid (during delivery); lanes 3 and 4, isolates from two different piglets; M, Lamda PFG Marker (New England Biolabs). In litter 1, band patterns of isolates from rectal (1) and amniotic fluid (2) of the sow and from one newborn piglet (3) are identical and distinct from that of the other piglet (4). This was observed in the same way in litter 2 (1, 2, and 4 are identical and 3 is different). In litter 3, patterns of both isolates from the sow resembled; but it failed to get the assay worked for isolates from her piglets.

Presence of antibodies against Arcobacter in colostrum

Western blot analysis showed reactivity in all 10 colostrum samples with whole cell lysates of the tested *A. cryaerophilus* isolate (data not shown). The reactivity could be detected up to a dilution of 1:1000. The colostrum samples also reacted with the *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* reference strains (Fig. 3B). Identical patterns were found for the tested *A. cryaerophilus* field isolate and the reference strain for this species, which were different to those of *A. skirrowii* and *A. butzleri* (Fig. 3B).

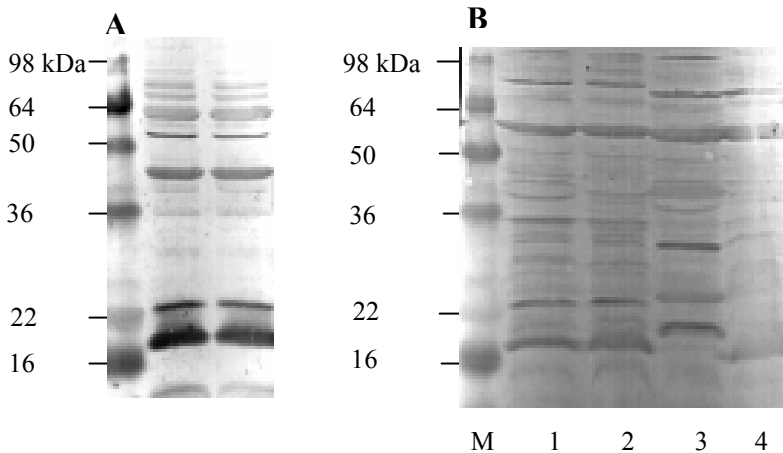


Fig. 3. Western blot analysis of colostrum to detect antibodies against *Arcobacter* spp. (A) Reactivity of one colostrum sample (dilution 1/500) to whole cell lysates of an *A. cryaerophilus* strain isolated from the same sow (duplicate). (B) A single colostrum sample was tested against a sow isolate of *A. cryaerophilus* (1), the reference strains of *A. cryaerophilus* (2), *A. skirrowii* (3), and *A. butzleri* (4). M is a molecular weight marker (SeeBlue Plus2 Pre-Stained Standard, Invitrogen).

Discussion

Prevalence of Arcobacter spp. on the farm

In the present study, *A. skirrowii* was the species most frequently detected in pig faeces. In other investigations, *A. butzleri* (9; 23) or *A. cryaerophilus* (24) were demonstrated to be the most prominent species detected in animals. Shifts in the species detected in sows that were sampled both in June and December were observed. The percentage with which the three species were detected also changed. Although *A. skirrowii* was still the most prominent species in December, *A. cryaerophilus* was more often detected. This could be explained by intermittent excretion. As for *Campylobacter* (28), discontinuous shedding of *Arcobacter* spp. has been suggested (8). Thus in hosts colonised by more than one *Arcobacter* strain or species, the shedding pattern of the strains may vary which could lead to one strain or species being detected at one time and other(s) at another. On the other hand, the circulation and reinfection of the strains throughout the stock ought to be taken into account.

Heterogeneous PFGE profiles of *A. cryaerophilus* strains isolated from newborn piglets in the same litter (which indicated the transmission of different strains of *A. cryaerophilus* from an individual sow to her newborns) and of strains from different litters demonstrated genetic variation among the *A. cryaerophilus* isolates present at the farm (Fig. 2). In a study by Hume *et al.* (2001), genotypic heterogeneity was also observed among *A. butzleri* isolates obtained from sows and

piglets on one farm. One of the possible explanations for this heterogeneity is genetic rearrangement between bacteria, which may occur during bacterial transmission throughout the stock, as was demonstrated for *Campylobacter* (27).

The possible bias in detection of *Arcobacter* species in the multiplex PCR should also be mentioned. Preferential amplification of one target sequence over another or others in the m-PCR has been reported. This bias may occur due to interaction of PCR reagents, low template concentration and chemical or radiation damage of templates (16). The ratios of different DNA templates in m-PCR can also have influence on the out-come products (13). In the present study, no matter what the DNA target was, the amplification favoured sequences with higher proportionality in the template mixtures. Therefore, strains that are present in faeces but in much lower numbers than others may not be simultaneously detected by m-PCR.

Transmission of Arcobacter species from pregnant sows to their piglets

In several studies, *Arcobacter* spp. have been detected in the reproductive organs (uterus, oviduct, placenta) of sows as well as in the internal organs of dead fetuses (2; 4). However, Hume *et al.* (2001) found no *Arcobacter* in nursing piglets of infected mothers. To our knowledge, here the first study of both congenital and post-natal transmission of *Arcobacter* spp. from carrying sows to their live piglets is described.

Arcobacter species were not found in vaginal swabs taken a few days before parturition, which excludes the possible contamination of the bacteria from the sow vagina into amniotic fluid samples. Therefore, the detection of *Arcobacter* in the amniotic fluid samples from the sows and in rectal samples from the newborn piglets clearly indicates transplacental transmission of the bacteria from mothers to their piglets. Homologous PFGE profiles of *A. cryaerophilus* isolates from a rectal sample (before birth) and the amniotic fluid of one sow and from the isolate of one of her piglets confirmed this congenital transmission.

A. cryaerophilus seems to be more invasive in the host than other *Arcobacter* species. Although *A. butzleri* and *A. skirrowii* were detected alone or simultaneously with *A. cryaerophilus* from sow rectal swabs, the latter was the only species found in all amniotic fluid samples and was the most predominantly identified species in newborn piglets both by PCR and culture. Although sharing with other siblings an all-embracing chorion, each pig foetus possesses its own amnion and allantois (14). Due to the possible differences in structure of these sacs, the resistance to transmission of substances and pathogens may not be the same for each individual sibling. This might explain the range of congenital infection of newborn piglets found in this study (from 38.5 to 83.3%). It also may explain why in some litters, one piglet showed congenital infection with *A. cryaerophilus* together with *A. butzleri* or *A. skirrowii* while its siblings were only infected with

A. cryaerophilus. Interestingly, the PFGE assay indicated that newborn piglets from the same mother can be infected with genetically heterologous *A. cryaerophilus* strains. Due to the intrusion of *A. cryaerophilus* in most placentas, whereas the other two species were only present in one or two, much higher numbers of *A. cryaerophilus* were released into the amniotic fluid when the piglets were delivered. Consequently, the bias in the m-PCR (discussed above) resulted in the fact that *A. cryaerophilus* was the only species detected by m-PCR in fluid samples although *A. skirrowii* and/or *A. butzleri* might also have been present, albeit in much lower proportions. This was supported by the observation in sow three from which *A. cryaerophilus* was detected by m-PCR in a rectal sample, while *A. skirrowii* was isolated from its amniotic fluid, and both *A. cryaerophilus* and *A. skirrowii* were present in one of her piglets, whereas *A. cryaerophilus* was present in the *Arcobacter*-positive siblings of this piglet.

Horizontal transmission was observed upon examination of rectal samples of piglets at 2–3 weeks of age. At this age, *A. cryaerophilus* no longer was the most predominant species but *A. skirrowii* or *A. skirrowii* together with *A. butzleri*. The recovery rates of *Arcobacter* spp. seemed to decrease at Week 2 and slightly increase again 1 week later. This could be related to the presence of antibodies against *Arcobacter* spp. in the colostrum, which may help piglets to inhibit colonisation or/and multiplication of bacteria acquired congenitally or post-natally from sows or from the environment. The level of maternal antibodies declines after a few weeks while the immune system of the young animals is not yet completely developed. Consequently, piglets may acquire *Arcobacter* spp. shed by the sow or by newcomers from other infected litters. This could be a problem at 3–4 weeks of age in particular, since at that time in addition to the declined serum levels of maternal antibodies piglets also undergo the stress of weaning, which provides an opportunity for microbial colonisation and multiplication. The observations of Hume *et al.* (2001) that no infection took place in nursing piglets from *Arcobacter*-positive sows and the later increase in prevalence due to shedding and prolonged exposure might be explained in this way.

In conclusion, the transmission of *Arcobacter* species from carrying sows to their offspring can occur both vertically and horizontally. Although sows were colonised by all three *Arcobacter* species, *A. cryaerophilus* was better able to invade through the intestine and placenta than *A. butzleri* and *A. skirrowii*. *Arcobacter*-positive sows provide some protection to their piglets by antibodies in the colostrum during the first few weeks after birth. Longer exposure to an environment that is contaminated open ways for the bacteria to establish themselves in the intestine of the piglets.

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Chapter 3

Vertical transmission of *Arcobacter* spp. in chickens

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Submitted for publication

Abstract

The aim of this study was to investigate the possibility of vertical transmission of *Arcobacter* species in chickens. The presence of *Arcobacter* spp. in two breeding-hen flocks was determined by examination of the gut contents of slaughtered chickens. The bacteria were detected in 85% of the sampled intestinal tracts of one flock and in 20% of the other. *Arcobacter* species were recovered in 6 oviduct magnums from the first flock. However, the bacteria could not be detected, neither by PCR nor by isolation, on 20 eggs collected on the farm of the first flock and on 20 eggs still remaining in the vagina hens from the other flock. Furthermore, none of 30 ovarian eggs sampled from each flock were positive. At a hatchery, fluff samples in hatching chambers, eggshells, unhatched eggs and newly hatched chicks were examined. Apart from one fluff sample from which *A. cryaerophilus* was isolated, no arcobacters were recovered from any of the hatchery samples. From these results there is no evidence of transmission of *Arcobacter* spp. from positive breeding hens to eggs and chicks.

Introduction

Arcobacter spp. have recently been considered to play a role as potential zoonotic microorganisms (11). *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have been detected in association with diseased as well as healthy humans and animals. In humans, the bacteria were isolated from several cases of bacteraemia (17; 21; 22; 35; 36). They were found in stool specimens of both healthy and diarrhoeic humans (15; 27; 33). In animals *Arcobacter* spp. were isolated from healthy animals (18; 30-32). *Arcobacter* species were also found in relation with reproductive disorders in cattle and swine (6-8; 23). In pigs, both vertical and horizontal transmission of *Arcobacter* spp. from sows to piglets has been demonstrated (12).

Like *Campylobacter*, *Arcobacter* species have been detected worldwide in meat, especially in chicken products (5; 19; 25; 28). The bacteria are present on almost all sampled broiler carcasses and at significantly higher recovery rates than *Campylobacter* spp. in the same samples (14). However, *Arcobacter* species are rarely isolated from live chicken and their intestinal contents (1; 29). The prevalence of *Arcobacter* spp. increases with the bird age (34). The aim of this study was to investigate the potential of vertical transmission of *Arcobacter* spp. from breeding hens to their chicks.

Materials and Methods

Sampling and sample processing

Viscera were taken from 2 broiler-breeding flocks at a slaughterhouse in the Netherlands. Gut contents and oviduct mucosa were sampled. The sampling area was flushed with 75% ethanol and scissors sterilised in a burning flame were used to open the intestines (ileum and caeca). Cotton swabs were used to transfer the gut contents into 10 ml enrichment medium (*Arcobacter* broth, Oxoid, CM965 supplemented with Cefoperazone, Teicoplanin, Amphotericin B (CAT), Oxoid, SR174). A 10 cm straight of the oviduct magnum mucosa was gently scalded with sterile scalpels and cotton swabs were used to transfer the samples into 10 ml enrichment broth. From each viscera, 1 ml content of a 2-cm ovarian egg was taken using a sterile syringe and added to 9 ml enrichment broth.

In addition to the viscera, 20 freshly laid eggs from one flock were collected on the farm and 20 eggs still remaining in vagina of the other flock were also collected. The eggs were randomly paired, the eggshell surface of each pair was wiped with a sterile cotton compress wetted in 20 ml *Arcobacter* broth and 1 ml of the wiping sample was inoculated in 9 ml enrichment medium. The egg content of each pair was pooled, homogenised and 1 ml was inoculated in 9 ml enrichment medium.

Various samples were taken at a hatchery. Fluff samples were taken of the floor inside three hatching chambers. From each of six different egg lots (including the ones hatched in the fluff-sampled chambers), eggshells and 10 unhatched eggs were collected. In addition, 80 newly hatched chicks that had been euthanised by CO₂ by the hatchery (which were the male chicks and some accidentally killed females) were sampled. All samples were transported to the laboratory on ice and processed within 24 hours. In the laboratory, the surface of 10 unhatched eggs from each lot was wiped with a sterile cotton compress wetted in 20 ml *Arcobacter* broth, from which 1 ml of liquid was inoculated in 9 ml enrichment medium. From the sixty unhatched eggs, 28 contained unhatched chicks and 32 were rotten. The contents of every 2 randomly paired eggs (containing unhatched chicks or rotten) were pooled and 1 ml egg juice sample was added to 9 ml enrichment medium. Eighty newly hatched chicks were randomly divided over eight groups. Each group was washed in 100 ml broth from which 1 ml was inoculated in 9 ml enrichment medium. From each group, 2 chicks were randomly paired. The contents from the yolk sac and minced intestines of each pair were pooled and 1 ml was inoculated in 9 ml enrichment medium. Twenty gram of hatchery fluff and eggshell samples were homogenized in 180 ml *Arcobacter* broth and 1 ml was transferred into 9 ml enrichment medium.

All enrichment samples were incubated for 48 hours at 30°C under microaerophilic conditions (generated by BD CampyPak™, Becton, Dickinson and Company).

Detection of Arcobacter spp.

The presence of *Arcobacter* species in enrichment samples was investigated both by PCR and isolation of the bacteria. DNA from each enrichment culture was extracted by the boil lysis method and used in a genus-specific PCR reaction (forward primer: 5' GAG ATT AGC CTG TAT TGT ATC 3', reverse primer: 5' TAG CAT CCC CGC TTC GAA TGA 3') (9). PCR-positive samples were then examined for the presence of *A. butzleri*, *A. cryaerophilus* or *A. skirrowii* by a multiplex PCR (m-PCR) (primer ARCO: 5' CGT ATT CAC CGT AGC ATA GC 3', primer BUTZ: 5' CCT GGA CTT GAC ATA GTA AGA ATG A 3', primer SKIR: 5' GGC GAT TTA CTG GAA CAC A 3', primers CRY1: 5' TGC TGG AGC GGA TAG AAG TA 3' and CRY2: 5' AAC AAC CTA CGT CCT TCG AC 3') (16). Samples that were negative from *arcobacter*-specific PCR were tested by PCR with eubacterial primers (27f: AGA GTT TGA TCM TGG CTC AG; 519r: GWA TTA CCG CGG CKG CTG; K = G, T; M = A, C; W = A, T) (20) for the possible presence of inhibitors. DNA from *A. butzleri* LMG 6620, *A. cryaerophilus* LMG 7537 and *A. skirrowii* LMG 6621 was used as positive control.

For bacterial isolation, 50 µl of each enrichment sample was dropped onto a cellulose-nitrate membrane filter (0.65 µm) (Sartorius, The Netherlands) which was placed on top of a blood agar plate (Brain Heart Infusion Agar, Oxoid, plus

5% horse blood) supplemented with CAT. The plates were incubated for 1 hour at 30°C in air, the filters were removed and the filtrates were evenly distributed on the agar surface with a sterile spreader. The agar plates were incubated for 48 hours at 30°C under microaerophilic conditions. Plates with no growth were incubated for another 48 hours. Colonies suspected to be *Arcobacter* spp. were transferred onto blood agar plates without CAT supplement and incubated for 48 hours at 30°C under microaerophilic conditions. *Arcobacter* isolates were confirmed by the m-PCR.

ERIC-PCR

To investigate the genetic relationship between isolates from gut contents and from samples of ovarian eggs and oviducts from a same individual, ERIC-PCR was performed (primer 1: ATG TAA GCT CCT GGG GAT TCA C and primer 2: AAG TAA GTG ACT GGG GTG AGC G) (13). DNA of the isolates was obtained by the boiled lysate method and 3 µl was used in 25 µl total reaction volume.

Results and Discussion

From one breeder flock, 34 out of 40 intestinal tracts (85%) were arcobacter-positive by PCR (Table 1). By m-PCR *A. butzleri* was found in 30 tracts, *A. cryaerophilus* in 2 and both *A. butzleri* and *A. skirrowii* in 2 tracts. Six oviducts were found positive by both PCR and isolation from which only *A. butzleri* was identified by m-PCR. The oviduct isolates had similar ERIC-PCR fingerprints with some isolates from the gut contents (data not shown). In the other flock, *Arcobacter* species were detected by PCR in 6 out of 30 intestinal tracts (20%), of which 5 were shown by m-PCR to contain both *A. butzleri* and *A. cryaerophilus* and only the latter was detected from the sixth. None of the oviduct samples from this flock were positive neither by PCR nor isolation (Table 1). None of the 20 eggs taken on the farm (flock 1), the 20 eggs from the vagina (flock 2) and ovarian follicles (40 from flock 1 and 30 from flock 2) were positive for *Arcobacter* spp. by PCR and isolation. Of the hatchery fluff samples, one was PCR-positive, from which *A. cryaerophilus* was isolated. However, no arcobacters were detected by PCR from hatchery eggshell, unhatched eggs and newly hatched chicks (Table 1). The samples that were negative for arcobacters by PCR were retested with PCR using eubacterial primers and gave positive results albeit different DNA band intensity. The PCR results were in accordance with the isolation since *Arcobacter* could not be isolated from these samples.

Table 1. The presence of *Arcobacter* spp. in breeder hens and in hatchery samples

Samples	Number of samples	Number of positive ^a
(i) Breeder flock 1		
Intestinal tracts	40	34 (85%)
Oviducts	40	6 (15%)
Ovarian follicles	40	None
Eggs (on farm)	20	
Surface wipe		None
Egg contents		None
(ii) Breeder flock 2		
Intestinal tracts	30	6 (20%)
Oviduct	30	None
Ovarian follicles	20	None
Eggs in vagina	20	
Surface wipe		None
Egg contents		None
(iii) Hatchery		
Hatchery fluff samples	3	1
Eggshells	6	None
Unhatched eggs		
Surface wipe	60	None
Dead chicks	28	None
Rotten eggs	32	None
Newly hatched chicks		
Whole chick rinse	80	None
Yolk sac contents	80	None
Intestine	80	None

^a The numbers of positive samples were determined by both PCR and isolation of the enrichment culture.

The significance of the observed vertical transmission of *Campylobacter* spp. from breeding hens to chicks is still controversial due to the inconsistency of the results and data interpretation between different studies. Some authors reported the presence of *Campylobacter* spp. in hen ovarian follicles and oviducts as well as in hatchery facilities and wastes, which conclusively indicated the potential of vertical transmission from breeders to broilers (2; 4; 10). In contrary, observations on parent breeders, broiler-breeding eggs, young broilers, hatchery samples and artificial infection of SPF eggs suggest that the vertical route of transmission of

Campylobacter spp. from breeders to broilers is rare and not of importance compared to the transmission from surrounding environments (3; 24; 26).

From the investigation described here it can be concluded that although *Arcobacter* spp. were present in breeding hens with a high prevalence and *A. cryaerophilus* was found in one hatching chamber, vertical transmission of *Arcobacter* spp. from breeding hens to chicks seems unlikely.

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Chapter 4

The introduction of *Arcobacter* spp. in poultry slaughterhouses

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Submitted for publication

Abstract

Despite the presence high levels of *Arcobacter* spp. on chicken carcasses, the source of arcobacter contamination in slaughterhouses still remains unclear. It has been hypothesised in the literature that *Arcobacter* species that contaminate carcasses originate in in-plant slaughterhouses and/or supply water. The present study aimed to determine the source of *Arcobacter* contamination in two poultry slaughterhouses in the Netherlands. Carcasses and intestinal tracts from 3 hen flocks and 2 broiler flocks were collected. Water drippings from carcasses in 2 slaughterhouses and supply water in one slaughterhouse were also taken. For one flock, cloacal swabs and faecal samples were taken on the farm before slaughtering. ERIC-PCR was applied to study the genetic diversity and relationship among the isolates. No *Arcobacter* spp. were found in the supply water but on almost all of the sampled carcasses and in water drippings arcobacters were identified. *Arcobacter* spp. were detected in the gut systems of chickens, ranging from 20% to 85% in hens and 3.3% and 51% in broilers. Similar ERIC-PCR genotypes were detected in gut contents as well as on carcasses from the same flock. The present study demonstrated that *Arcobacter* spp. inhabit the chicken intestines and probably in this way are imported into slaughterhouses where the bacteria contaminate carcasses during processing.

Introduction

The genus *Arcobacter* was proposed in 1991 and belongs to the family *Campylobacteraceae* (31; 32). Like *Campylobacter*, *Arcobacter* species have been indicated as foodborne microorganisms since they have been detected in food of animal origin, especially in products from chicken all over the world (6; 19; 26; 28). The bacteria are found on almost all sampled broiler carcasses with a significantly higher recovery rate than *Campylobacter* spp. for the same samples (15). Up to several thousand bacteria were present per gram of neck skin prior to evisceration. *Arcobacter* spp. are recovered from carcasses at different processing stages (e.g. pre- and post-scald, pre- and post- evisceration and pre- and post chill) (15; 29) as well as along the slaughterhouse processing line, equipment and processing water (11; 15; 16). The organisms are hardly isolated from the gut contents of birds belonging to the same flock where arcobacter-positive carcasses come from (3; 15). It has been hypothesised that *Arcobacter* spp. are probably not normal inhabitants of chicken intestines (7; 10; 11). The source of arcobacter contamination on carcasses would be the slaughterhouse environment and/or water used during processing (3; 11; 30).

The aim of this study was to determine the source(s) of introduction of *Arcobacter* species in chicken slaughterhouses by examining the presence of *Arcobacter* spp. on chicken carcasses, in the content of different parts of the chicken intestine and in the water supply of two poultry slaughterhouses in the Netherlands.

Materials and Methods

Sampling

Carcasses and intestinal tracts of chickens were obtained from two slaughterhouses in the Netherlands (with a capacity of 10000-12000 birds per hour). Both slaughterhouses are operated under the EU hygiene regulations 852, 853 and 854/2004. At slaughterhouse 1, three flocks of laying hens were sampled (on different days). From flock 1, twenty pre-chilling carcasses and 40 intestinal tracts were randomly taken. Five days prior to slaughtering of this flock, 50 cloacal swabs and 6 faeces pools (each containing 5-6 different faecal droppings) were collected randomly on the farm. Thirty pre-evisceration carcasses were collected from flock 2 and 40 intestinal tracts were collected from flock 3. At slaughterhouse 2, samples were taken randomly from 2 different broiler flocks on 2 different days. Thirty pre-evisceration carcasses were collected from one flock. Twenty pre-evisceration carcasses and 100 intestinal tracts were collected from the other. In addition, approximately 100 ml of water drippings from hanging broiler carcasses at different processing stages was collected: (1) from carcasses immediately after leaving the scalding tank (operated at 52°C/3 minutes), (2) from carcasses after

defeathering and washing and (3) after the last wash before entering the chilling room. Tap water used as processing water in the slaughterhouse was also sampled (500 ml).

Sample processing

Cloacal swabs taken on the farm were immediately placed in 10 ml *Arcobacter* broth (Oxoid, CM965) supplemented with CAT supplement (Cefoperazone, Teicoplanin, Amphotericin B, Oxoid, SR174). Other samples, collected on the farm or in slaughterhouses, were transported on ice to the laboratory and were processed within a few hours. *Arcobacter* broth without CAT supplement was used for processing samples (for homogenisation, rinsing or wiping). The processed samples were inoculated in *Arcobacter* broth supplemented with CAT, (from now on referred to as enrichment medium). Twenty gram of each faecal pool was homogenised (by vortex) in 40 ml *Arcobacter* broth and 1 ml of the suspension was added to 9 ml enrichment broth. Post-evisceration carcasses were rinsed with 100 ml *Arcobacter* broth in sterile plastic bags and pre-evisceration carcasses were wiped with a sterile cotton compress wetted in 100 ml *Arcobacter* broth. One ml of the samples obtained by rinsing or wiping of carcasses was then inoculated in 9 ml enrichment medium. The sampling area of the intestinal tracts was flushed with 75% ethanol and scissors sterilised in a burning flame were used to open the guts. The content of 10-cm duodenum, 10-cm ileum or 5-cm caeca was collected using a sterile scalpel, mixed with sterile cotton swabs and placed in 10 ml enrichment broth. One ml of dripping water was inoculated in 9 ml enrichment medium. Fifty ml of tap water was centrifuged for 20 minutes at 4000 \times g and 15°C. Forty nine ml of the supernatant was gently removed and 9 ml enrichment medium was added and mixed with the remaining 1-ml sample.

All enrichment samples were incubated for 48 hours at 30°C under microaerophilic conditions (generated by BD CampyPak™, Becton, Dickinson and Company).

Detection of Arcobacter species

The presence of *Arcobacter* spp. was investigated both by PCR and isolation of the bacteria. After 48-hour incubation, DNA from each enrichment culture was extracted by the boiled lysate method. The presence of *Arcobacter* DNA was examined by a genus-specific PCR reaction (primer 219f: GAG ATT AGC CTG TAT TGT ATC, primer 1427r: TAG CAT CCC CGC TTC GAA TGA) (13). PCR-positive samples were retested with a multiplex PCR (m-PCR) for species specification (using primers ARCO: CGT ATT CAC CGT AGC ATA GC; BUTZ: CCT GGA CTT GAC ATA GTA AGA ATG A; SKIR: GGC GAT TTA CTG GAA CAC A; CRY 1: TGC TGG AGC GGA TAG AAG TA; CRY 2: AAC AAC CTA CGT CCT TCG AC) (17). Samples that were negative in the *arcobacter*-specific PCR were tested by PCR with eubacterial primers (27f: AGA GTT TGA

TCM TGG CTC AG; 519r: GWA TTA CCG CGG CKG CTG; K = G, T; M = A, C; W = A, T) (20) for the possible presence of inhibitors. DNA from *A. butzleri* LMG 6620, *A. cryaerophilus* LMG 7537 and *A. skirrowii* LMG 6621 was used as positive controls.

For isolation of bacteria, 50 µl of each enrichment sample was dropped on a cellulose-nitrate membrane filter (0.65 µm) (Sartorius, The Netherlands) which was placed on blood agar plates (Brain Heart Infusion Agar, Oxoid, plus 5% horse blood) supplemented with CAT (Cefoperazone, Teicoplanin, Amphotericin B, Oxoid). After 1-hour incubation at 30°C in air, the filters were removed, the filtrates were evenly distributed over the agar surface with a sterile spreader, and the plates were incubated for 48 hours at 30°C under microaerophilic conditions. Plates with no growth were incubated for two more days. Three to five colonies from each plate suspected to be *Arcobacter* spp. were transferred to blood agar plates without antimicrobial supplement and incubated for 48 hours at 30°C under microaerophilic conditions. Isolates were purified by passing onto blood agar plates at least 3 times. The isolates were confirmed to be *Arcobacter* spp. by the m-PCR (17).

ERIC-PCR

To investigate the genotypic diversity and the genetic relationship among the isolates, ERIC-PCR was performed on the isolated *Arcobacter* strains (primer 1: ATG TAA GCT CCT GGG GAT TCA C and primer 2: AAG TAA GTG ACT GGG GTG AGC G) (14). DNA of the isolates was obtained by the boiled lysate method and 3 µl was used in 25 µl total reaction volume.

Survival of Arcobacter isolates under scalding conditions

Five *Arcobacter* isolates, two *A. butzleri*, two *A. cryaerophilus* and one *A. skirrowii*, were randomly chosen for testing their survival after 3 minutes in water at 52 and 58°C. The origin of the isolates is shown in Table 3. One ml of a 10-fold, 100-fold or 1000-fold dilution of an overnight broth culture of each strain was inoculated in 9 ml sterile water that had been placed in a water bath of 52 or 58°C at least 1 hour prior to the assay. After 3-minute incubation, 100 µl from each sample was transferred onto blood agar plates and another 100 µl was added into 900 µl BHI broth (Brain Heart Infusion, Oxoid) at room temperature, for serial dilution. One hundred µl of each dilution was then spread onto blood agar plates. The plates were incubated for 2 days at 30°C under microaerophilic conditions and colonies were counted.

Table 1. Presence of *Arcobacter* spp. in samples as determined by PCR and by isolation

	Numbers of positive samples/ total	Numbers of positive samples by PCR/ by isolation
Slaughterhouse 1-Flock 1		
Cloacal swabs	0/50	0/0
Faecal droppings	2/6	1/2
Carcasses	20/20 (100%)	20/20
Intestinal tracts	34/40 (85%)	34/33
Duodenum		9/8
Ileum		6/6
Caeca		5/5
Duodenum + ileum		2/2
Duodenum + caecum		1/1
Ileum + caecum		7/7
3 positions		4/4
Slaughterhouse 1- Flock 2		
Carcasses	30/30 (100%)	30/24
Intestinal tracts	6/30 (20%)	6/4
Ileum		3/3
Caeca		1/1
Ileum + caecum		2/0
Slaughterhouse 1- Flock 3		
Intestinal tracts	13/40 (43.3%) *	5/10*
Ileum		3/8
Caeca		2/0
Ileum + caecum		0/2
Slaughterhouse 2- Flock 4		
Carcasses	30/30 (100%)	30/28
Intestinal tracts	1/30 (3.3%)	1/1
Ileum		1/1
Caeca		0
Ileum + caecum		0
Drippings 1 (post-scalding)	1/2	1/0
Drippings 2 (post-defeathering)	2/2	2/2
Drippings 3 (pre-chill)	2/2	2/0
Tap water	0/2	0/0
Slaughterhouse 2- Flock 5**		
Carcasses	15/20 (75%)	15
Intestinal tracts	51/100 (51%)	51
Ileum		26
Caeca		8
Ileum + caecum		17

Drippings 1 (post-scalding)	0/1	0
Drippings 2 (post-defeathering)	1/1	1
Drippings 3 (pre-chilling)	2/2	2
Tap water	0/2	0

* For this flock, 5 tracts were PCR-positive, but arcobacters were isolated from 10 tracts including 2 PCR-positive tracts

** For this flock, the investigation was done by PCR only.

Results

Presence of Arcobacter species in samples

The presence of *Arcobacter* spp. in samples detected both by PCR and isolation is presented in Table 1 and 2. In general, the PCR technique yielded a higher prevalence of *Arcobacter* spp. than the isolation method. The isolation of *Arcobacter* species from some PCR-positive samples was unsuccessful due to overgrowth by other enteric bacteria or fungi. Arcobacters were also isolated from a few PCR-negative samples. Therefore, the number of positive samples was calculated by combination of the results from both PCR and isolation.

Almost all sampled carcasses were *Arcobacter*-positive. From flock 1 (hens), 1 faecal sample (out of 6) taken on the farm was PCR-positive and *A. skirrowii* was isolated. In addition, *A. cryaerophilus* was isolated from 1 PCR-negative sample. None of the 50 cloacal swab samples taken on the farm were *Arcobacter*-positive either by PCR or isolation. However, Arcobacters were detected in the content of 34 out of 40 intestinal tracts (85%) taken from the same flock. The majority of the isolates from these samples were identified as *A. butzleri*. Both *A. butzleri* and *A. cryaerophilus*, but not *A. skirrowii*, were detected in the intestinal content of 6 of the 30 hens from flock 2. In flock 3 (hens), only 5 intestinal tracts were found positive by PCR. *A. butzleri* and *A. cryaerophilus* were isolated from 10 tracts (including two PCR-positive ones). From one broiler flock (flock 4) *A. butzleri* was detected by PCR and isolated from 1 of the 30 intestinal tracts. To reconfirm the presence of *Arcobacter* species in broiler intestines, carcasses and intestinal tracts were collected from another broiler flock (flock 5) on another visit and the presence of *Arcobacter* spp. was detected only by PCR. Fifty one out of the 100 intestinal tracts were found positive.

At the broiler slaughterhouse, various water samples were taken during the 2 visits and examined for *Arcobacter* species. *Arcobacter* spp. were not detected by either PCR or isolation in all supply water samples. In contrast, the bacteria were detected by PCR in water dripping samples from all sampling sites (Table 1). Unfortunately, due to the overgrowth by some swarming microorganisms *Arcobacter* species were

only isolated from two dripping samples taken after defeathering and identified as *A. cryaerophilus*.

Table 2. Identification of *Arcobacter* species in enrichment samples by m-PCR and by isolation

	Species ^{a, b}				
	butz	cry	skir	butz + cry	butz + skim
Slaughterhouse 1-Flock 1					
Faecal droppings		0/1	1/1		
Carcasses	9/8	0/5		11/7	
Intestinal samples ^c	48/45	2/2	2/1	0/3	
Slaughterhouse 1- Flock 2					
Carcasses	11/2	1/16		18/6	
Intestinal samples	1/0	2/4		5/0	
Slaughterhouse 1- Flock 3					
Intestinal samples ^c	4/11	0/1		1/0	
Slaughterhouse 2- Flock 4					
Carcasses	23/21	0/2		0/5	7/0
Intestinal samples ^c	1/1				
Drippings 1 (post-scalding)					1/0
Drippings 2 (post-defeathering)		0/2		2/0	
Drippings 3 (pre-chilling)				2/0	
Slaughterhouse 2- Flock 5 ^d					
Carcasses	15				
Intestinal samples	68				
Feather 1	1				
Feather 2	1				
Drippings 2 (post-defeathering)	1				
Drippings 3 (pre-chilling)	2				

^a Data are presented as numbers of samples detected by mPCR/ by isolation.

^b butz, *A. butzleri*; cry, *A. cryaerophilus*; skim, *A. skirrowii*.

^c Samples from different parts of the intestinal tracts (duodenum, ileum, caeca).

^d For this flock the investigation was done by PCR only.

ERIC-PCR fingerprinting of Arcobacter isolates

ERIC-PCR fingerprints of *Arcobacter* isolates from carcasses and intestinal content are shown in Figure 1. In general, in each flock a genotypically diverse set of isolates were detected on the carcasses. Genetic heterology was also observed among isolates from the gut content of chickens from the same flock. In each flock

several similar genotypes were detected on different carcasses and in different intestinal tracts but only one or two isolates from carcasses had similar patterns as the intestinal isolates. Furthermore, the fingerprint of the *A. cryaerophilus* isolate from faeces on the farm of flock 1 (lane 1, Fig. 2A1) was different from that of all carcass and gut isolates from this flock. Similarly, the faecal *A. skirrowii* isolates from this farm (lanes 32 and 33, Fig. 2A1) had a pattern different from that of the *A. skirrowii* isolate in a caecal sample (lane 34, Fig. 2A1).

Survival of Arcobacter species under scalding conditions

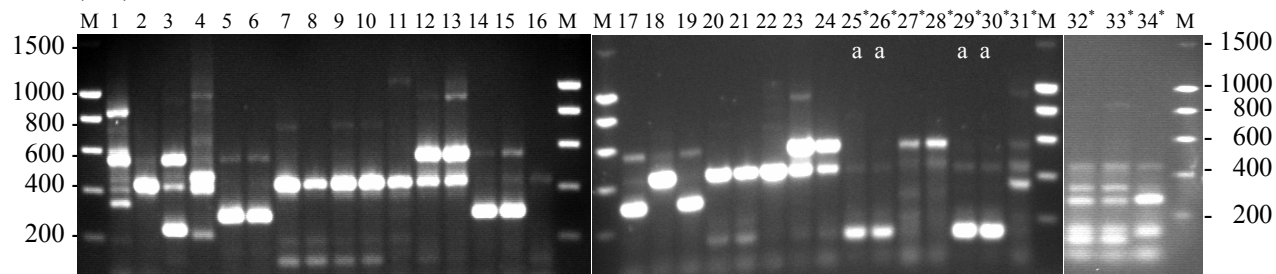
Since *Arcobacter* species are mesophilic and unable to grow at 42°C (32), the ability of five *Arcobacter* isolates to survive the scalding conditions was examined with different bacterial inocula (10^6 , 10^5 , 10^4 CFUs/ml). After exposure to 52°C for 3 minutes, the bacterial numbers were reduced approximately 0.6-4.0 log₁₀ CFU/ml (Table 3). At 58°C, at least 3 log₁₀ CFUs/ml reduction was demonstrated. Heat resistance seemed strain-dependent.

Discussion

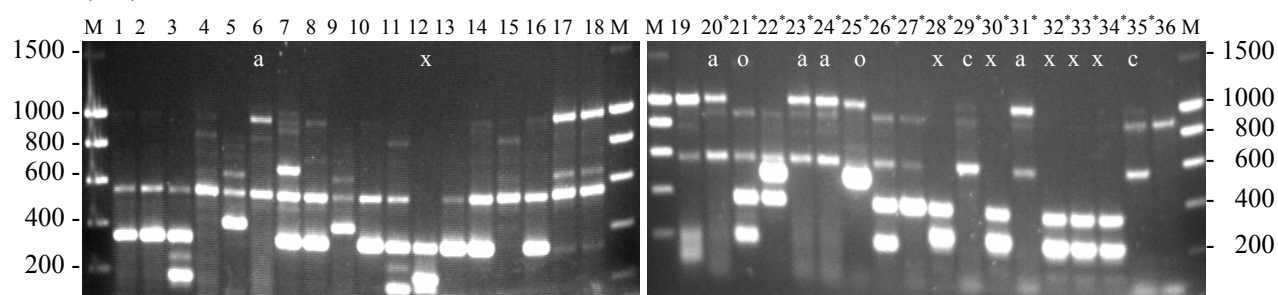
The present study was performed to determine the sources of introduction of *Arcobacter* spp. in slaughterhouses. *Arcobacters* could not be recovered from the supply water whereas almost all sampled carcasses and water drippings from carcasses at different processing stages were positive. It seems impossible that the slaughterhouse supply water is the source of *arcobacter* contamination on carcasses. The results demonstrated the presence of *Arcobacter* spp. in the intestinal content of all sampled hen and broiler flocks albeit in different numbers (Table 1 and 2). Likewise, *Arcobacter* species were isolated from 1/15 intestinal tracts in a study by Atabay and Corry (1997) and from 1/10 intestinal tracts for each of two flocks in a study by Van Driessche and Houf (2007). In Japan, *arcobacters* were isolated from 14.6% of cloacal swabs taken from 234 chickens on farms (18).

Furthermore, the ERIC-PCR results demonstrated that a few *arcobacter* genotypes in the intestines were similar to genotypes on carcasses of the same flock. This indicates that *Arcobacter* spp. can be introduced in a slaughterhouse by the gut contents of chickens. The finding of extreme genetic diversity of isolates on carcasses in the present study as well as in the literature (16; 28; 30) can be explained by cross-contamination among slaughtered chickens within one flock and from flocks of different farms. Spreading of *Campylobacter* spp. from previously slaughtered birds to subsequent flocks has been clearly demonstrated (21; 22; 27). This cross-contamination can already start during transportation if already-contaminated crates are used.

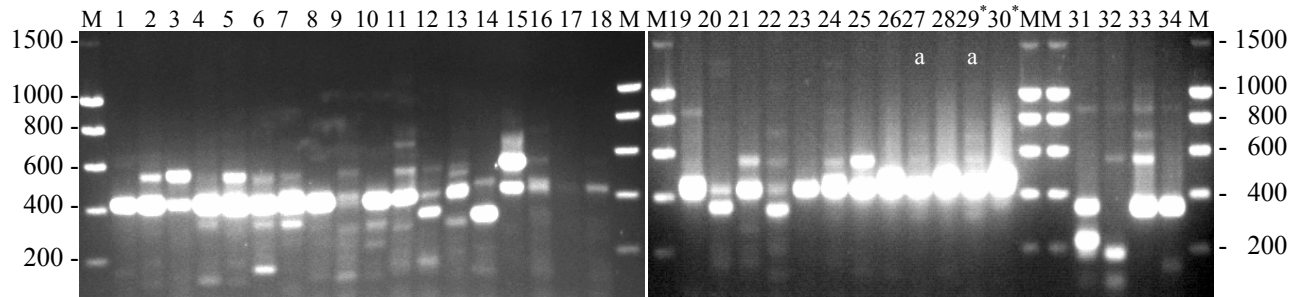
(A1)



(A2)



(B)



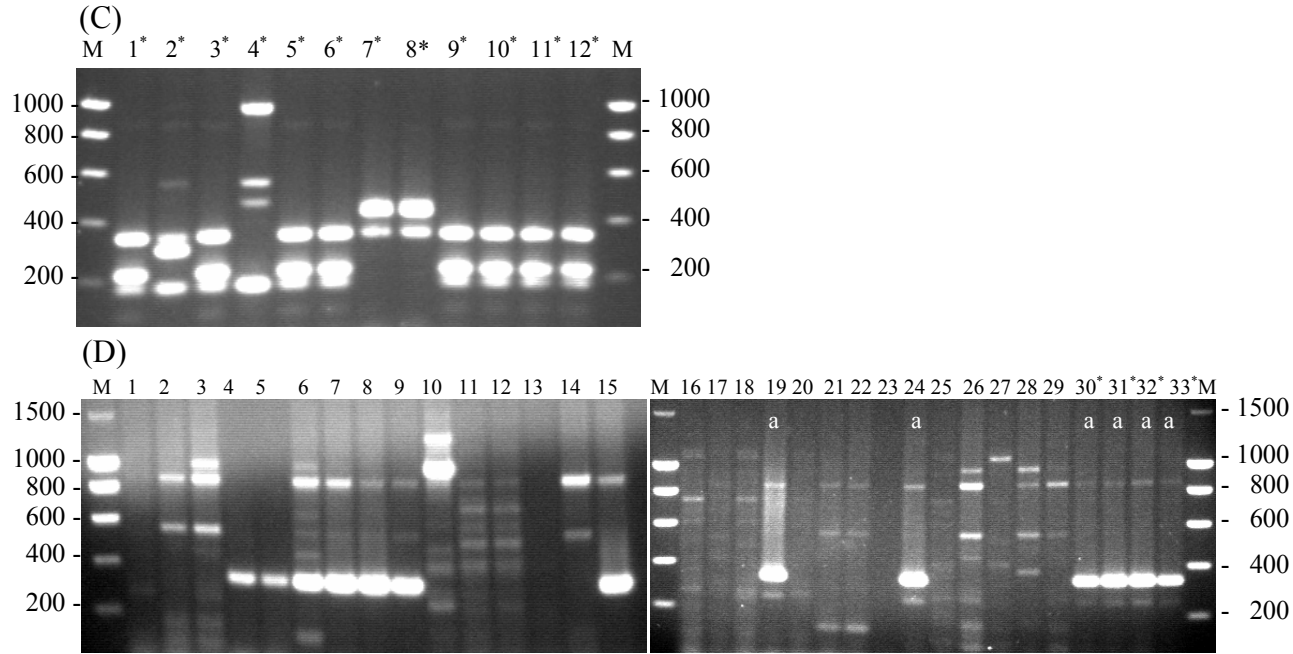


Fig. 1. ERIC-PCR fingerprints of *Arcobacter* isolates; M: DNA marker (Smartladder, Eurogentec); lane numbers labeled with asterisk * are of isolates from intestinal tracts; lanes marked with the same sign of a, c, o, or x show similar fingerprints among isolates from carcasses and intestinal samples; A1: *A. cryaerophilus* (lanes 1-31) and *A. skirrowii* (lanes 32-34), and A2: *A. butzleri* isolates from flock 1; B: *A. cryaerophilus* (lanes 1-30) and *A. butzleri* (lanes 31-34) isolates from flock 2; C: *A. butzleri* isolates gut contents from flock 3; D: *A. butzleri* isolates from broilers.

Table 3. Assay on survival of *Arcobacter* isolates at scalding conditions

	Log ₁₀ CFUs per ml (52°C/ 3 minutes)		Log ₁₀ CFUs per ml (58°C/ 3 minutes)	
	Inoculum	Survival	Innoculum	Survival
<i>A. skirrowii</i> 9C1 (hen caecal isolate)	6.6	4.1	6.4	2.7
	5.4	1.6	5.4	ND
	4.4	ND	4.4	ND
<i>A. cryaerophilus</i> K21a (hen carcass isolate)	6.1	3.1	6.1	2.8
	5.1	4.4	5.1	2.1
	4.6	2.0	4.1	ND
<i>A. cryaerophilus</i> K141b (hen carcass isolate)	6.4	5.8	6.4	3.3
	5.4	3.9	5.4	1.9
	4.3	2.4	4.4	ND
<i>A. butzleri</i> K21c (hen carcass isolate)	6.5	5.6	6.6	3.9
	5.6	4.2	5.6	2.8
	4.6	1.9	4.6	ND
<i>A. butzleri</i> K429a (broiler carcass isolate)	6.1	4.3	6.8	ND
	5.8	2.2	5.8	ND
	4.8	1.8	4.8	ND

ND: Not detected

It can also occur during scalding, defeathering and evisceration through contaminated equipment, air and personal(1; 5; 8; 12; 23; 24). The results in the present study indicated that these transmission routes seem the same for arcobacters. *Arcobacter* spp. are found on equipments along the processing line and in processing water: water in the scalding tank, water outlets of the defeathering machine and washers (11; 15). The bacteria were also isolated from the transportation crates used by flocks from which no *Arcobacter* species were detected in their caecal contents (15). In our study, the bacteria were present in dripping water from carcasses just leaving the scalding tank (before entering the defeathering stage), after defeathering and before going to the chilling room. The survival test on some *Arcobacter* isolates at a scalding temperature of 52°C in tap water for 3 minutes indicated that a proportion of the *Arcobacters* that contaminated the scalding water can survive these conditions and cause cross-contaminating within and between flocks in the scalding tank and in later processing stages.

The recovery of *Arcobacter* species in live birds or intestinal contents depends on sampling size and place. In most studies in the literature only a small numbers of samples were taken (3; 11; 30). The sampling site also appears to be important. In the present study, *Arcobacter* spp. were detected more often in ileal samples than in caecal samples. For salmonellae, it has been demonstrated that different results can be obtained by samplings at different parts of the chicken intestines (25). The survival of campylobacters in parts of the intestines is depending on the ecology of

that part of the intestines. It has been indicated that caeca and cloaca are the primary colonisation sites of *Campylobacter* spp. in chicken intestines (2; 4). *Arcobacter* species being aerotolerant could favour the ileum instead of the more anaerobic caeca.

Finally, the prevalence of *Arcobacter* spp. could be underestimated due to difficulties in isolation of these bacteria. In particular, the intestinal flora harbours larger numbers of enteric bacteria and fungi and yeasts compared to carcasses. In the present study *Arcobacter* spp. could not be isolated without filtration, due to overgrowth of other enteric microorganisms. *A. skirrowii* was detected in some samples by m-PCR but could not be isolated. The co-existence of more than one species in a sample was shown at higher rates by m-CR than by isolation. 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 (9; 10). However, as shown in Table 1 the PCR method sometimes also failed to detect the bacteria in enrichment samples. Since these samples also yielded negative results for the PCR using eubacterial primers, the presence of inhibiting factors in the samples can be assumed.

The results from this present study clearly indicate that *Arcobacter* spp. are imported into slaughterhouses by the gut contents of chickens. Processing in the slaughterhouses seems to be responsible for the spread of *Arcobacter* species from the intestines to carcasses. The supply water does not seem to play a role in introducing arcobacters in a slaughterhouse. The observed large genetic diversity of *Arcobacter* spp. present on carcasses can be explained by cross-contamination between chickens of one flock and between chickens of different flocks.

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Chapter 5

Interaction of *Arcobacter* spp. with human and porcine intestinal epithelial cells

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Abstract

Little is known about the pathogenic mechanisms or potential virulence factors of *Arcobacter* spp. The aim of the study described here was to obtain more insights in the pathogenicity mechanisms of *Arcobacter* spp. by testing their ability to adhere to, invade and induce interleukin-8 expression in human Caco-2 and porcine IPI-2I cell lines. Eight *Arcobacter* strains were tested. Four strains were obtained from a culture collection, and represent the four *Arcobacter* spp. known to be associated with animals and humans. The other four strains were field isolates from the amniotic fluid of sows and from newborn piglets. All eight *Arcobacter* strains were able to adhere to both cell lines, and induced interleukin-8 production as early as 2 hours after a 1-hour incubation period. This production was still increased 6 hours post-infection. Differences in the cell association of the eight strains were obvious, with *A. cibarius* showing the highest adhesion ability. Invasion of intestinal epithelial cells was only observed for *A. cryaerophilus* strains. No correlation between invasiveness or strong adhesion of the tested strains and the level of interleukin-8 induction was observed.

Introduction

The genus *Arcobacter* was introduced into the family *Campylobacteraceae* in 1991 (32). Currently, the genus *Arcobacter* comprises six species. *Arcobacter butzleri*, *A. cryaerophilus* and *A. skirrowii* have been isolated from humans (31; 40), nonhuman primates (1), and other animals (chickens, pigs, cattle, horses, and sheep) (13; 30). These bacteria have also been recovered worldwide from chicken, pork, and beef (3; 14; 22; 36). In 2005, a new species, *A. cibarius*, isolated from chicken carcasses, was added to the genus (11). Two other species were isolated in the environment: *A. nitrofigilis*, a free-living nitrogen-fixing organism (19), and *A. halophilus*, isolated from a hypersaline lagoon (6).

Recently, there has been public concern about *Arcobacter* as a potential zoonotic foodborne and waterborne pathogen (2; 25). Besides the abundance of *Arcobacter* spp. in meat, *A. butzleri* has recently been isolated from raw milk samples (28). Associations of *A. butzleri*, *A. skirrowii* and *A. cryaerophilus* with illness in humans and animals have been reported (5; 18; 33; 39). These three species were detected in stool samples of hospital patients with gastrointestinal disorders and in healthy children (27). *Arcobacter butzleri* was found to be the fourth most common *Campylobacter* or *Campylobacter*-like organism isolated from human diarrheic stool specimens (34). The isolation of *A. butzleri* from a neonate made *in utero* infection seem likely (24).

However, despite reports of its association with human and animal diseases, knowledge on the pathogenicity of *Arcobacter* is still very limited. *In vivo* studies on the invasion and virulence of *Arcobacter* strains in 1-day-old piglets and in chickens several days of age have been performed (37; 38). The results varied with the bacterial strains, as well as with the species and breed of the animals. Besides reports on *in vitro* cytotoxicity (7; 12; 20; 35; 36), a few studies of the adhesive and invasive abilities of *A. butzleri* and *A. cryaerophilus* have been carried out in the HEp-2, Hela and INT 407 cell lines (4; 7; 20; 35).

The present study was performed to obtain more information on the pathogenicity of *Arcobacter* strains associated with human and animals by studying their capacity for adhesion to and invasion of human and pig intestinal cell lines (Caco-2 and IPI-2I, respectively). We also report the ability of these *Arcobacter* strains to induce expression of the proinflammatory cytokine interleukin-8 (IL-8), which is considered to be a major virulence factor in *Helicobacter pylori* and *Campylobacter* spp. (9).

Materials and Methods

Bacterial strains and culture

The experiments were carried out on four *Arcobacter* strains obtained from the LMG collection (Laboratory of Microbiology, Gent University, Belgium) – *A. butzleri* LMG 6620, *A. skirrowii* LMG 6621, *A. cryaerophilus* LMG 7537, and *A. cibarius* LMG 21996 – and four *Arcobacter* strains isolated on a pig farm in the Netherlands; one of these was *A. skirrowii*, and the three *A. cryaerophilus* strains could be genetically distinguished by pulsed-field gel electrophoresis (10). A *Salmonella enterica* strain was used as a control in the adhesion, invasion and IL-8 assays (29) (Table 1).

Table 1. Bacterial strains used in this study

Strains	Origin of isolates
<i>A. butzleri</i> LMG 6620	Blood sample of a 65-year old woman with cellulites
<i>A. skirrowii</i> LMG 6621	Feces of a lamb with history of persistent diarrhea
<i>A. cryaerophilus</i> LMG 7537	Aborted ovine fetus
<i>A. cibarius</i> LMG 21996	Chicken carcass
<i>A. skirrowii</i> 125	Field strain from sow amniotic fluid
<i>A. cryaerophilus</i> 141	Field strain from newborn piglet
<i>A. cryaerophilus</i> 112	Field strain from sow amniotic fluid
<i>A. cryaerophilus</i> 1038	Field strain from newborn piglet
<i>S. enterica</i>	Chicken, serotype Enteritidis 90-13-706 (ID-DLO, Lelystad, The Netherlands)

To prepare inocula for adhesion and invasion assays, *Arcobacter* strains from stock cultures were streaked onto blood agar plates (Brain Heart Infusion agar, Oxoid, supplemented with 5% horse blood) and incubated for 48 hours at 30°C under microaerophilic conditions (5% O₂, 10% CO₂, and 85% N₂). A single colony of each strain was transferred into Brain Heart Infusion broth (Oxoid) and cultured overnight at 30°C under microaerophilic conditions. The broth cultures were centrifuged (5 minutes, 6000 × g, 4°C) and resuspended in warm (37°C) plain Dulbecco's modified Eagle's medium (DMEM; see following section for a description of the medium). Overnight cultures of *S. enterica* in Luria–Bertani (LB) broth were diluted 1/100 in LB broth and grown at 37°C for 2 hours. Bacteria were collected by centrifugation (15 minutes, 1800 × g, at room temperature) and resuspended in warm plain DMEM.

Cell culture

Human enterocyte-like Caco-2 cells (ATCC HTB 37) were grown in DMEM (Flow Laboratories, Amstelslad BV, Amsterdam, the Netherlands) supplemented with 1% (v/v) nonessential amino acids, 50 µg/ml gentamicin, 10 mM NaHCO₃, 1.7 mM glutamine, 25 mM HEPES, and 20% (v/v) foetal calf serum. The IPI-2I cell line (ECACC 93100622) was established from the ileum of an adult boar (d/d haplotype) and immortalized by transfection with an SV40 plasmid (pSV3-neo) (15). IPI-2I cells were grown in the same supplemented DMEM as described above, except for the use of 10% (v/v) foetal calf serum and the addition of 0.024 IU/ml bovine insulin (Sigma). Both cell lines were grown at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The plain DMEM used in the experiments is referred to as supplemented medium without gentamicin and foetal calf serum. Caco-2 and IPI-2I cells were seeded into 12-well plates at 4×10^4 and 5×10^4 cells/cm², respectively. Differentiated Caco-2 cells were obtained after 19 days of culture (5×10^5 cells/cm²). A time-course experiment was carried out to determine the confluency stage of IPI-2I cells as described below. At least 1 hour before adhesion and invasion assays were started, the monolayers were washed twice with 1 ml of warm plain DMEM. Plates were then incubated in 1 ml of warm plain DMEM per well until the assays of bacterial infection started. All incubation steps of the following assays were carried out at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Characterization of the IPI-2I cell line

To determine the growth curve of the IPI-2I cells, a culture was seeded in six-well plates (5×10^4 cells/cm²) and followed for 4 weeks. Three times a week, the culture medium was collected and total cell protein was measured with the BCA™-Protein Assay Kit (Pierce). Cell numbers were counted by microscopy using haemocytometers. IL-8 production was measured in the supernatants (as described below). The experiment was performed in triplicate with one particular passage of the cell line.

Adherence and invasion assays

The assays were performed essentially as described by (17). Briefly, the two cell lines were infected with 1 ml of culture of each bacterial strain for 1 hour. On the basis of a cell-associated saturation study with *C. jejuni* and Caco-2 cells (26), $1\text{--}3 \times 10^9$ CFUs of *Arcobacter* strains were added per well for both adhesion and invasion assays. Inocula of the *S. enterica* strain were 10^8 CFUs per well (17; 29). To determine the number of cell-associated *Arcobacter* organisms (adhering and invading), the monolayers were washed three times with plain DMEM and lysed in 10% Triton-X in phosphare-buffered saline at room temperature for 5 minutes, in order to release the bacteria. The suspensions were serially diluted 10-fold, and

100 µl of each dilution was plated on BHI agar supplemented with 5% horse blood. The plates were incubated for 48 hours at 30°C under microaerophilic conditions. Lysates of cells infected by *S. enterica* were plated onto LB agar and incubated for 24 hours at 37°C. The numbers of cell-associated bacteria were calculated as total (adhering and intracellular) bacterial CFUs recovered per 10 Caco-2 or IPI-2I cells. In the invasion assay, the monolayers in the wells were washed once with 1 ml of warm plain DMEM after 1 hour of incubation with bacteria, and then incubated for 2 hours with 1 ml of 300 µg/ml colistin in warm plain DMEM to kill extracellular bacteria. Cells were washed three times with plain DMEM, and finally lysed in 1% Triton X-100. The number of intracellular bacteria was determined by plating as described above. Cell association and invasion assays were performed in triplicate per passage on two different cell passages.

Enzyme-linked immunosorbent assay for IL-8

Monolayers in 12-well plates were infected with bacteria and incubated for 1 hour as described above. The plates were washed once with 1 ml of warm plain DMEM. One microliter of 300 µg/ml colistin in warm plain DMEM was added, and the plates were incubated for 2 hours. In order to monitor the kinetics of IL-8 induction vs. incubation time, the monolayers in the wells were incubated for 2, 6 or 24 hours with *A. butzleri* LMG 6620, *A. skirrowii* LMG 6621, *A. cryaerophilus* LMG 7537 and *S. enterica*, respectively. The medium was then collected for testing of IL-8 production. The cytokine concentration was determined using the IL-8 Cytosets™ antibody pair kit containing matched, pretitered and fully optimized capture and detection antibodies, and recombinant standard and streptavidin–horseradish peroxidase's (Biosource Europe SA, Nivelles, Belgium). The assay was performed following the manufacturer's instructions.

Scanning electron microscopy (SEM)

Both cell lines were seeded on plastic slides in 12-well tissue culture plates. Differentiated cells were incubated with the various *Arcobacter* strains for 1 hour. The cells were then washed three times with plain DMEM and fixed with Karnovsky's glutaraldehyde fixative for more than a week. After being washed with 0.1 ml of cacodylate buffer (pH 7.4), the samples were post-fixed with 2% OsO₄ in 0.1 ml of cacodylate buffer (pH 7.4) for 2 hours. The specimens were dehydrated in serial solutions of 50%, 70%, 80%, 96% and 100% acetone, and then critical-point-dried using a Bal-Tec CPD 030 system. Finally, the slides were coated with platinum to a thickness of about 10 nm, and examined by SEM (Phillips XL 30 SFEG).

Statistical analysis

One-way ANOVA and Tukey's multiple comparison tests were used to analyze differences in cell association and IL-8 induction among *Arcobacter* strains (using Minitab version 12.32). Repeated-measures analysis was performed for the monitoring of IL-8 induction vs. incubation time (SPSS version 10.0.5). A confidence level of 95% was defined for these analyses.

Results

Growth curve of the IPI-2I cell line

The growth and the confluency of the IPI-2I cells were determined over a 28-day period of culturing. Confluent monolayers were reached on day 5, as judged by microscopic examination. Both the cell number and the total cell protein of the IPI-2I cells increased initially but levelled off after about 7 days of culture (10^7 cells per well were obtained with a total protein content of 1 mg per well). On the basis of these results, IPI-2I cells were grown for 5 days to obtain confluent monolayers of $0.75\text{--}1.00 \times 10^6$ cells/cm² to be used in the adherence and invasion assays.

Cell association and invasion

All eight *Arcobacter* strains demonstrated the ability to adhere to Caco-2 and IPI-2I cells Table 2. Scanning electron micrographs of *Arcobacter* organisms adhering to the epithelial cells are shown in Fig. 1. In general, the association of each strain with both cell lines was comparable. Tukey's pairwise comparison indicated a significantly stronger association of the *A. cibarius* strain with both Caco-2 and IPI-2I cells than that of the other *Arcobacter* strains (more than 10 bacteria per Caco-2 cell). The capacity of *A. skirrowii* strains to adhere to both cell lines appeared to be lower than that of the other strains, although a statistically significant difference was only detected in comparison with the *A. cibarius* strain. Of the eight *Arcobacter* strains, only *A. cryaerophilus* LMG 7537 invaded both human and pig epithelial cells Table 2. *Arcobacter cryaerophilus* 1038 was the only porcine strain that exhibited an ability to invade Caco-2 cells, although it did not invade IPI-2I cells. When the assay was repeated, this capacity was lost.

Table 2. Interaction of *Arcobacter* strains with Caco-2 and IPI-2I cells^a

Strains	Cell-association ^b		Invasion ^b	
	Caco-2	IPI-2I	Caco-2	IPI-2I
<i>A. butzleri</i> LMG 6620	49 ± 15	38 ± 20	No invasion	No invasion
<i>A. skirrowii</i> LMG 6621	18 ± 12	8 ± 5*	No invasion	No invasion
<i>A. cryaerophilus</i> LMG 7537	53 ± 15	56 ± 12	237 ± 179	44 ± 20
<i>A. cibarius</i> LMG 21996	123 ± 26**	78 ± 49*	No invasion	No invasion
<i>A. skirrowii</i> 125	18 ± 10	11 ± 10*	No invasion	No invasion
<i>A. cryaerophilus</i> 141	28 ± 26	32 ± 29	No invasion	No invasion
<i>A. cryaerophilus</i> 112	18 ± 13	42 ± 38	No invasion	No invasion
<i>A. cryaerophilus</i> 1038	42 ± 37	49 ± 42	130 ± 53 ^c No invasion ^d	No invasion
<i>S. enterica</i>	68 ± 25	54 ± 5	265 ± 93	49 ± 25

^a The numbers are presented as mean ± standard deviation of bacterial CFUs.

^b Data are presented as bacterial CFUs per 10 Caco-2 or IPI-2I cells in cell association and CFUs per 10⁴ Caco-2 or IPI-2I cells in invasion.

^{c, d} *A. cryaerophilus* 1038 showed invasive ability into Caco-2 cells in the first assay ^(c) but did not invade in the repeated assay ^(d).

** Tukey's test demonstrated significantly higher cell-associated levels of the *A. cibarius* strain to Caco-2 than those of the other arcobacter strains ($P < 0.001$).

* Significant differences were observed between *A. cibarius* LMG 21996 and the two *A. skirrowii* strains ($P < 0.01$).

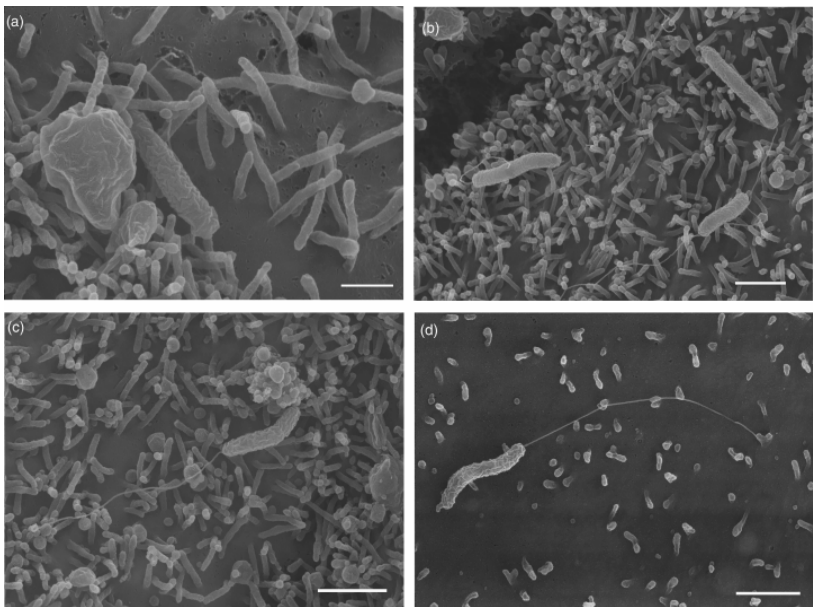


Fig. 1. Scanning electron micrographs of cell-associated *Arcobacter*: (a, b), *Arcobacter cryaerophilus* LMG 7537 on Caco-2; (c), *Arcobacter skirrowii* LMG 6621 on Caco-2; (d) *Arcobacter skirrowii* LMG 6621 on IPI-2I. Scale bar = 0.5 µm (a) or 1 µm (b, c, d).

IL-8 production

All *Arcobacter* strains tested induced IL-8 production in both Caco-2 and IPI-2I cells Fig. 2. Two hours post-infection, the up-regulation of IL-8 production by Caco-2 induced by *Arcobacter* strains was lower than that induced by the control *Salmonella* strain (Fig. 2a). *A. butzleri* LMG 6620 isolated from a female patient caused significantly higher induction of IL-8 in Caco-2 cells than the other *Arcobacter* strains. Pairwise comparison showed that, among the LMG strains, *A. cibarius* induced Caco-2 to produce the lowest levels of IL-8. The porcine isolates were seen to up-regulate IL-8 secretion at higher levels in IPI-2I than the LMG strains, especially *A. cryaerophilus* 141, for which the IL-8 measures were statistically higher than those of *A. butzleri* LMG 6620, *A. skirrowii* LMG 6621, and *A. cryaerophilus* LMG 7537. It should be noted that cytokine expression by IPI-2I cells varied remarkably between the different experiments, and even among the triple incubations of one experiment (as demonstrated by the high SD).

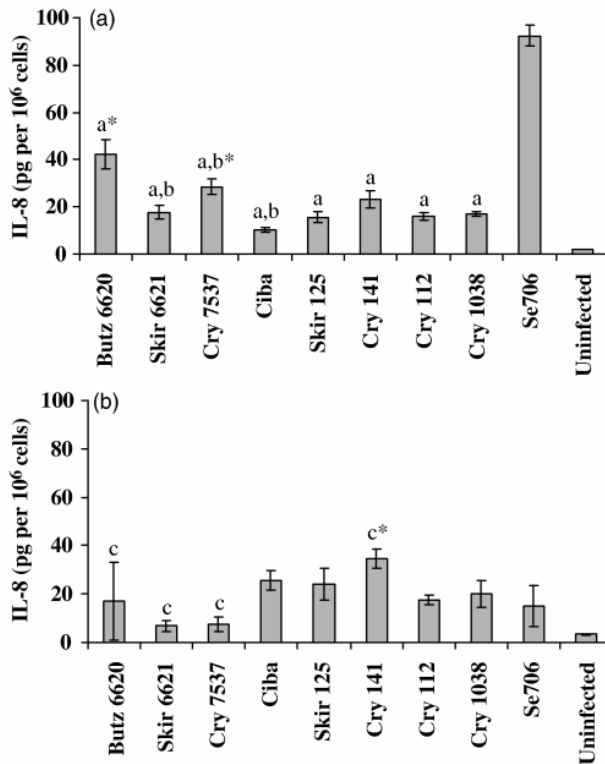


Fig. 2. IL-8 production by Caco-2 (a) and IPI-2I (b) cells 2 hours after a 1-hour exposure to *Arcobacter* strains (error bar = SD). Butz, *Arcobacter butzleri*; Skir, *Arcobacter skirrowii*; Cry, *Arcobacter cryaerophilus*; Ciba, *Arcobacter cibarius*; Se706, *Salmonella enterica*, used as a control; Uninfected, samples without exposure to bacteria. Statistical indicators: a*, b* and c* indicate significant differences ($P<0.05$) compared to (a), (b) and (c), respectively.

A kinetic study of IL-8 secretion by Caco-2 and IPI-2I cells upon bacterial stimulation was done with the three LMG strains by measuring IL-8 production at 2, 6 and 24 hours after a 1-hour incubation with bacteria. The analysis indicated significant increases in IL-8 measures for both cell lines at these time points. Accumulation of IL-8 in the medium of infected Caco-2 cells increased for up to 24 hours after bacterial challenge, whereas IL-8 production by uninfected cells could hardly be detected Fig. 3a. At 2-hour post-infection, IL-8 production by Caco-2 cells induced by *A. skirrowii* LMG 6621 was lower than that induced by *A. butzleri* LMG 6620 and *A. cryaerophilus* LMG 7537. Nevertheless, the IL-8 concentrations in the 6- and 24-hour samples of *A. skirrowii* were higher than those for the two other strains. However, the difference was not statistically significant. During the first 6-hour post-exposure, the IL-8 contents in the medium of infected IPI-2I cells rose remarkably, whereas those of the untreated cells remained at low levels Fig. 3b. However, at 24-hour post-exposure, IL-8 accumulation in all samples, including those from uninfected cells, was still rising.

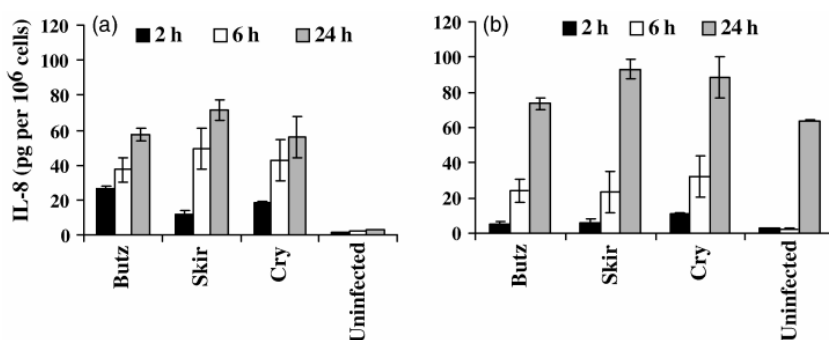


Fig. 3. IL-8 production by Caco-2 (a) and IPI-2I (c) cells 2, 6 and 24 hours after a 1-hour exposure to *Arcobacter* strains (error bar = SD). Butz, *A. butzleri* LMG 6620; Skir, *A. skirrowii* LMG 6621; Cry, *A. cryaerophilus* LMG 7537; Uninfected, samples without exposure to bacteria.

Therefore, a kinetic study of IL-8 secretion by IPI-2 cells over time without bacterial infection was performed (in triplicate of one cell passage). It was shown that without bacterial infection, IPI-2I cells expressed IL-8, and the cytokine content in 2–3-day-old medium ranged from 1.22 to 1.57 ng per 10⁶ cells.

Discussion

As pigs are hosts and important reservoirs for a number of zoonotic pathogens, including *Arcobacter* species, we intended in this study to further characterize porcine IPI-2I intestinal epithelial cells and to investigate their use as a model in the study of swine-associated pathogens. Although the cells possess microvilli,

they are poorly differentiated (Fig. 1d); this is related to the lack of villin expression (15). In comparison to the well-established human Caco-2 cell line, IL-8 production by porcine IPI-2I cells in response to bacterial infection varied among cells in different wells seeded from the same cell culture of one particular passage. According to the authors, who established this cell line, IPI-2I cells are genetically unstable, and this can lead to phenotypic variation and heterogeneity. Importantly, IL-8 production was observed after prolonged incubation of uninfected cells. Apparently, the cells were under some kind of stress that may have come from the growth conditions or some component(s) in the growth medium, although the medium was prepared as indicated by the supplier. Therefore, in order for the IPI-2I cell line to be used as a model in the study of porcine-associated intestinal pathogens, more research is needed to optimize this cell line for this purpose.

Two studies have been reported on the adhesive ability of *A. butzleri* strains isolated from environmental (sea water and river water) samples (4; 20). In one, only one of 18 strains adhered to Hela and INT 407 cells (20). In the other, only one-third of the strains (six of 17) tested were found to be adhesive to HEp-2 and Hela cells (4). However, all 12 *A. butzleri* strains isolated from human stool specimens were able to adhere to HEp-2 cells (35). Similarly, all eight strains of the four *Arcobacter* species tested in the present study showed the ability to adhere to both human Caco-2 and porcine IPI-2I cells. Probably, the different results obtained in studies of the adhesive ability of *Arcobacter* strains may be partly attributed to the origin of the strains, i.e. human- or animal-associated strains vs. environmental isolates. The different cell lines used and the assays employed could also influence the outcome. In the present study, a well-established adhesion and invasion protocol was used on Caco-2 and IPI-2I cells. With this protocol, live adhering bacteria are counted, using an adhesive and invasive wild-type *S. enterica* strain as a control.

In the study of *A. butzleri* strains isolated from river water (20), none of the 18 tested isolates invaded Hela and INT 407 cells. Nevertheless, four of 12 *A. butzleri* strains isolated from human stools were able to invade HEp-2 cells (35). Of eight *Arcobacter* strains tested in the present study, only two strains of *A. cryaerophilus* showed the ability to invade the human Caco-2 cell line. The ability of *A. cryaerophilus* strains isolated from an aborted bovine foetus and from swine faeces to invade HEp-2 cells was demonstrated (7). Evidently, *A. cryaerophilus* strains are more invasive than those of other *Arcobacter* spp., which allows them to penetrate the porcine intestinal tissue and placenta and to invade foetuses (10). However, upon repeated testing, the invasiveness of the porcine *A. cryaerophilus* strain 1038 was lost. Apparently, *in vitro* subculture of the strain had resulted in a change in virulence; this has also been reported for other pathogenic bacteria, including *Helicobacter* and *Campylobacter* (8; 16; 21; 23).

Up-regulation of IL-8 expression by Caco-2 and IPI-2I cells was seen by 2 hours after infection with all of the tested *Arcobacter* strains. However, the up-regulation in Caco-2 cells was less than that induced by the control *Salmonella* strain. The results showed no correlation between cell invasion or level of adhesion and IL-8 induction by the tested *Arcobacter* strains. Thus different species and strains of *Arcobacter* may possess different virulence mechanisms (36). This is not unlikely, as the tested strains were isolated under different circumstances and from different host species. The *A. cryaerophilus* strains used in the present study were isolated from an aborted foetus (*A. cryaerophilus* LMG 7537), amniotic fluid samples and newborn piglets, for which a vertical transmission route has been demonstrated (10). Therefore, the ability to invade epithelial cells would be a prerequisite for these *Arcobacter* strains. On the other hand, for strains isolated from a case of persistent diarrhoea, e.g. *A. skirrowii* LMG 6621, intestinal association and the ability to trigger local inflammation but not invasion would be more appropriate for their successful survival. It is difficult to draw conclusions on the correlation between invasion ability, level of cell association and level of IL-8 induction of the tested *Arcobacter* strains.

Finally, our study clearly demonstrated that all of the tested strains of the four human- and animal-associated *Arcobacter* species possess the ability to adhere to and to induce IL-8 production by human Caco-2 and porcine IPI-2I intestinal epithelial cells. Two *A. cryaerophilus* strains, isolated from an aborted ovine fetus and a newborn piglet, were able to invade both cell lines.

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Chapter 6

***Arcobacter* spp. possess two very short flagellins of which FlaA is essential for motility**

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Submitted for publication

Abstract

The genus *Arcobacter* belongs to the epsilon subdivision of the Proteobacteria that comprises both human- and animal-associated as well as free-living motile bacteria living in a wide range of habitats. The aim of this study was to characterize the flagellin proteins of *Arcobacter* spp., since these proteins are known virulence factors in other epsilonproteobacteria. Like *Campylobacter* and *Helicobacter* spp., *Arcobacter* spp. possess two flagellin genes (*flaA* and *flaB*) located adjacent to each other. With the exception of *A. nitrofigilis*, arcobacter flagellins are almost half the size of those in other epsilonproteobacteria. *Arcobacter* flagellin proteins lack a large part of the variable central region. The low homology observed among flagellins of different *Arcobacter* species indicates genetic heterology between the members of this genus. Unlike in other epsilonproteobacteria, the transcription of flagellin genes is not regulated by σ^{28} - or σ^{54} -dependent promoters, which suggests that transcription might be regulated in a different way in *Arcobacter* spp.. Mutational study revealed that only FlaA is needed for the motility of *Arcobacter* spp.. Quantitative PCR analysis showed that transcription of *flaB* was higher at 30°C than at 37°C. Mutation of *flaB* had no effect on motility or on *flaA* transcription while mutation of *flaA* abolished motility and increased the transcription of *flaB*. These results underline that the genus *Arcobacter* is an unusual taxon in the epsilon subdivision of the Proteobacteria.

Introduction

Based on rRNA gene analysis *Arcobacter nitrofigilis* and *A. cryaerophilus* have been placed in a separated cluster of the rRNA superfamily VI in 1991 apart from *Campylobacter* spp., *Helicobacter* spp. and *Wolinella succinogenes* (45). The genus *Arcobacter* was assigned comprising two species: *A. nitrofigilis* and *A. cryaerophilus*, of which the former is the type species. *Arcobacter* spp. are Gram-negative, non-spore-forming rods, generally 0.2-0.9 μm wide and 0.5-3 μm long. They are motile and have one unsheathed single polar flagellum at one or both ends of the cells (44). At the moment the genus contains six species which inhabit extremely diverse environments. *A. nitrofigilis* is present in the roots of a marsh plant (24) and *A. halophilus* was isolated from a hypersaline lagoon (6). Other *Arcobacter* species were found in association with humans and animals: *A. cibarius* was isolated from chicken carcasses (15); *A. butzleri*, *A. skirrowii* and *A. cryaerophilus* were found in food from animal origin (19; 37), in the stool of healthy as well as sick animals and humans (2; 18; 31; 46; 47). This mixture of species with very different habitats makes *Arcobacter* an “unusual” (44) and probably heterogeneous genus.

Several studies on adhesion, invasion, and cytotoxicity of *A. butzleri* and *A. cryaerophilus* have been performed, but the knowledge on their pathogenic potential still is limited (14). In fact there are only a few reports on the molecular properties of the genus. Genome mapping of an *A. butzleri* strain has shown that this strain has a larger genome than the other members of the Epsilonproteobacteria (39). Despite being a member of *Campylobacteraceae* the draft genome of an *A. butzleri* strain reveals the highest similarity to *W. succinogenes* (26).

Much research concerning *Campylobacter* and *Helicobacter* spp. has been done on the flagellum. Bacterial flagella are involved in cell motility and chemotaxis. Their role in colonization and invasion of host cells has been extensively studied. Flagellin, the subunit of the flagellar filament is one of the best characterized protein antigens of *Campylobacter* spp. (10). Flagellins are important virulence factors and a primary target for the immune system (8; 33). Apart from studies on their role in pathogenesis and their immunological properties, flagellins and the genes encoding them have been used to develop molecular methods for bacterial identification and diagnostics (50). Usually the flagellar filament is constituted of a single type of flagellin. However, some bacteria flagella contain several different flagellin subunits (50). In *Campylobacter* spp., *Helicobacter* spp., and *W. succinogenes*, there are two homologous flagellin genes of which one is predominantly expressed. The regulation of these genes and the significance of their co-existence is still an important subject of research.

Here, the genes encoding flagellins and their expression in several *Arcobacter* strains are described.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

All bacterial strains and plasmids used are listed in Table 1. *Arcobacter* strains were grown in BHI broth (Brain Heart Infusion, Oxoid) under shaking conditions or on BHI agar supplemented with 5% horse blood and incubated overnight or for 48 hours, respectively, at 30°C under microaerophilic conditions (BD CampyPak™, Becton, Dickinson and Company) unless special growth conditions were required (see below). *Escherichia coli* strains were grown in LB (lysogeny broth) broth or agar at 37°C (5). When antibiotic selection was required, ampicillin (100 µg/ml) or chloramphenicol (25 µg/ml) was added.

Table 1. Bacterial strains and plasmids used in this study

Bacteria and plasmids	Origin/function	Source
Bacterial strains		
<i>A. butzleri</i> LMG 6620	Blood sample of a woman with cellulites	BCCM*
<i>A. butzleri</i> UU-K32	Chicken carcass	Laboratory collection
<i>A. skirrowii</i> LMG 6621	Feces of a lamb with persistent diarrhea	BCCM
<i>A. cryaerophilus</i> LMG 7537	Aborted ovine fetus	BCCM
<i>A. cibarius</i> LMG 21996	Chicken carcass	BCCM
<i>A. nitrofigilis</i> LMG 7604	Isolation from the root of a mash plant	BCCM
<i>E. coli</i> DH5α, NCCB 2955	Competent cells for cloning	NCCB**
Plasmids		
pGEM-T Easy	Cloning vector	Promega
pAV35	Plasmid with chloramphenicol resistance (Cm ^r) gene	van Vliet et al. (1998)

* BCCM: The Belgian Co-ordinated Collections of Microorganisms.

** NCCB: The Netherlands Culture Collection of Bacteria.

Isolation of flagellin proteins

To estimate the size and to isolate the *Arcobacter* flagellins, crude flagellin proteins were extracted by the acid pH disaggregation method (16). Overnight broth cultures were harvested by centrifugation at 5000 × *g* for 30 minutes at 20°C. The pellet was resuspended in 0.9% NaCl. The suspension was adjusted to pH 2.0 with 1M HCl and centrifuged for 15 minutes at 8000 × *g* at 4°C. After ultra-centrifugation for 1 hour at 100000 × *g*, at 4°C, the supernatants were adjusted to pH 7.2 with 1M NaOH and proteins were precipitated with ammonium sulfate. The samples were dialysed against running tap-water for 2 hours and next against

distilled water containing activated charcoal for 18 hours at 4°C. Samples were stored at -70°C for further analysis.

Polyacrylamide-SDS gel electrophoresis and N-terminal amino acid sequencing

N-terminal amino acid sequencing was performed on the putative flagellin extracted from *A. butzleri* strain UU-K32. The crude flagellin samples were electrophoresed on 12% polyacrylamide SDS gel. After blotting the proteins from the gel onto nitrocellulose membrane (Schleicher & Schuell, BioScience) overnight at 20 V, 4°C, using a Tank Transfer System (Bio-Rad) bands were visualized by Coomassie Brilliant Blue staining. The correct bands were excised, and sent to the Sequence Centre Utrecht (Faculty of Chemistry, Utrecht University, The Netherlands) for N-terminal amino acid sequence determination. This amino acid sequence was analyzed with BLAST (see below) and degenerated primers were designed in order to amplify a part of the gene encoding the flagellin.

Detection of protein glycosylation

The glycosylation of *A. butzleri* flagellin proteins was examined using the DIG Glycan Detection kit (Roche). To detect the presence of the N-glycosyl linkages the samples were treated with N-glycanase (Prozyme) and electrophoresed on 12% polyacrylamide SDS gel. The N-glycosylated surfactant protein D (SP-D) was used as a positive control. All assays were performed according to the manufacturers' instructions.

Determination of the nucleotide sequence of the genes encoding Arcobacter flagellin

The nucleotide sequence of *Arcobacter* flagellin genes was determined in three steps:

(i) *Degenerate PCR*: A degenerate forward primer based on the N-terminal amino acid sequence of the *A. butzleri* UU-K32 flagellin and degenerate reverse primers based on an alignment of the major flagellin genes in *Arcobacter*-related organisms (FlaA in *Campylobacter* and *Helicobacter* spp., FlaG in *W. succinogenes*) were designed and used in a PCR to generate an amplicon from the flagellin gene of *A. butzleri* UU-K32. The PCR product was sequenced. The deduced amino acid sequence was aligned with those in related bacteria to design a second set of degenerated primers for PCR to amplify the flagellin gene of other *Arcobacter* strains.

(ii) *Southern blot hybridization*: Southern hybridization was performed in order to determine the number of flagellin genes present in *Arcobacter* species. Total genomic DNA was restricted with the endonucleases *EcoR* I, *Hind* III or *Pst* I

(New England Biolabs). DNA fragments were transferred from agarose gels to Hybond-N+ membranes (Amersham Biosciences) by capillary blotting (38). The DNA was immobilized on the filters using a Stratalinker® UV Crosslinker (Stratagene).

Hybridization was performed overnight at 60°C with DIG-labeled probes. Species-specific probes were synthesized by standard PCR reactions with the PCR DIG Probe Synthesis kit (Roche), using primers that were designed based on the sequences generated with the degenerated PCR reactions in step (i) (Table 2). Colour was developed using an anti-DIG-alkaline phosphatase antibody and NPT/BCIP substrates (Roche). All steps were performed following the manufacturers' instructions.

(iii) *Sequence extending*: With the first fragment of each strain, obtained by the degenerated PCR in step (i), DNA walking was carried out in order to obtain the entire gene(s) sequence using the TOPO® Walker Kit (Invitrogen). Total genomic DNA was digested with the restriction enzyme *Pst* I or *Nsi* I (New England Biolabs) that leave a 3'- overhang and nucleotide sequence extension was performed according to the manufacturer's instructions.

Cloning for nucleotide sequencing

To determine the nucleotide sequences, PCR amplicons were ligated into the pGEM-T Easy vector (Promega) and the plasmids were transformed into *E. coli* DH5 alpha (Table 1). The transformation was performed either by electroporation or by heat-shock method depending on the length of the insert. Plasmids with inserts were purified (Miniprep, QIAGEN) and sent to BaseClear (The Netherlands) for nucleotide sequence determination.

Preparation of A. butzleri LMG 6620 competent cells

A. butzleri LMG 6620 competent cells were prepared for transformation as described for *C. jejuni* (49) with some modifications. An overnight culture was diluted (1:100) in pre-warmed (30°C) BHI broth and incubated for 4 hours under shaking conditions. Bacterial cells were harvested by centrifugation for 3 minutes at $11000 \times g$ at 4°C. Cell pellets were gently resuspended in ice-cold glycerol water (15% glycerol and 7% sucrose) and centrifuged for 3 minutes at $11000 \times g$ (4°C). This washing step was repeated twice. Finally, cells were resuspended in ice-cold glycerol water and left on ice for 1.5 hours before electroporation. Forty µl of competent cells (containing approximately $3\text{-}5 \times 10^9$ colony-forming units - CFUs) was used for each transformation.

Construction of flagellin mutants

Mutants in the flagellin genes of *A. butzleri* LMG 6620 were generated using the allelic exchange method (51). The target gene was amplified and the amplicon was ligated into the pGEM-T Easy vector. The plasmids containing an insert were linearised by a reverse amplification using primers with a *Bam*H I site at the 5' end in order to introduce a unique *Bam*H I restriction site into the PCR products. The linear plasmid was digested with *Bam*H I (New England Biolabs) and dephosphorylated with calf intestine alkaline phosphatase (Fermentas). Finally, the dephosphorylated plasmid was ligated to an 850 bp fragment containing the chloramphenicol resistance gene (*Cm^r*) obtained by digesting the pAV35 plasmid (43) with *Bam*H I. All primers used to construct mutants are shown in Table 2.

Table 2. Primers used for synthesis of hybridization probes, construction of *fla* mutants, and qPCR

Primer	DNA sequences (5'to 3')	Primer	DNA sequences (5'to 3')
Synthesis of probes (Southern hybridization)		Quantitative PCR	
Probe-F	agcagcagatgatgcttctgg	Arco- <i>gyrA</i> -F	gaaagaaccagcaggttctctctaca
Probe-R-BUTZ	ttgattctctaccatcagctg	Arco- <i>gyrA</i> -R	cgccaagattatgaggtggaat
Probe-R-SKIR	ctgctggtagagttgctattg	6620- <i>fla</i> F1	gcagcaacaggacaaaactgaa
Probe-R-CRY	tcagctatttgtaaaagagcaactg	6620- <i>fla</i> R1	agctgattcaacttggttttgagtag
Probe-R-CIBA	tagcttctctaccatcagcag	6620- <i>fla</i> F2	tctttaacaggtttagcggtttca
		6620- <i>fla</i> R2	atatccattaagtgttgtagtgcaacta
Construction of <i>flaA</i> mutant		Construction of <i>flaB</i> mutant	
6620-MF1	tgagaattaatacaaacgtttcatctt	6620-MF2	agcagggtcttatgcgattagcc
6620-MR1	gagaacctaacttttgctaagac	6620-MR2	tggagcatttcttaatggagcaactcc
6620-BamHIF1	cgaggatccagtagcacatgttttcc*	6620-BamHIF2	cgaggatccgactaatacagcaggttggt*
6620-BamHIR1	cgaggatcctaccagaagttgcatcagc*	6620-BamHIR2	cgaggatcccgaagcagtagaagcagcacc*

* The *Bam*H I site is underlined

The constructed plasmid was introduced into *A. butzleri* LMG 6620 competent cells by electroporation with settings of 2.25 kV, 300 Ω , and 25 μ F. After stabilization of the bacterial cells in 100 μ l SOC medium (13) for 10 minutes at room temperature, the cells were transferred to 2 ml pre-warmed BHI broth and incubated for 3 hours at 30°C under microaerophilic and shaking conditions and spread onto blood agar plates containing 25 μ g/ml chloramphenicol. The plates were incubated for 2 days at 30°C under microaerophilic conditions. The resulting mutants with their target flagellin gene disrupted by double crossover events were verified by PCR.

Motility assay

To test for bacterial motility, individual colonies were spotted onto 0.4% thioglycolate plates (Fluka). The plates were incubated at 30°C and 37°C under microaerophilic conditions, and the growth and expansion of colonies was examined.

Electron microscopy

Overnight cultures were inoculated into pre-warmed (30°C) BHI broth (1:100) and incubated for 4 hours. Carbon-coated copper grids were floated for 5 minutes on top of 20 μ l drops of broth culture and then immediately stained for 1 second with 2% potassium phosphotungstate pH 7.0. The grids were examined with a Philips electron microscope CM10.

Quantitative PCR (qPCR) and study of flagellin gene expression

The transcription of flagellin genes in *A. butzleri* LMG 6620 at different growth temperatures (30°C vs 37°C) and at different oxygen concentrations (aerobic vs microaerophilic) was examined. Overnight cultures (grown at 30°C under microaerophilic conditions) of the wild type and mutant strains of *A. butzleri* LMG 6620 were diluted in pre-warmed BHI broth (1:100) and incubated at the experimental conditions to obtain OD₆₀₀ = 0.6-0.8 (mid-log phase). Total bacterial RNA was isolated using TRIzol[®] reagent (Sigma). For each sample, 3 μ g of total RNA was treated in 60 μ l with DNase I (Invitrogen) prior to qPCR amplification and consequently 4 μ l of treated RNA was used in 25 μ l qPCR reaction mixture. The qPCR was performed using the One step RT qPCR MasterMix Plus for SYBR[®] Green I kit (Eurogentec) on an ABI Prism 7000 sequence detection system (Applied Biosystems). All procedures were performed as instructed by the manufacturers.

Relative gene expression to a housekeeping gene- *gyrA* (gyrase A from *Arcobacter* strains of which sequences were obtained from the NCBI database) was calculated in order to compensate for the variance in the amount of mRNA in the reactions. The results are presented in mRNA-fold difference between two growth conditions (30°C vs 37°C; microaerophilic vs aerobic; wild type vs mutant) according to the $\Delta\Delta$ Ct method (32). All qPCR reactions were performed in triplicate.

Sequence and data analysis

Known amino acid sequences used for comparison of similarity and designing degenerated primers were obtained from National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>). Similarities between amino acid sequences obtained in this study and known sequences were determined using

The Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov/blast/>). Multiple sequence alignments were performed by ClustalW (<http://align.genome.jp/>). Phylogenetic trees were constructed using Mega 3.1 (21).

Nucleotide sequence accession numbers

Flagellin gene sequences of *Arcobacter* spp. in this study have been assigned Genbank accession numbers from EF667144 to EF667148 and EU056204.

Results

Identification of arcobacter flagellins

To isolate the *Arcobacter* flagellins an acid pH disaggregation method was used to obtain crude flagellin protein extracts. Only extracts from *A. butzleri* strains contained a major band upon polyacrylamide-SDS gel electrophoresis, with a molecular weight between 36 and 43 kDa.

Amino acid sequence determination of the purified flagellin protein from strain *A. butzleri* UU-K32 resulted in an N-terminal sequence of twenty nine amino acids: M R I N T N V S S L T A Q E A A V N T N K N I S S S L E K which resembled the N-terminal sequences of flagellin subunits from *Helicobacter* spp. and *W. succinogenes*.

Nucleotide sequence of arcobacter flagellin genes

To obtain the nucleotide sequence of the flagellin genes PCR reactions with degenerated primers were performed on *A. butzleri* UU-K32 using a forward primer based on the sequence A Q E A A V N T of the N-terminal sequence above and a reversed primer based on the sequence A Q A(S) G S Y A(S) of the conserved C-terminal sequence of the flagellins in *Campylobacter* spp., *Helicobacter* spp., and *W. succinogenes*. The reaction produced an amplicon of 840 nucleotides. TBLASTX analysis (search translated nucleotide database using a translated nucleotide query) of the nucleotide sequence of this amplicon demonstrated a resemblance with sequences coding for flagellins in the organisms mentioned above.

The deduced amino acid sequence of *A. butzleri* UU-K32 flagellin was aligned with flagellin sequences of closely related bacteria and a conserved sequence - A A D D A S G was chosen to design another degenerated forward primer which was used together with the previous degenerated reverse primers to amplify the nucleotide sequences encoding flagellins of other *Arcobacter* strains. This PCR

produced fragments of 678 to 786 nucleotides for all strains except *A. nitrofigilis* for which an amplicon of 1332 bp long was obtained, approximately twice as long as that the others. Again, TBLASTX analysis of the sequences obtained from these amplicons showed resemblance with sequences coding for flagellin subunits in *Campylobacter* spp., *Helicobacter* spp., and *W. succinogenes*. Based on the obtained sequences, primers were designed to synthesize species-specific probes and for gene walking.

To determine the number of flagellin genes present in *Arcobacter* species Southern blots were performed (data not shown). In the hybridization one band was visible in all DNA samples cleaved with *EcoR* I. Hybridization of DNA cleaved with *Hind* III showed two bands for *A. cryaerophilus* and one band the for other strains. Hybridization of DNA digested with *Pst* I showed two bands for *A. butzleri* and *A. skirrowii*. Only one band was detected for *A. cibarius* DNA restricted with either *Hind* III or *Pst* I. These results indicated the existence of at least two flagellin genes in *A. butzleri*, *A. skirrowii*, and *A. cryaerophilus* as seen for other members of the epsilon subdivision.

In order to obtain the entire gene sequence, the TOPO[®] gene walker kit was used for each strain. The walking process was repeated until the complete desired sequence was obtained. The primer set for the next walking was designed based on the sequence generated in the previous step. In this way the two adjacent *fla* genes for *A. butzleri*, *A. skirrowii*, *A. cryaerophilus*, and *A. nitrofigilis* were completely sequenced. In the case of *A. cibarius*, the walking process failed to yield the second gene due to difficulties with non-specific amplification.

Analysis of Arcobacter flagellin genes

Like in *Campylobacter* species, the two flagellin genes of *Arcobacter* species are orientated, head-to-tail separated by a non-coding region of 158 to 168 nucleotides, which is 217 nucleotides in *A. nitrofigilis*. The deduced amino acid sequences of arcobacter flagellins, with the exception of *A. nitrofigilis*, are half the size of those in *Campylobacter* spp. (Table 3). Promoter elements resembling conserved promoter sequences in other bacteria could not be identified from the sequences upstream of the start codon of the various genes, but a well-positioned ribosome-binding site was present.

The amino acid sequence homology between the two flagellin subunits varies between 80 to 90% in *A. butzleri*, *A. skirrowii*, and *A. cryaerophilus* strains and is 62% in *A. nitrofigilis*. The similarity of each subunit ranged from 73 to 79% among human/animal-associated arcobacter species and 61 – 72% between this group and *A. nitrofigilis*. Furthermore a relatively low homology (75%) was observed between the flagellins of two different *A. butzleri* strains.

Table 3. Similarity of the amino acid sequences between FlaA and FlaB subunits

Strains	Length (amino acid)		Homology between FlaA & FlaB
	FlaA	FlaB	
<i>A. butzleri</i> LMG 6620	283	282	84 %
<i>A. butzleri</i> UU-K32	307	307	90 %
<i>A. skirrowii</i> LMG 6621	300	302	81 %
<i>A. cryaerophilus</i> LMG 7537	316	316	90 %
<i>A. cibarius</i> LMG 21996	280	-	-
<i>A. nitrofigilis</i> LMG 7604	498	495	62 %
<i>C. jejuni</i> RM1221	573	573	95 %
<i>H. pylori</i> 26695	510	514	59 %
<i>W. succinogenes</i> DSM 1740	518	513	69 %

In *Campylobacter* species *flaA* and *flaB* are flanked by genes coding for motility accessory factors (*maf*). In *A. cryaerophilus* a gene encoding a plasmid stabilization system protein (RelE/ParE family) is located upstream of the *flaA* gene, while in *A. skirrowii* and *A. nitrofigilis*, the gene coding for RNA methyltransferase (TrmA family) was found. No homology was found for the 571 nucleotides upstream of the flagellin in *A. cibarius*.

Glycosylation of A. butzleri flagellins

The DIG Glycan Detection assay showed that the flagellin protein of *A. butzleri* strains were glycosylated. The assay with N-glycanase indicated that *A. butzleri* flagellin did not contain glycan N-linkages (data not shown).

Phenotypic characterization of the flaA and flaB mutants of A. butzleri LMG 6620

Mutants in *flaA* or *flaB* of *A. butzleri* LMG 6620 were obtained by insertion of a chloramphenicol resistance gene cassette in the opposite orientation to the *fla* genes.

It was noted that natural chloramphenicol resistance for *A. butzleri* LMG 6620 was present at a rate of 1 in $1-2 \times 10^6$. The natural resistance was still present when the concentration of the antibiotic in the medium was increased to 40 µg/ml. The same was observed for the other *A. butzleri* and *A. cryaerophilus* strains. The use of a kanamycin resistance gene for selection of mutants was contemplated but was disregarded since the same problems with natural kanamycin resistance as with chloramphenicol resistance was encountered. This phenomenon caused difficulties

in the selection of the correct mutant colonies. Therefore, one hundred colonies from each transformation (for either *flaA* or *flaB* disruption) were screened by PCR and subsequently three transformants for each were obtained, in which the target *fla* gene was interrupted by the Cm^r gene.

The motility of these mutants was examined on thioglycolate agar plates under different temperature and oxygen tension. *flaA* mutants were non-motile under all growth conditions (30°C and 37°C and aerobic and microaerophilic). In contrast, *flaB* mutants exhibited comparable motility to the wild type strain as seen on the agar plates under all growth conditions (Fig. 1). Electron microscopy showed that the *flaA* mutant did not possess a flagellum while the *flaB* mutant still produced a flagellum with a comparable length to that of the wild type strain (Fig. 2).

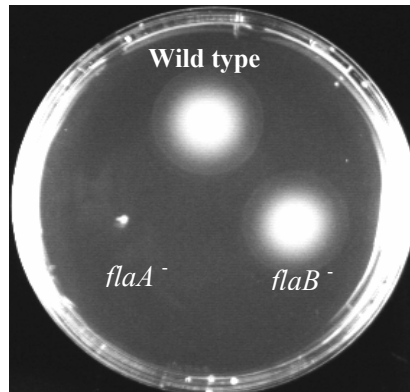


Fig. 1. Motility phenotypes of the wild type, *flaA*, and *flaB* mutants of *A. butzleri* LMG 6620 on thioglycolate medium.

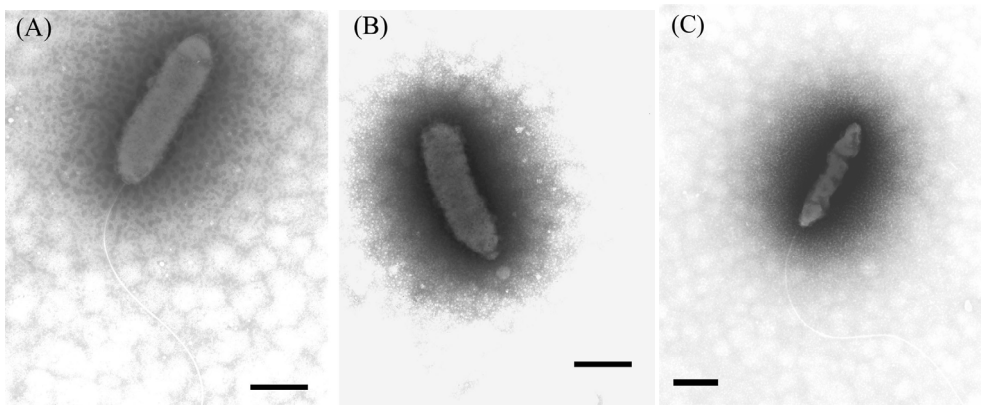


Fig. 2: Electron micrographs of *A. butzleri* LMG 6620 wild type (A), *flaA* mutant (B), and *flaB* mutant (C). Bar = 500 nm.

Transcription of the flagellin genes in *A. butzleri* strain LMG 6620

To study the transcription of the two *fla* genes, the mRNA levels of the *flaA* and *flaB* genes were analyzed by qPCR. It has been reported that the regulation of *flaB* in *Campylobacter* spp. is modulated by the growth environment (1). Therefore, the wild type and mutants of *A. butzleri* LMG 6620 were grown under different conditions with respect to temperature and oxygen tension. Low quantities of *flaB* mRNA were detected compared to those of *flaA* in the wild type strain. Oxygen had no significant influence on the *flaA* and *flaB* mRNA levels. Temperature did not have any influence on the *flaA* transcription. In contrast, the mRNA levels of *flaB* were approximately 3- to 6-fold higher at 30°C than at 37°C (Fig. 3A) suggesting that the flagellin genes are differentially regulated. Comparison of the transcription of each *fla* gene in the wild type and mutant strains demonstrated that mutation of *flaB* did not influence *flaA* transcription but mutation of *flaA* caused an increase in the expression *flaB* (Fig. 3B).

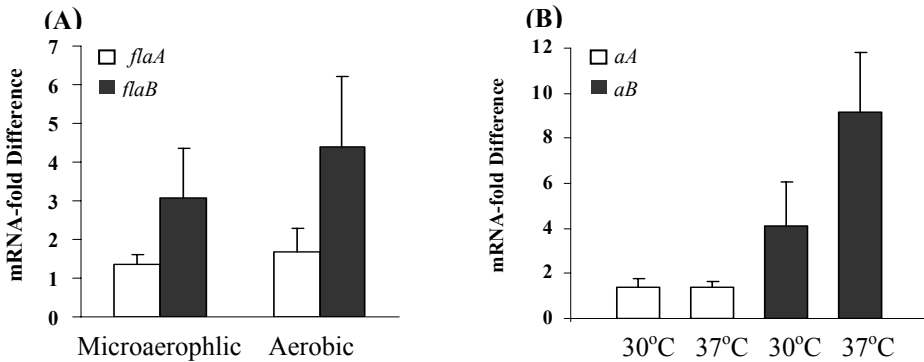


Fig. 3. Comparing relative expression of flagellin genes by qPCR. (A): difference in mRNA levels at 30°C vs 37°C in the wild type; (B): difference in mRNA levels of *flaA* or *flaB* between the wild type and *flaB* mutant or *flaA* mutant, respectively (see Results). The result is expressed as mean of triplicate (error bar = standard deviation).

Discussion

The genus *Arcobacter* has been considered a “peculiar” group since both plant- and human/animal- associated organisms are present in this group (44). The present study showed that the plant-associated *A. nitrofigilis* possesses remarkably different flagellins, both in size and amino acid sequence, compared to the human- and animal-associated *Arcobacter* species. Only low homology between each flagellin subunit was found among the members of the genus and even between two strains of one species. Furthermore, the protein encoding sequences found upstream of *flaA* in different *Arcobacter* strains resembled genes encoding various proteins in *Campylobacter* and *Helicobacter* spp., which may indicate a

dissimilarity in the gene order among these strains. This underlines the presence of substantial genetic variation among members of the genus, which has been observed before in studies on the prevalence of *Arcobacter* spp. in animals (41; 42). Nevertheless, the flagellins of all *Arcobacter* species form a separate group apart from other related genera including *Campylobacter* spp. in a phylogenetic tree derived from flagellin amino acid sequences of these species (Fig. 4).

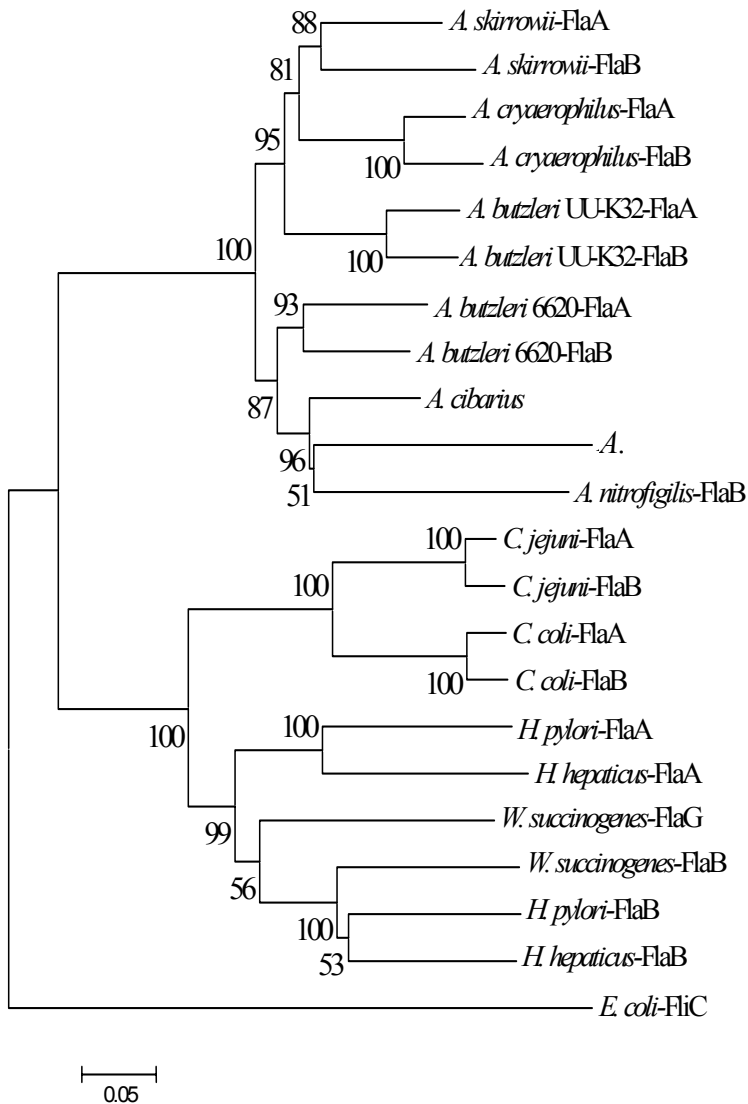


Fig. 4. Phylogenetic tree (Neighbor-Joining with boot strap value) of flagellins of *Arcobacter* spp. and the related organisms (*C. jejuni* RM1221, *C. coli* VC167, *H. pylori* J99, *H. hepaticus* ATCC51449, *W. succinogenes* DSM 1740), and *E. coli* K12.

Except for *A. nitrofigilis*, the flagellins in *Arcobacter* species associated with humans and animals are almost half the size of those in *Campylobacter* spp., *Helicobacter* spp., and *W. succinogenes*. Small flagellin proteins (< 300 amino acids) have been found in several Gram-positive and Gram-negative bacteria (50). A functional flagellin should have a minimum size of approximately 250 amino acids with conserved N- and C- terminal sequences of about 140 and 90 residues, respectively (4). These conserved regions, which are embedded in the inner core of the flagellar filament play important roles in the filament structure and flagellar motility (52). Among bacterial flagellins the central domain of the protein is highly variable, both in amino acid sequence and length (50). Experiments with insertions and deletions in the central domain of the flagellins in *Salmonella* and *E. coli* demonstrate that modifications in amino acid sequence and size of this region do not affect the motility of flagella (22; 23; 29). The 142-N- and 87-C- terminal amino acid residues of the *Arcobacter* flagellin proteins share high sequence similarities with those in related bacteria. In conclusion the short length of *Arcobacter* flagellins is fully accommodated in the center part of the proteins.

Apart from the N- and C-termini, flagellins in other genera of the Epsilonproteobacteria share two other conserved sequences within the central region, from residues 210 to 229 and 280 to 311 (residue numbers are according to the *W. succinogenes* FlaG sequence) (36). These two regions were also found in the flagellin sequences of *A. nitrofigilis* (albeit with a lower level of similarity than among *Campylobacter* spp., *Helicobacter* spp., and *W. succinogenes*). However they are scarcely present in the protein sequences of the other *Arcobacter* species, where substantial deletions occurred (Fig. 5).

The differences in *Arcobacter* flagellins compared to related bacteria can be explained by gene shortening and genomic shaping during evolution of bacterial genomes (3; 27; 35). It has been observed that members of the alphaproteobacteria associated with mammals- and arthropods possess considerably smaller genomes than plant-associated or aquatic relatives (3). Genomic analysis suggests that mutation by deletion rather than insertion is the primary force that drives the streamlining the bacterial genomes (27). This would be beneficial for bacteria in terms of metabolic efficiency, growth rate and avoidance of accumulation of deleterious mutations. It may also explain why despite high frequencies of horizontal gene transfer and gene duplication, bacterial genomes still remain small in size (27).

A difference was also found with respect to the regulation of *Arcobacter fla* gene expression compared to that in *Campylobacter* and *Helicobacter* spp.. In these organisms *flaA* is transcribed from a typical σ^{28} - type promoter while *flaB* transcription is under control of a σ^{54} promoter (12; 17; 20; 30). However, the consensus promoter elements for those sigma factors were not detected upstream of *fla* genes in any of the *Arcobacter* species. A phylogenetic tree derived from the

<i>A. butzleri</i> -UU-K32	M--RINTNVSSSLTAQEAAVNTNKNISSSLEKLSTGLRINKAADDASGLAIADKLRTQATSINQGISNGNSAVALLQ	74
<i>A. butzleri</i> -6620	M--RINTNVSSSLTAQESTSQTNKSISSSLEKLSSGLRINKAANDASGLAIADKLRTQASSINQGISNGNSAVALLQ	74
<i>A. skirrowii</i>	M--RINTNVSSSLTAQEASTNTNKYISNSLEKLSSGLKINKASDDASGLAIADKLRTQVTSINQGVANGNSAVALLQ	74
<i>A. cryaerophilus</i>	M--RINTNISSSLGAQEAANTNKSISNSLEKLSTGLAINKASDDASGLAIADKLRTQVTSINQSSISNGNSTVALLQ	74
<i>A. cibarius</i>	M--RINTNVSSSLTAQEAAQNTTKTITGSLEKLSTGLKINKASDDASGLAIADKLRTQATSINQGGIANGNSAVALLQ	74
<i>A. nitrofigilis</i>	M--RINTNIASLNAQEAAANTNKKIATSLERLSTGLKVNKASDDASGLAIADKLRTQASSISQGDIDNGNSAVALLQ	74
<i>C. jejuni</i>	MGFRINTNVAALNAKANADLNSKSLDASLSRLSSGLRINSAADDASGMAIADSLRSQANTLQQAISNGNDALGILQ	76
	* ****::* *: : ..*: **.*:**.*.*::*****.***.*:.....*: **:::.*	
<i>A. butzleri</i> -UU-K32	ITDKSMAEQSTILDTIKSKLIQANTDTTSVAGRTAIAKDITKLLQQLNNGEQTNYNGTNLL-----	136
<i>A. butzleri</i> -6620	IADKSMAEQSNILDTVKAKLIQANTATTADGRESIRKIDIKLLNQLDNIQKQTNYNGTYLL-----	136
<i>A. skirrowii</i>	IADKSMAEQSTILDTIKAKLIQANTDTTSTAGRVIAIKDVTKLLDQLNNAIQKQTNYNGTALL-----	136
<i>A. cryaerophilus</i>	IADKSMAEQSKILDTIKSKLIQANSDDTTSDDGRTAIAKDVTKLLQQLNNAIEQTNYNGTNLL-----	136
<i>A. cibarius</i>	IADKSMGEQSNILDTVKAKLIQANTDTTSDADGREAIRKIDITKLLKQLDNIATQTNYNGTALL-----	136
<i>A. nitrofigilis</i>	IADKSMSEQSNILDTVKAKLIQANTDTTSPDGREAIRKIDIVKLEQLDNIQQTSYNGITLL-----	136
<i>C. jejuni</i>	TADKAMDEQLKILDTIKTKATQAAQDQGSLKTRTMLQADINRLMEELDNIANTTSFNGKQLLSGNFINQEFQIGAS	152
	::**:* ** .***:*.* ** * * :*: :*:::**.*. *:*** **	
<i>A. butzleri</i> -UU-K32	--QNARTTANA-S-TE---GNL---TATR-TALGGLSF-----Q-----IG-----	166
<i>A. butzleri</i> -6620	--QAD-AT---S-----G---TAAA-ST-AHV-F-----Q-----IG-----	156
<i>A. skirrowii</i>	--QAAPDS---S-TA---G---IA-K-S-E-G-LKF-----Q-----IG-----	158
<i>A. cryaerophilus</i>	--QSARTTGGATSLTAS--G---AGNK-AK-GELSF-----Q-----IG-----	166
<i>A. cibarius</i>	--QAS--ATSN-AIS---G---G---LN--F-----Q-----IG-----	154
<i>A. nitrofigilis</i>	--QDSYDSMSS-SIELKFQIG---GNK-DDIMKSSF-I-----Q-----SNTVGLG-G-----GQGAL	180
<i>C. jejuni</i>	SNQTVKATIGA-TQSSK--IGLTRFETGGRIITSGGEVQFTLKNYNGIDDFQFKVVISITSVGTGLGALADEINKNA	225
	* : : * . * * * *	
<i>A. butzleri</i> -UU-K32	EG-----TQ-D-LIK-----TK-----T-----INSN----	181
<i>A. butzleri</i> -6620	EK-----TT-D-TIT-----MN-----T-----VQAN----	171
<i>A. skirrowii</i>	ES-----TS-D-IIE-----TK-----T-----IQAN----	173
<i>A. cryaerophilus</i>	EG-----TK-D-LIK-----TK-----T-----IQSN----	181
<i>A. cibarius</i>	EK-----AT-D-VI-----TN-----TG-----IRSN----	169
<i>A. nitrofigilis</i>	DQV-----SGSSTRNN-VISQGLSTVISGD-----GTNPITLDGYATGT-A-IKVN----	223
<i>C. jejuni</i>	DKTGVRATFTVETRGMAAVRAGTTSDNDFAINGVITGQVAYEDGDGNGALVAAIN---SVKDTTGVEASIDANGQL	298
	: * : * :	
<i>A. butzleri</i> -UU-K32	VA----GLK-----LSA-----LAK-----A-VRSG-----	197
<i>A. butzleri</i> -6620	TT----GLG-----LTT-----LAG-----LA-----	184
<i>A. skirrowii</i>	VT----GLE-----LTT-----LKT-----A-ASTG-----	189
<i>A. cryaerophilus</i>	VL----GLE-----LKT-----LAK-----N-ASTGS-----	198
<i>A. cibarius</i>	TQ----GLS-----LT--G-----LS-----LA-----	181
<i>A. nitrofigilis</i>	IT----GIPGD-----ITANGPT-----ELSKVDSTTQLVL-EALANATSGVNGSPATVEVKDINDR-VIGT	279
<i>C. jejuni</i>	LTSREGRGIKIDGNIGGGAFINANMKENYGRLSLVKNDGKDIIVSGTGLSFAGFGANSFISQASISLRESKGQLDA	374
	*: :. *	

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A. butzleri-UU-K32 -----GKMSAGA-----TAG-----TTG-----VFT-----RTMAQ 218
A. butzleri-6620 -----ASGL-----T-----TA-----VAA 194
A. skirrowii -----ANATLGA-----D-G-----KYTGT-----VFT-----KANAS 211
A. cryaerophilus ----AAKAASAAFAL-----SMGA-----KSAAK-----FFT-----RAQAS 227
A. cibarius -----ASGL-----T-----KTV-----AS 191
A. nitrofigilis FTLETTPPGTSIASGLVM--HSTPGLNGAEPVVFSLASGATIGMGGKTIINTVEIAGDPLNTFTIGAMTI-SQTSAS 352
C. jejuni NIADAMGFGSVNKGVVIGGFSTVSAYMSSESGSGFSAGSG-----YSIGS---GKGYSATLTGNATFISTASAAS 440
          *                                     *

A. butzleri-UU-K32 -----SG-----Q-----K-----AIDKAISILNG 233
A. butzleri-6620 -----TG-----Q-----T-----EVDAAITKLNG 209
A. skirrowii -----AG-----Q-----A-----AIDKAITQLNG 226
A. cryaerophilus -----AG-----Q-----I-----AIDKAITKLNG 242
A. cibarius -----AQ-----Q-----T-----IVDKAITTLNG 206
A. nitrofigilis GNITFDANAQNLN---IQNLNSANGQELKVTTATGSITGGGTLAGLKALQAGQLTQKQAVKYQGIVDAALSQMNI 424
C. jejuni RVYNVSSGSGFSTGSNLSQFAT-----MKTS-----VLGVKDETAGVTTLKGAMAVMDIAETAITNLNDQ 499
          :               *                               : *:: ::

A. butzleri-UU-K32 YRGDIGSTQNQVESAIRNLTTQATNIKNAESTIRDVDYAQESANYNKLNIIAQAGSYAISQANSTQQNVLRLLQ 307
A. butzleri-6620 YRGDIGSTQNQVESAIRNLTTQATNIKNAESTIRDVDYAAESANYNKLNIIAQAGSYAISQANSTQQNVLRLLQ 283
A. skirrowii YRGDIGSTQNQVESAIRNLMTQSTNIKAAESVIRDVDYAQESANFNKLNIISQAGSYAISQANATQQNVLRLLQ 300
A. cryaerophilus YRGDIGSTQNQVESAVRNLMTQATNIKAAESVIRDVDYAQESANFNKLNIISQAGSYAISQSNATQQNVLRLLQ 316
A. cibarius YRGDIGSTQNQVESAVRNLMTQATNVKAAESIIRDVDYAAESANFNKQNIIAQAGSYAISQANAVQQNVSRLLQ 280
A. nitrofigilis YRSDLGSTQNQVESAVRNLMTQKTNVTAAESVIRDVDYAQEFANFNKQNIIAQAGNYAMSQANSVAQNVLRLQ 498
C. jejuni IRADIGSVQNVQVSTSTINNITVTVQNVNKAASQIRVDVFAAESANYSKANILAQSGSYAMAQANSVQQNVLRLLQ 573
          *.*:**.**** *:.:*: . .*: .*** *****: * *:.* **::*:*.**.:*:. ** **

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Fig. 5. Alignment of FlaA of *Arcobacter* spp. and of *C. jejuni* RM1221. Consensus positions: “*” indicates identical residues in all sequences; “:”, conserved substitutions; “.” Semi-conserved substitutions; shadowed residues are found conserved within the central region of flagellin among the Epsilonproteobacteria.

120-nucleotides upstream of the start codon (ATG) of *fla* genes in *Arcobacter* spp. and related organisms (the putative promoter regions) yielded three well-separated clusters (Fig. 6). One includes all sequences of the *fla* genes regulated by σ^{28} and another consists of those from *flaB*, regulated by σ^{54} in *Campylobacter* spp., *Helicobacter* spp., and *W. succinogenes*. All sequences upstream *Arcobacter* *fla* genes cluster in the third group.

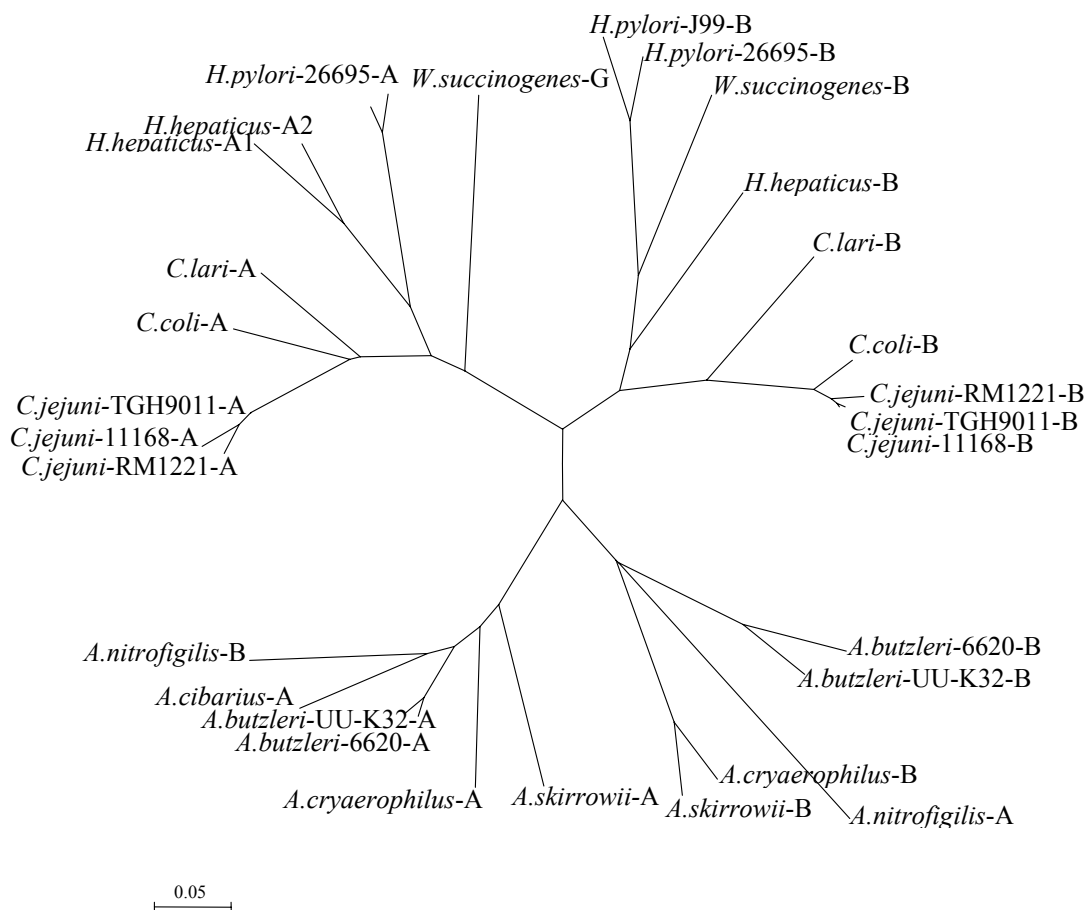


Fig. 6. Phylogenetic tree (Neighbor-Joining) of flagellin promoter regions (120 nucleotides upstream the start codon ATG) of *Arcobacter* spp. and their related organisms (A: *flaA*; B: *flaB*; G: *flaG* in *W. succinogenes*; *H. hepaticus* ATCC 51449 possesses 2 identical *flaA* genes, *flaA*-1 and *flaA*-2).

Although different systems may regulate their gene expression, the study on motility phenotype and transcription of *fla* genes in wild type and *flaA* or *flaB*

mutants of *A. butzleri* LMG 6620 indicates a similar pattern as found in *Campylobacter* spp. (11; 48). In the wild type *flaB* was expressed at low levels. The transcription of *flaB* was influenced by the growth temperature, but not that of *flaA*. Mutation of *flaB* had no influence on the transcription of *flaA*, the mutant still produced a flagellum with a comparable length and was equally motile as the wild type strain. In contrast, mutation of *flaA* clearly increased the transcription of *flaB* indicating a compensation effect which is often seen for duplicate genes (9). However, some amino acid substitutions in the N- and C-terminal-coding parts of *flaB* may prevent the incorporation of the FlaB protein into functional flagella. Consequently, the *flaA* mutant was aflagellated and non-motile.

The simultaneous shortening of both flagellin subunits and the clustering of both FlaA and FlaB shown in the phylogenetic tree (Fig. 4) suggest a concerted evolution of the two *Arcobacter fla* genes. This was also observed in *C. jejuni* (25). So far, the significance of concerted evolution in multigene families still needs to be clarified (40). It has been suggested that unequal crossover or/and gene conversion are involved in the process of evolution of duplicated genes (7; 28), which assures the preservation of functional parts of the gene while still allowing genetic diversity (34). The co-existence of two paralogous genes in tandem and the indications for their concerted evolution demonstrate that although *flaB* may not play an essential role in motility, the gene itself and its protein might serve for antigenic variation which is important in host-parasite interaction or/and might be involved in bacterial pathogenicity (such as adhesion and invasion).

In conclusion, in the present study some specific features of the genes encoding flagellins in *Arcobacter* spp. are described and compared to those in related organisms. Human- and animal- associated *Arcobacter* species possess much smaller flagellins than *A. nitrofigilus*. A similar transcription pattern for the two *fla* genes in *A. butzleri* as in related bacteria was found. However, in the expression of the genes in *Arcobacter* spp. different sigma factors than those employed in other related bacteria seem to play a role.

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Chapter 7

***Arcobacter*, what has been done and should be done
on a potential foodborne zoonotic agent
- Summarising Discussion**

In the 1910s Gram-negative curved organisms were isolated in association with abortion in animals and assigned to the genus *Vibrio*. Four decades later these organisms were transferred to the genus *Campylobacter* (reviewed in Neill et al., 1979). In the late 1970s, the organisms were isolated in high numbers from aborted foetuses of bovine and porcine origin. This time their properties were intensively characterised (9; 10; 24; 25). The bacteria appeared to be atypical *Campylobacter* spp.. They were aerotolerant and grew optimally at 30°C. In 1985, the new species *Campylobacter cryaerophila* was introduced for these bacteria (23). A few years earlier, in 1983 another new species *Campylobacter nitrofigilis* was announced, a nitrogen-fixing organism isolated from the roots of a marsh plant *Spartina alterniflora* Loisel (22). Studies based on DNA-DNA and DNA-rRNA hybridisation of *Campylobacter* spp. and related-organisms led to the revision of the taxonomy of this group and subsequently to the proposal of the new genus *Arcobacter* with *A. nitrofigilis* as the type species in 1991 (35).

As reviewed in **Chapter 1**, the genus *Arcobacter* at the moment includes 6 species: two are environmental inhabitants – *A. nitrofigilis*, a nitrogen-fixer as mentioned above and *A. halophilus* that is isolated in a hypersaline lagoon; the three most popular arcobacter species (at least among research microbiologists) - *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* are associated with human and animals and *A. cibarius* is only found on chicken carcasses. Recently, the presence of *A. cibarius* in pigery effluent and effluent-irrigated soil is reported in Australia (6). More than a decade after the introduction of the genus, publications are still limited to the description of isolation procedures and identification methods as well as to the prevalence in human and animals, in food and in various environments. No optimal media for isolation of all *Arcobacter* species have been established, which contributes to the problems encountered with respect to further studies of these bacteria. One study on artificial infection of piglets and two of chickens have been conducted to investigate the distribution and pathogenicity of *Arcobacter* spp. in animals, but the results were inconclusive.

Studies on *Arcobacter* spp. in animals with respect to animal health and production

Understanding the routes of transmission of an organism is an important step in studies of its epidemiology, pathogenicity and of the control of the organism in causing disease. In **Chapter 2** of this thesis the investigation of the transmission routes of *Arcobacter* spp. in pigs which can be either clinical hosts of *Arcobacter* spp. and/or reservoirs for shedding the bacteria is described. The results indicated that newborn piglets can acquire *Arcobacter* spp. both by vertical and horizontal routes. It was demonstrated that all three species *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* can colonise and co-exist in the pig intestine and be shed intermittently by clinically healthy sows and piglets. Pregnant sows excreted all three species of

which *A. skirrowii* was detected at the highest rates. Nevertheless, *A. cryaerophilus* was often the sole *Arcobacter* species to be recovered from amniotic fluid and newborn piglets. Similarly, *A. cryaerophilus* was the only *Arcobacter* species isolated from bovine, ovine and porcine aborted fetuses by Ellis and co-workers (9; 10; 24; 25). *A. cryaerophilus* was also the predominant organism isolated from aborted fetuses of pigs in studies from Brazil (7) and the U.S. (29). In a study from Denmark, it was reported that *Arcobacter* spp. could be recovered from 23/55 aborted pig fetuses, of which 40% was identified as *A. cryaerophilus* and 37% as *A. skirrowii*. Hence, the following questions can be raised:

- (i) whether *A. cryaerophilus* is the *Arcobacter* species mainly associated or responsible for animal abortion, like i.e. in *Campylobacter* spp., *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* are known as primary pathogens, of which the former causes abortion in cattle and sheep and infection in human (isolates have been obtained from blood, aborted fetuses and abscesses), while the latter causes abortion and infertility in cattle (34);
- (ii) *A. butzleri* and *A. skirrowii* are just co-existing or inhabiting pigs as a reservoir;
- (iii) whether the association of *Arcobacter* species with reproductive disorders in pigs affects the productivity; and
- (iv) since the transmission patterns of *Arcobacter* spp. have been clarified in pigs and the prevalence of these bacteria in sows and pigs is considerably high, whether or not action should be employed to reduce the risk of spreading the bacteria to the public.

Furthermore, apart from reproductive disorders, *Arcobacter* spp. have also been linked to enteritis and diarrhoea in animals (3; 15; 34; 37). It would be interesting to know how severe this problem is in terms of production loss caused by *Arcobacter* species compared to other well-known enteric pathogens in farm animals.

Finally, there are no reports at the moment on the presence of *Arcobacter* spp. in pets. In our initial investigation, *A. butzleri* and *A. cryaerophilus* were isolated from the dog rectum (unpublished results) This study will be continued in Vietnam in order to determine the prevalence as well as the possible illness caused by these bacteria in dogs and cats.

To answer all the questions above, more efforts than currently exerted is needed, especially if one takes into account that answering the questions will not be easy. With respect to pig production, the effects of reproductive problems caused by *Arcobacter* species appear not to have been recognised so far, as significant. Also, the ethical restriction on animal experiments nowadays could hinder artificial

infections to study the pathogenic role of each species in association with reproductive disorders. Nevertheless, some practical observations can be made which could be feasible for further studies to be conducted:

(i) assessment of the severity of the disorders caused by *Arcobacter* species by determination of the correlation of the presence of arcobacters in sows with abortion and other reproductive disorders (e.g. numbers of aborted fetuses, dead piglets and mummies per litter as well as the productivity record of arcobacter-positive sows or cows compared to arcobacter-free individuals);

(ii) with these observations, statistical analyses can be performed that hopefully reveal which *Arcobacter* species is/are the dominant cause(s) of the disorders (a combination of PCR and isolation from enriched samples is recommended for species detection, see i.e. **Chapter 2** and **Chapter 4**); and

(iii) evaluation of the long-term economic impacts caused by reproductive disorders linked to arcobacters on the productivity of the pig industry.

Finally, further interest and investment in studies of animals as *Arcobacter* reservoirs is depending on the pathogenicity and virulence of the bacteria in human, which will be discussed later.

***Arcobacter* spp. in chicken, from farm or slaughterhouse to table**

Like for *Campylobacter* spp., the interest in *Arcobacter* spp. in chickens is driven by their potential importance for public health. The results in **Chapter 3** indicated that unlike in pigs, vertical transmission of *Arcobacter* spp. in chickens does not seem to be an important route for acquisition of *Arcobacter* by chickens. Various samples of different batches in a hatchery were analysed for the presence of arcobacters and found negative. The bacteria were found with high frequency in the intestinal tract of hens but not in ovarian eggs and mature eggs of the same individuals. However, to confirm our preliminary data infection experiments of arcobacter-free breeders with arcobacter isolates from chickens should be conducted and the transmission of the bacteria to the hen reproductive organs and eggs should be determined.

The horizontal route of transmission of arcobacters in chicken still remains to be verified. Horizontal transmission of *Campylobacter* spp. in chickens is suggested to be primarily responsible for the spread of the bacteria in a flock, rather than the vertical route (5; 26; 27). The major source of introduction of *Campylobacter* in broilers are depopulation movements, between houses on a farm and spreading by on-farm staffs (1). One would expect to obtain similar observations for *Arcobacter* spp.. However, the results in **Chapter 4** indicated certain differences in the ecology of *Arcobacter* spp. compared to campylobacters, which in our opinion, has resulted

in the discrepancy in the studies on arcobacter prevalence in chickens as well as the source of arcobacter contamination on carcasses.

The colonisation of *Arcobacter* spp. in chickens, especially in broilers has been under discussion in the literature. Apart from a report on a prevalence of 14.5% in chickens in Japan (19), *Arcobacter* spp. have been rarely found in the intestinal content of broilers in other studies (4; 8; 17; 40). From this observation a hypothesis was derived that *Arcobacter* spp. may not be natural inhabitants in the chicken intestinal microflora but live in the surrounding environment on farms, such as litters, waste waters, etc. (8). A similar theory has been proposed for the presence of *Arcobacter* in slaughterhouses. Since the majority of carcasses is heavily contaminated with arcobacter but the bacteria are not frequently isolated from the gut contents, it was hypothesised that *Arcobacter* spp. present on carcasses would originate from slaughterhouse environments and a role of supply water as reservoir has been suggested (13; 14; 31).

However, the results described in **Chapter 4** strongly indicated that arcobacters contaminating almost all carcasses are not from the in-plant supply water. *Arcobacter* spp. were not detected in supply water which was sampled twice on different visits. In contrast, it was demonstrated that one of the sources of *Arcobacter* contamination was the gut content of the chickens. The hypothesis that *Arcobacter* spp. contaminating carcasses are natural inhabitants of the slaughterhouse environment can also be excluded because of their slow and fastidious growth, which can not explain the observed widespread contamination and extreme genetic diversity of *Arcobacter* isolates on carcasses as demonstrated in **Chapter 4** and other studies (30; 31). Moreover, in this Chapter the presence of *Arcobacter* spp. in chicken intestinal contents was confirmed. The prevalence ranged from 3.3% (1/30) in one broiler flock to 53% (53/100) in another. A higher prevalence was detected in hens. Therefore, like in the case of *Campylobacter* spp., *Arcobacter* species in slaughterhouses are undoubtedly introduced from arcobacter-positive chickens, resulting in cross-contamination within and between slaughtered flocks. It should be noted that although *Arcobacter* spp. are slow-growing and fastidious they are aerotolerant mesophiles and therefore are able to survive in the environment better than the *Campylobacter* spp. Higher detection rates of *Arcobacter* spp. compared to that of *Campylobacter* spp. on the same chicken carcasses have been reported (17). In one of our experiments, *A. butzleri* could still be recovered 12 hours post-exposure on pork skin but not *C. jejuni* (unpublished data).

One of the possibilities resulting in the discrepancy mentioned above is that unlike *Campylobacter* spp., *Arcobacter* species seem to favour the upper part of the chicken intestines. Our results indicated higher recoveries of arcobacters from the ileum than from the caeca. Furthermore, from one flock of laying hens, 50 cloacal swabs were negative for arcobacters whereas the bacteria were found in 34 out of

40 intestinal tracts. Therefore, a study on the ecology of *Arcobacter* spp. in broilers should be conducted to confirm our explanation that the aerotolerant arcobacters do not prefer strict anaerobic niches like the caeca for their colonisation.

Are *Arcobacter* spp. human pathogens?

The results in **Chapter 5** indicated that *Arcobacter* spp., like *Campylobacter* spp. and other enteric pathogens, possess the ability to adhere and/or invade the host intestinal epithelial cells and to initiate inflammatory responses by the induction of IL8 production. This was the first demonstration of the induction of IL8 production by *Arcobacter* species. The adhesion and invasion of *Arcobacter* spp. to other human cell lines has also been described in the literature and was reviewed in **Chapter 1**. In humans, there have been several reports of bacteraemia linked to *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* (18; 20; 41). An outbreak of recurrent abdominal cramps without diarrhoea was reported in an Italian school, of which *A. butzleri* was confirmed to be the cause (36). The bacteria were isolated from diarrhoeal patient stools in South Africa together with *H. pylori* and *Campylobacter* spp. (28). However, statistical analysis of the results indicated that only *C. jejuni* was significantly associated with diarrhoea. *A. butzleri* was found as the fourth most common *Campylobacter*-related bacterium in patient stool samples in a study in Belgium and more frequently associated with a persistent, watery diarrhoea (38).

Therefore, from these clinical studies and reports as well as from *in vitro* pathogenicity studies there is no doubt that *Arcobacter* spp. are human pathogens. So far, it seems that the virulence and severity of the illness caused by *Arcobacter* species is less compared to *C. jejuni*. More clinical observations are necessary to clarify the involvement of *Arcobacter* spp. in human illness. Insight should be obtained in which diseases they can cause apart from enteric disorders and in the role of each species. In developing and highly populated countries like Vietnam where 24 public food poisoning cases, some of which made more than 1000 victims, were reported in Ho Chi Minh City in 2006, it is necessary to determine the cause of the epidemic in order to establish good strategies for the prevention. This situation also provides opportunities to investigate the role of *Arcobacter* spp. as foodborne pathogens.

However, it should be realised that a significant disadvantage and restraint which might lead to the neglect of the investigation of the pathogenicity of *Arcobacter* spp. in human and animals will be their slow and fastidious growth. The studies described in **Chapter 2** and **Chapter 4** showed that PCR gave a higher number of positive isolates for *Arcobacter* spp. than isolation methods, especially for *A. cryaerophilus* and *A. skirrowii*, which is in agreement with data in the literature (11; 12). Since isolates are needed for further *in vitro* bacterial characterisation and

pathogenicity studies, an optimal procedure for the isolation of all species is needed. It is known that different detection techniques like PCR vs isolation or direct isolation vs isolation after enrichment do not always result in the same species that are recovered with the same incidences (11; 16; 32; 33). This, therefore, makes it more difficult to determine the exact incidence and role of each *Arcobacter* species in relation to humans and animals.

Characterisation of flagellins in *Arcobacter* spp.: is *Arcobacter* an unusual taxon in the Epsilonproteobacteria?

In **Chapter 6** the characterisation of flagellins in five *Arcobacter* species, including the type species *A. nitrofigilis* is described. The results revealed differences in this genus compared to related bacteria. Although *Arcobacter* spp. possess two *fla* genes which are tandemly adjacent like in *Campylobacter* spp., the molecular weight of the encoded flagellins and regulation of the gene expression were found to be different.

While the flagellins of the type species *A. nitrofigilis* have a molecular mass comparable to those in *Campylobacter* and other related organisms, the flagellins in the other *Arcobacter* species are only half in size. The shortened region is located in the centre of the molecule which is known to be responsible for flagellar antigenic variability. Therefore it would be interesting to continue the study of *Arcobacter* flagellins by looking at the structure-function relation of flagellins from different *Arcobacter* species and strains.

The regulation system for *fla* genes in *Arcobacter* spp. was also found to be different from that in *Campylobacter* and *Helicobacter* spp. Consensus promoter sequences recognised by sigma factors σ^{28} and σ^{54} that regulate the expression of *flaA* and *flaB*, respectively, in *Campylobacter* spp. and other epsilonproteobacteria could not be identified in front of the *fla* genes in *Arcobacter* species. A phylogenetic tree made with the sequence of the 120-nucleotides upstream the start codon ATG (which is assumed to contain the promoter sequence) of each *fla* gene in *Arcobacter* spp. and their related organisms was constructed. This tree contains three main branches: one for the σ^{28} and another for the σ^{54} promoter sequences in *Campylobacter*, *Helicobacter* spp. and *W. succinogenes*, and a third for the *Arcobacter* sequences. Furthermore, the *arcobacter* branch is divided into 2 groups: one for sequences upstream *flaA* (the first *fla* gene of the tandem pair) and the other for sequences upstream *flaB* (the second *fla* gene of the pair) in all *Arcobacter* spp. except *A. nitrofigilis*. The phylogenetic positioning unexpectedly revealed that the sequence upstream *flaA* of *A. nitrofigilis* is grouped with those upstream *flaB* of the other *Arcobacter* species and vice versa. Similar to *Campylobacter* spp., in *A. butzleri* LMG 6620 the first gene in the tandem pair is mainly responsible for the cell motility since the deletion mutation of this gene

resulted in aflagellated and non-motile cells (discussed later). Therefore, the exchange between the sequences upstream *flaA* and *flaB* in *A. nitrofigilis* on the phylogenetic tree is unusual. This could mean that:

- (i) the second *fla* gene in the tandem, and not the first one, is responsible for motility in *A. nitrofigilis*;
- (ii) a completely different regulation pattern is involved in the expression of *fla* genes in this organism; or
- (iii) the promoter sequences of the *fla* genes in this species are located outside the 120-nucleotide sequence upstream the *fla* genes, which would be highly unlikely.

To partly answer these questions, mutants with disfunctioning *fla* genes of *A. nitrofigilis* have to be conducted. The motility of these mutants should be assayed in combination with qPCR for the gene expression, like this was done for *A. butzleri* LMG 6620 in **Chapter 6**. However, the growth of this environmental strain in the laboratory is very slow and difficult, which will make genetic manipulation and other assays difficult to perform.

In **Chapter 6**, the first time genetic manipulation of *Arcobacter* spp. was described. The function of each *fla* gene in motility and their transcription in *A. butzleri* LMG 6620 was investigated by the mutation of each gene. Using a qPCR assay, it was shown that in the wild type strain both *fla* genes are transcribed, of which *flaB* was transcribed at lower levels. In *flaA* mutants, the transcription of *flaB* increased, but the cells did not possess flagella and were non-motile. In contrast, *flaB* mutants had a long flagellum and their motility was comparable to that of the wild type as shown in the semi-solid agar assay. Therefore, it is an interesting question why the *flaB* gene is present in *Arcobacter* and *Campylobacter* spp.

In the literature, the evolution and significance of duplicate genes has been analysed and some theories have been put forward. If the gene duplication is not needed for the quantity of their products (for example in the case of rRNA genes), three alternative fates for duplicate genes have been suggested (21; 42): (i) one copy could be degenerated by mutations and finally removed (non-functionalisation); (ii) while one copy retains the original function, the other copy could evolve to a novel and beneficial function (neo-functionalisation); or (iii) due to mutation accumulation each copy can not maintain the original function but adopts different parts of the functions (sub-functionalisation). The third destination probably is the case of the flagellins in *Helicobacter* spp. of which the homology between *flaA* and *flaB* is low and the expression of both gene products is needed for full cell motility. At the moment the most common and acceptable explanation for the existence of two highly homologous *fla* genes in *Campylobacter* spp. and

possibly in *Arcobacter* spp. is that *flaA* is responsible to the cell motility and the products of both genes together contribute to the pathogenicity such as adhesion and invasion of the host epithelium as well as immunogenic variability. It is also hypothesised that *flaB* may serve as a genetic reservation for reassortment and recombination of the flagellin DNA as a mechanism for antigenic variation (2; 39). This would come close to the second theoretical fate for duplicate genes mentioned above. In our additional experiments FlaB was found to be secreted in the growth medium of *flaA* mutants as well as in cell surface-detached fractions (unpublished data). Therefore, it will be interesting to investigate the function of FlaB, for example in adhesion and antigenicity, by comparison assays between the wild type, the *flaA* mutant and a *flaAflaB* mutant.

Conclusion

In this thesis the presence of *Arcobacter* spp. in amniotic fluid of sows and two routes of *Arcobacter* acquisition in piglets were demonstrated. With respect to the pig production, further investigation of the possible correlation between the presence of *Arcobacter* spp., (especially of each species), and reproduction disorders as well as economic impacts is suggested. In chickens, we found no evidence for the threat of vertical transmission of *Arcobacter* spp. from hens to their eggs and chicks. Furthermore, our data strongly indicated that *Arcobacter* spp. inhabit the chicken intestines and are introduced into slaughterhouses with the chickens, which excludes the theory that arcobacter contamination originates from the slaughterhouse environment. Further investigation of the ecology of *Arcobacter* spp. in chicken intestines is badly needed.

The ability of *Arcobacter* isolates from humans and animals to adhere to human and porcine intestinal epithelial cell lines and to induce inflammatory responses via stimulation of IL-8 production was demonstrated. Additional investigation of the link between *Arcobacter* spp. (again taking each *Arcobacter* species into account) and human illness as well as the severity of the disorder caused, is needed. However, one should keep in mind the slow and fastidious growth of these bacteria which will hinder such investigations and result in underestimation of their importance.

The characterisation of flagellins in *Arcobacter* spp. in Chapter 6 added more unusual and worth-to-be-studied properties to this genus compared to related organisms. The finding of differences in the flagellin protein mass as well as the regulation of expression of the *fla* operon opens the way to more studies of flagellin properties, the regulation of the flagellar gene expression, as well as of the function of the flagellin B protein. The construction of *fla* mutants in this thesis should provide a useful tool for further genetic studies of these microorganisms. Finally, in this thesis it is shown that the type species *A. nitrofigilis* is not only

different from other *Arcobacter* members in its ecological but also in genetic properties. Whether this difference is in the flagellins only or also in other genetic properties is unknown at the moment. It will be interesting to study the mechanism by which this differentiation is driven.

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Samenvatting

In 1991 is een nieuw geslacht van bacteriën, het genus *Arcobacter* dat verwant is aan *Campylobacter* beschreven. Voor die tijd werden deze bacteriën aangeduid als *Campylobacter* achtige organismen op grond van hun morfologie. *Arcobacters* zijn Gram-negatieve gebogen staafvormige bacteriën die tussen 0.2 en 0.9 µm breed zijn en 0.5-3 µm lang. De bacteriën vormen geen sporen en bezitten een enkele polaire flagel aan één of beide uiteinden van de bacteriecel. In tegenstelling tot *Campylobacter* zijn de *Arcobacter* spp. aerotolerant en is hun optimale groei temperatuur 30°C. De leden van het genus *Arcobacter* komen op veel verschillende plaatsen voor. De “prototype” stam van de soort *Arcobacter*, *A. nitrofigilis* is een stikstof fixerende bacterie, die in symbiose leeft met de wortels van de in zoutwater moerassen voorkomende plant *Spartina alterniflora*. *A. halophilus* is een obligaat halofiele bacterie, die uitsluitend bij hoge zout concentraties groeit en geïsoleerd is uit een lagune op Hawaï met extreem hoge zoutconcentraties. Drie andere soorten, *A. butzleri*, *A. cryaerophilus* en *A. skirrowii* worden voornamelijk aangetroffen bij zowel zieke als gezonde dieren en mensen. In het geval van ziekte worden deze bacteriën voornamelijk geassocieerd met enteritis en soms met bacteremie. De eerst beschreven isolatie van *Arcobacter* was echter van geaborteerde foetussen van runderen en schapen. *A. cibarius* is tot nu toe uitsluitend geïsoleerd van karkassen van kippen. In **Hoofdstuk 1** wordt een overzicht gegeven van de kennis over het geslacht *Arcobacter* toen het in dit proefschrift beschreven onderzoek werd begonnen. Dat *A. butzleri*, *A. cryaerophilus* en *A. skirrowii* in grote aantallen voorkomen in zowel voedingsmiddelen van dierlijke oorsprong als drink - en grondwater en dat zij in verband gebracht kunnen worden met ziekte bij mens en dier was de reden dat deze bacteriën worden geacht een rol te spelen als voedsel of water gerelateerde ziekteverwekkers. Niettemin, zijn meer dan een decennium na de beschrijving van het genus de wijze waarop deze bacteriën worden overgedragen en hun ziekteverwekkende eigenschappen grotendeels onbekend. Het doel van het onderzoek beschreven in dit proefschrift was om de overdrachtsroutes van *Arcobacter* in en tussen dieren te onderzoeken, evenals sommige aspecten van hun ziekteverwekkende eigenschappen.

In **Hoofdstuk 2** is de mogelijkheid van verticale- (van moeder op nakomeling) en horizontale (van het ene individu op het andere) overdracht van *Arcobacter* spp. in varkens bestudeerd. Analyse van rectaal genomen monsters toonde aan dat *Arcobacter* spp. in hoge mate voorkomen bij zeugen (ongeveer 42% van de onderzochte zeugen was drager van één of meer *Arcobacter* soorten). Het met tussenpozen uitscheiden van een bepaalde *Arcobacter* soort en het verschuiven in de tijd bij individuele dieren van de uitscheiding van een bepaalde soort *Arcobacter* naar een andere werd tijdens dit onderzoek waargenomen. *Arcobacter* spp. werden ook aangetroffen in het vruchtwater van de zeugen en in rectaal afgenomen

monsters van pasgeboren biggen (variërend van 38.5- 83.3% per toom). De grote overeenkomst tussen de PFGE profielen (een moleculaire typeringsmethode, gebaseerd op DNA fragmenten, waarmee verwantschappen van bacteriën kunnen worden bepaald) van de *Arcobacter* isolaten afkomstig van de zeugen en hun respectievelijke pasgeboren biggetjes maakt het bestaan van een intra-uterine overdrachtsroute waarschijnlijk. Antilichamen specifiek gericht tegen *Arcobacter* spp konden worden aangetoond in het colostrum van de zeugen middels Western blotting. Op grond van deze resultaten werd ook het bestaan van een postnatale infectie route duidelijk, waarbij arcobacters worden over gedragen van zeugen op nieuwkomers in een toom en/of de omgeving op de biggetjes. Dus is in dit hoofdstuk het voorkomen van twee verschillende manieren waarop biggetjes met *Arcobacter* kunnen worden besmet door drachtige zeugen (zowel horizontaal als vertikaal) aangetoond.

In **Hoofdstuk 3** wordt beschreven dat er een hoge prevalentie is van *Arcobacter* spp. in hennen. Niet werd waargenomen dat de bacteriën ook aanwezig waren in of op de eieren van verschillende koppels hennen, zelfs niet als bij deze experimenten de heel gevoelige polymerase ketting reactie werd gehanteerd. Evenmin konden de bacteriën worden aangetoond door middel van isolatie in of op eieren van twee verschillende positieve koppels hennen. Behalve uit een dons monster, kon in verschillende monsters genomen in broedmachines, géén *Arcobacter* worden aangetoond. Door deze resultaten kon worden aannemelijk gemaakt dat in tegenstelling tot sommige andere bacteriën, zoals Salmonella geen overdracht plaats vindt van *Arcobacter* spp. van positieve hennen naar hun eieren of kuikens.

In **Hoofdstuk 4** wordt het onderzoek beschreven dat verricht is om het mysterie achter de bron van *Arcobacter* besmetting op karkassen in slachthuizen te ontrafelen. De karkassen en het maagdarkanaal van dieren die zijn onderzocht, waren afkomstig uit drie verschillende koppels hennen en twee verschillende koppels slachtkuikens. Er werden geen arcobacters in het toevoer water bij de slachtlijn aangetoond, maar wel op alle onderzochte karkassen en in het water dat van deze karkassen afdroop. *Arcobacter* spp. waren aanwezig in de darmen van kippen, het voorkomen ervan verschilde van koppel tot koppel en varieerde van 20-80% in de hennen en van 3.3-51% in de slachtkuikens. Door middel van genotypische karakterisering met behulp van een ERIC PCR kon worden aangetoond dat binnen een koppel dezelfde stammen voorkwamen in de darm inhoud en op de karkassen. Uit deze studie werd de conclusie getrokken dat *Arcobacter* spp. voorkomen in het darmkanaal van kippen en dat van via deze route de bacteriën in het slachthuis worden geïmporteerd waar zij de karkassen verontreinigen gedurende het slachtproces.

In **Hoofdstuk 5** worden een aantal ziekteverwekkende eigenschappen van *Arcobacter* stammen afkomstig uit mens en dier onderzocht door hun vermogen

om aan te hechten en binnen te dringen in cellen van de humane Caco-2 cellijn en de varkens cellijn IPI-2I te bestuderen. Alle acht onderzochte *Arcobacter* stammen waren in staat om aan te hechten aan beide typen cellen en ze induceerden al twee uur nadat ze gedurende een uur met de cellen in contact waren geweest de productie van interleukine -8 (een eiwit dat geproduceerd wordt als sprake is van ontstekingsreacties). Invasie van darmepitheel cellen werd slechts waargenomen voor *A. cryaerophilus* stammen. Een correlatie tussen het vermogen om cellen binnen te dringen en meer of minder sterke aanhechting van de onderzochte stammen werd niet waargenomen. Deze correlatie was er evenmin met de productie van interleukine-8.

In **Hoofdstuk 6** worden de verschillende flagel eiwitten van *Arcobacter* spp. gekarakteriseerd. Evenals de bacteriesoorten *Campylobacter* en *Helicobacter* bezitten *Arcobacter* spp. twee flagelline genen (*flaA* en *flaB*), die in het genoom van de bacterie direct naast elkaar gelokaliseerd zijn. Met uitzondering van de flageleiwitten van *A. nitrofigilis*, zijn deze eiwitten ongeveer half zo groot als in de voornoemde bacteriën. Bij de *Arcobacter* flagel eiwitten mist een groot gedeelte van het centrale en meest variabele deel van het eiwit. De lage homologie die werd waargenomen tussen de flageleiwitten van de verschillende *Arcobacter* soorten wijst op grote genetische verschillen tussen de leden van het genus *Arcobacter*. In tegenstelling tot de verwante bacteriën *Campylobacter* en *Helicobacter* worden de genen die coderen voor de flagel eiwitten afgeschreven van tot nu toe niet bekende promotoren bij dit soort bacteriën. Dit zou er op kunnen duiden dat de transcriptie van deze genen in *Arcobacter* op een andere wijze gereguleerd is dan bij verwante bacteriën is waargenomen. Studies waarin de genen coderend voor de twee flageleiwitten door mutatie kapot gemaakt waren toonde aan dat alleen FlaA nodig is voor de beweeglijkheid van *Arcobacter* spp. Kwantitatieve polymerase ketting reactie studies lieten zien dat de transcriptie van het *flaB* gen hoger was bij 30°C dan bij 37°C. Mutatie van het *flaB* gen had geen invloed op de beweeglijkheid van de *Arcobacter* stammen en ook niet op de transcriptie van het *flaA* gen, terwijl mutatie van het *flaA* gen de bacteriën onbeweeglijk maakte en de transcriptie van *flaB* deed toenemen. Deze resultaten onderstrepen de uitzonderingspositie die het genus *Arcobacter* inneemt binnen de epsilon subdivisie van de Proteobacteriën.

Op grond van de in voorgaande hoofdstukken verkregen resultaten wordt in **Hoofdstuk 7** besproken hoe het toekomstige onderzoek naar *Arcobacter* spp. er uit zou moeten zien. Dit onderzoek zou zich moeten richten op de betrokkenheid van elke *Arcobacter* soort afzonderlijk bij stoornissen in de voortplanting bij verschillende diersoorten. Ook zal onderzocht moeten worden wat de economische gevolgen zijn van dit soort stoornissen voor de varkenshouderij Het onderzoek naar de ecologie van *Arcobacter* spp. in de darmen van kippen dient te worden uitgebreid. Bij de mens dient nader onderzoek te worden gedaan naar de relatie tussen de aanwezigheid van *Arcobacter* spp. en het voorkomen van ziekte, evenals naar de ernst van de ziekte die door de verschillende soorten veroorzaakt wordt. De

waarneming dat er grote verschillen zijn in de grootte van de flageleiwitten van *Arcobacters* en de regulatie van de synthese van deze eiwitten opent de weg voor nadere studie van de eigenschappen van het *Arcobacter* flagelline eiwit. Dit is van belang omdat van flagel eiwitten is aangetoond dat zij een rol spelen bij het ziekteverwekkende vermogen van de bacterie, niet alleen omdat ze de bacterie beweeglijk maken, maar ook omdat zij betrokken zijn bij aanhechting van bacteriën aan epitheelcellen van de gastheer en het bij de gastheer naar binnen brengen van eiwitten die het metabolisme van de gastheercel verstoren. Het is tevens van belang om de regulatie van de synthese van flagel eiwitten te bestuderen om er achter te komen welke bestanddelen uit de omgeving van de bacterie hier eventueel van invloed op zijn. De functie van het FlaB eiwit verdient eveneens nader onderzoek (er is voor dit eiwit immers nog geen functie bekend). Inmiddels is bekend geworden dat de volledige nucleotide volgorde van het genoom van *A. butzleri* bepaald is. Deze volgorde is nog niet publiekelijk beschikbaar, maar zodra dit het geval is zal dit het onderzoek naar *Arcobacter* en met name naar de ziekteverwekkende eigenschappen van *Arcobacter* enorm stimuleren.

Tóm tắt

Arcobacter, thuộc họ *Campylobacteraceae*, được công nhận là một giống mới vào năm 1991. Tuy nhiên, không giống *Campylobacter* - một vi khuẩn vi hiếu khí có nhiệt độ phát triển tối ưu là 42°C, *Arcobacter* spp. có thể chịu được trong không khí và phát triển tốt nhất ở 30°C. Các thành viên của giống này có môi trường cư trú rất đa dạng. Loài điển hình của giống vi khuẩn này là vi khuẩn cổ đậm *A. nitrofigilis*. Một loài khác - *A. halophilus* cư trú bắt buộc trong vùng biển mặn. Ba loài khác, *A. butzleri*, *A. cryaerophilus* và *A. skirrowii* được phát hiện trên người và động vật khỏe mạnh cũng như mắc bệnh. *A. cibarius* được phân lập từ quầy thịt gà.

Tổng quan các nghiên cứu về *Arcobacter* spp. được trình bày trong **Chương 1**. *A. butzleri*, *A. cryaerophilus* và *A. skirrowii* hiện diện khá phổ biến trong thực phẩm có nguồn gốc động vật và trong các nguồn nước và nước uống. Các vi khuẩn này được phát hiện trong phân người và động vật (bò, heo, gà) khỏe mạnh. Mối liên quan giữa các vi khuẩn này với các trường hợp bệnh lý ở người và động vật cũng được báo cáo. Cả ba giống *Arcobacter* này đều được phân lập từ các trường hợp xảy thai và tử phôi chết của bò, cừu và heo. Trong các trường hợp này, người ta không thấy sự hiện diện các vi sinh vật thường liên quan đến các rối loạn sinh sản khác. Trên người, chúng được phân lập từ phân tiêu chảy và một số trường hợp nhiễm trùng huyết được ghi nhận, trong đó có một trường hợp tử vong. Từ mối liên quan đến các trường hợp bệnh lý trên người và sự hiện diện phổ biến của chúng trong các loại thực phẩm có nguồn gốc động vật, cũng như trong các nguồn nước, hiện nay, *Arcobacter* spp. được xem như một vi khuẩn có khả năng gây ngộ độc thực phẩm và lan truyền giữa gia súc và người (zoonosis).

Mặc dù có rất nhiều báo cáo về sự phân bố của *Arcobacter* spp. và về các phương pháp phân lập và xác định sự hiện diện của chúng, hiện nay chưa có nhiều nghiên cứu về tính gây bệnh, độc lực và con đường truyền lây trong động vật cũng như giữa động vật và người. Do đó, mục tiêu của luận án này là khảo sát đường lan truyền vi khuẩn trong động vật, chủ yếu trên heo và gà, đồng thời nghiên cứu một số đặc tính liên quan đến độc lực của chúng.

Chương 2 trình bày kết quả nghiên cứu sự lan truyền *Arcobacter* từ heo nái sang heo con. Khảo sát được thực hiện ở 1 trại heo của khoa Thú Y, Đại học Utrecht. Vi khuẩn được phát hiện trong mẫu ngoáy trực tràng của khoảng 42% heo nái, ở cả 2 lần lấy mẫu - mùa hè và mùa đông. Nhiều cá thể thải ra cùng lúc 2 hay 3 loài khác nhau. Tuy nhiên, có sự thay đổi trong thành phần các loài được phát hiện trên cùng 1 cá thể trong 2 lần lấy mẫu khác nhau. *Arcobacter* spp. được phát hiện trong dịch ối và trong mẫu ngoáy hậu môn của heo con vừa sinh ra (38.5–83.3% mỗi lứa). Đường truyền lây từ mẹ sang bào thai được xác định qua sự tương đồng về kiểu gen (áp dụng kỹ thuật PFGE) giữa các khuẩn lạc phân lập từ heo mẹ và heo con. Kháng

thể chống lại arcobacters cũng được tìm thấy trong sữa đầu bằng phương pháp Westren blot. Kết quả cũng cho thấy heo con sau khi sinh có thể nhiễm vi khuẩn từ heo mẹ hay từ môi trường xung quanh.

Kết quả khảo sát khả năng truyền lây *Arcobacter* spp. từ gà mái sang trứng và gà con được trình bày trong **Chương 3**. Mặc dù vi khuẩn này được phát hiện với tỷ lệ khá cao ở gà mái, chúng không được tìm thấy trong trứng có nguồn gốc từ những những cá thể và những đàn gà dương tính. Trong một nhà máy ấp trứng, mặc dù *A. cryaerophilus* được phân lập từ 1 phòng ấp, *Arcobacter* spp. đã không được phát hiện từ gà con mới nở, trứng không nở và các rác thải từ nhiều phòng ấp khác nhau, kể cả các mẫu có nguồn gốc từ phòng ấp phân lập được *A. cryaerophilus*. Kết quả này cho thấy chưa có bằng chứng về khả năng truyền vi khuẩn này từ gà mái sang trứng hay gà con.

Như được trình bày ở Chương 1, mặc dù *Arcobacter* spp. được tìm thấy trên các quầy thịt gà với tỷ lệ khác cao (gần như 100% trong 1 số lò mổ ở Bỉ), nguồn truyền lây vi khuẩn này trong các lò mổ vẫn chưa được xác định. Để trả lời câu hỏi về nguồn gốc xuất phát của *Arcobacter* spp. nhiễm trên quầy thịt gà và trên dây chuyền giết mổ, chúng tôi đã thu thập mẫu trong 2 lò mổ gà, bao gồm quầy thịt gà, chất chứa ruột gà, nước cung cấp cho vệ sinh dây chuyền giết mổ và nước rửa gà ở các giai đoạn khác nhau. Kết quả được trình bày trong **Chương 4**. Vi khuẩn arcobacters đã không được tìm thấy trong các mẫu nước máy (được dùng cho toàn bộ các hoạt động trong dây chuyền). Tuy nhiên, arcobacters được phát hiện trong hầu hết các mẫu nước rửa quầy thịt, trong hầu hết các quầy thịt kiểm tra, và trong chất chứa ruột (20%-85% đối với gà mái; 3.3% và 51% đối với gà giò). Kết quả phân tích bằng phương pháp ERIC-PCR cho thấy sự đa dạng về kiểu gene của các gốc vi khuẩn arcobacters được phân lập. Tuy nhiên, sự tương đồng về kiểu gene của một số gốc vi khuẩn phân lập từ quầy thịt với các gốc phân lập từ chất chứa ruột của các cá thể cùng một đàn đã cho thấy các vi khuẩn nhiễm trên quầy thịt có nguồn gốc từ chất chứa ruột gà. Kết quả khảo sát đã xác nhận sự cư trú của *Arcobacter* spp. trong ruột gà và sự vấy nhiễm vi khuẩn từ chất chứa ruột sang dây chuyền giết mổ và quầy thịt có ý nghĩa quan trọng trong vệ sinh giết mổ.

Trong **Chương 5**, khả năng bám của arcobacters trên niêm mạc ruột và xâm nhập qua tế bào niêm mạc được khảo sát trên 2 dòng tế bào Caco-2 của người and IPI-2I của heo. Kết quả cho thấy cả 8 chủng khảo sát đều có khả năng bám trên cả 2 loại tế bào và kích thích các tế bào này tiết interleukin-8. Tuy nhiên, chỉ có các chủng *A. cryaerophilus* có khả năng xâm nhập.

Một trong các yếu tố độc lực được nghiên cứu nhiều nhất trên *Campylobacter* và *Helicobacter* – 2 vi khuẩn có họ hàng gần với *Arcobacter*, là flagellins. **Chương 6** của luận án này trình bày nghiên cứu đặc tính của arcobacter flagellins. Giống như đối với *Campylobacter* và *Helicobacter* spp., trong *Arcobacter* spp. tồn tại 2 genes cùng mã hóa flagellin (*flaA* and *flaB*). Các genes này nằm kề nhau và có cùng

hướng mã hóa. Ngoại trừ *A. nitrofigilis*, flagellins của các arcobacters khác có kích thước chỉ bằng phân nửa kích thước phân tử flagellins của các vi khuẩn khác trong cùng nhóm epsilonproteobacteria. Phần ngắn đi nằm ở vùng trung tâm phân tử. Phân tử flagellin của các loài arcobacters khác nhau có sự tương đồng di truyền khá thấp. Điều này cho thấy sự đa dạng di truyền trong các vi khuẩn thuộc giống này. Một điều khác biệt nữa về flagellin của arcobacters so với các vi khuẩn cùng nhóm epsilonproteobacteria là các promoters σ^{28} - or σ^{54} – điều khiển sự giải mã các gene flagellins trên các vi khuẩn cùng nhóm epsilonproteobacteria đã không được tìm thấy ở arcobacters. Như vậy, quá trình giải mã các genes này có lẽ được điều khiển bởi một hệ thống khác. Quá trình tạo các chủng đột biến *flaA* hay *flaB* trên chủng *A. butzleri* LMG 6620 được trình bày trong chương này và phản ứng qPCR đã được sử dụng trên các chủng đột biến để nghiên cứu cơ chế giải mã của các genes này. Kết quả cho thấy chỉ có *flaA* đóng vai trò quan trọng trong việc tạo thành flagella và di động của *A. butzleri*. Đột biến trên gene *flaA* làm vi khuẩn không tạo được flagella và mất tính di động. Trong khi đó, chủng đột biến gene *flaB* vẫn có flagella và có khả năng di động tương đương với chủng tự nhiên.

Từ các kết quả nghiên cứu trên, một số đề nghị đối với việc nghiên cứu *Arcobacter* spp. được trình bày trong **Chương 7**. Trên heo, có thể nghiên cứu vai trò của vi khuẩn này (đặc biệt là vai trò của từng loài) trong việc gây rối loạn sinh sản trên các động vật, cũng như tổn thất kinh tế do chúng gây ra trong chăn nuôi. Trên gà, cần hiểu rõ đặc tính tồn tại của *Arcobacter* spp. trong đường ruột. Tương tự, vai trò và mức độ gây bệnh của từng loài *Arcobacter* trên người cần được tìm hiểu rõ. Kết quả nghiên cứu flagellin genes trong luận văn này cho thấy cần tiếp tục nghiên cứu cơ chế điều hành việc giải mã các genes này cũng như vai trò của flagellin B đối với vi khuẩn.

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List of publications

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