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Characterization of the virulence potential of *Arcobacter butzleri*

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List of Abbreviations

ATCC	American Type Culture Collection
Caco-2	Human colorectal adenocarcinoma cell line
CCUG	Culture Collection of the University Gothenburg
CDT	Cytolethal distending toxin
CECT	Spanish Type Culture Collection
CHO	Chinese hamster ovary cell line
CIP	Collection de l'Institut Pasteur.
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FCS	Fetal calf serum
FHA	Filamentous hemagglutinin
HeLa	Human cervix carcinoma cell line
Hep-2	Human cervix carcinoma cell line (HeLa contamination)
HT-29	Human colorectal adenocarcinoma cell line
HT-29/B6	Human colorectal adenocarcinoma cell line
ICMSF	International Commission on Microbiological Specifications for Foods
IL	Interleukin
INT407	HeLa derivative
IPEC-J2	Porcine intestinal epithelium cell line
IPI-2I	Porcine intestinal epithelioid cell line
KCCM	Korean Culture Center of Microorganisms
KCTC	Korean Collection for Type Cultures.
LMG	Culture Collection of the Laboratory for Microbiology Gent
MTCC	Microbial Type Culture Collection and Gene Bank,
ND	Not determined
TER	Transepithelial electrical resistance
THP-1	Human acute monocytic leukemia cell line
TJ	Tight junctions
TNF-a	Tumour necrosis factor-a
Vero	Kidney epithelial cell line
MLN	Mesenthial lymphnodes

Chapter 1: Literature Review

1.1 The genus *Arcobacter* spp.

1.1.1 Taxonomy

Arcobacter spp. have taken on increasing importance as members of the genus have been recognised as emerging enteropathogens and potential zoonotic agents (Collado and Figueras, 2011; Ho et al., 2006a; Ho et al., 2006b; Snelling et al., 2006). This genus was first described by Ellis in 1977 as a taxon that contains gram-negative Spirillum/Vibrio-like organisms isolated from aborted bovine and porcine foetuses (Ellis et al., 1977). The first classification of *Arcobacter* was proposed by Vandamme et al. (1991) to accommodate two "aerotolerant *Campylobacter*" species: *Campylobacter cryaerophila* (now *Arcobacter cryaerophilus*) and *Campylobacter nitrofigilis* (now *Arcobacter nitrofigilis*) (Vandamme et al., 1991). In 1992, the genus was amended with the reclassification of *Campylobacter butzleri* [described by Kiehlbauch *et al*. (1991)] as *Arcobacter* (*A*.) *butzleri* and enlarged with a new species: *Arcobacter skirrowii*. *A. butzleri* was isolated from humans and animals with diarrhea. *A. skirrowii* was recovered from feces of lambs with diarrhea, and aborted porcine, ovine, and bovine foetuses (Kiehlbauch et al., 1991; Vandamme et al., 1992b).

As *Arcobacter* and *Campylobacter* share similar genotypic and phenotypic characteristics, both genera have been classified within the same bacterial family. Now, the genera *Arcobacter, Campylobacter* and *Sulfurospirillum* belong to the family of *Campylobactereaceae*, which belongs to the epsilon division of the class *Proteobacteria*, also known as ribosomal RNA superfamily VI (Vandamme and Ley, 1991; Miller et al., 2007). The genus *Arcobacter* comprises 18 recognised species, which has been based i.a. on the analysis of the 16S rRNA gene. They show a similarity of interspecies levels of the 16S rRNA genes ranging from 92.0 to 98.8 % (Collado and Figueras, 2011, http://www.bacterio.net/arcobacter.html. Status: October 2015). The taxonomy of *Arcobacter* spp. is illustrated in Figure 1. Compared to other members of the epsilon subdivision, rare information about *Arcobacter* spp. is available. But the genomic information from other epsilonproteobacterial taxa provides a solid foundation to compare and analyse *A. butzleri* to its taxonomic relatives (Miller et al., 2007). Miller et al. (2007) published the whole genome sequence of the *A. butzleri* strain RM4018. Although, *A. butzleri* is a member of the *Campylobacteraceae*, the majority of its proteome is most similar to those of *Sulfurimonas (S.) denitrificans* and *Wolinella* (*W.*) *succinogenes*, both members of

the *Helicobacteraceae*, and those of the deep-sea vent Epsilonproteobacteria *Sulfurovum* and *Nitratiruptor* (Miller et al., 2007). The high level of similarity of *A. butzleri* RM4018 with *Nitratiruptor*, *Sulfurovum* or *Sulfurimonas* suggest that the "close relatedness" of *Arcobacter* with the family *Campylobacteraceae* should be reviewed (Miller et al., 2007).

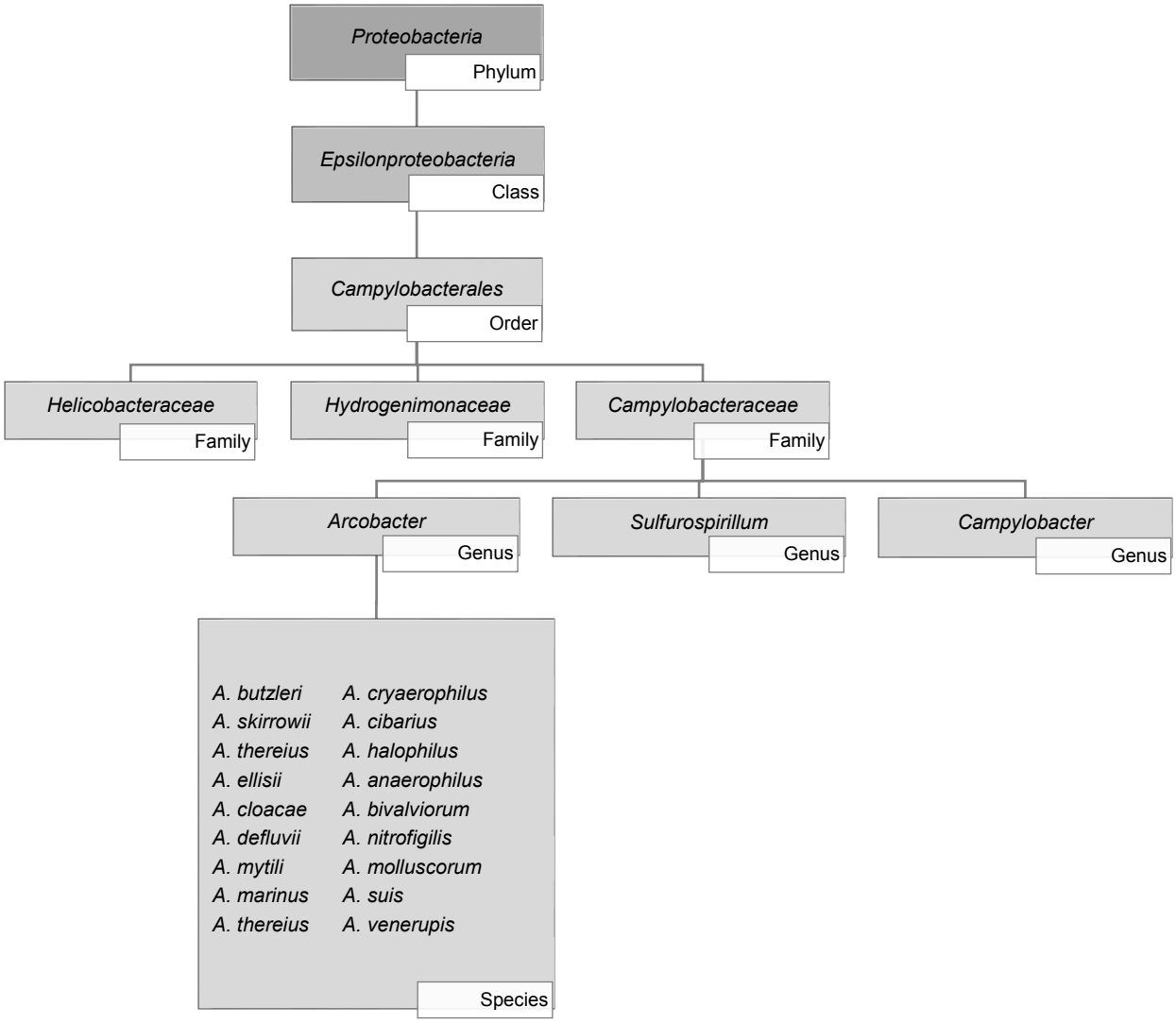

Figure 1: Taxonomy of *Arcobacter* spp.

1.1.2 Genomics

Five *Arcobacter* genomes have been completely sequenced, of which three were *A. butzleri* strains*: A. butzleri* RM4018, *A. butzleri* ED-1 and *A. butzleri* 7h1h (Miller et al., 2007; Toh et al., 2011; Merga et al., 2013). The genome sequence is an important first step in understanding the physiology and genetics of this species, which constitutes a bridge between the environment and mammalian hosts (Miller et al., 2007). *A. butzleri* RM4018 is a human clinical isolate (Miller et al., 2007). *A. butzleri* strain ED-1 is a microaerobic exoelectrogenic epsilonproteobacterium isolated from the electrode of an acetate-fed microbial fuel cell (Toh et al., 2011). *A. butzleri* strain 7h1h was isolated from the feces of a clinically healthy dairy cow (Merga et al., 2013). The genome *A. butzleri* RM4018 comprises 2,341,251 bp. It is considered to be the second largest epsilonproteobacterial genome; smaller than the genome of *Sulfurovum* strain NBC37-1 but larger than *S. denitrificans* strain ATCC 33889 and *W. succinogenes* strain DSM 1740. The RM4018 strain genome encodes 2259 coding sequences (CDSs), of which 1011 (45 %) were assigned a specific function, 505 (22 %) only a general function and 743 (33 %) an unknown function (Miller et al., 2007). The ED-1 genome consists of a circular 2,256,675-bp chromosome and contains 2,158 predicted protein-coding genes. 1454 (67 %) of the predicted proteins were assigned a known function, 639 (30 %) as conserved hypothetical genes and 65 % (3 %) as novel hypothetical genes (Toh et al., 2011). The *A. butzleri* 7h1h genome size is 2,253,233 bp and is predicted to carry 2199 genes, 5 ribosomal RNA operons and 54 tRNAs (Merga et al., 2013). The G+C content of the RM4018 genome (27 %) is remarkably low. The ED-1 genome consists a G+C content of 27.1 % and *A. butzleri* 7h1h a G+C content of 27.06 % (Merga et al., 2013; Miller et al., 2007; Toh et al., 2011).

The 7h1h genome is highly syntenic to both genomes (RM4018 and ED-1); no large-scale rearrangements were observed with respect to the other two genomes. 1,946 (88 %) of 2,199 genes of strain 7h1h were also identified in either RM4018 or ED-1 (Merga et al. 2013). ED-1 and RM4018 share 1,950 orthologous genes. The 16S rRNA gene sequence of *A. butzleri* ED-1 is identical to that in the sequenced genome of *A. butzleri* strain RM4018 (Toh et al., 2011). *A. butzleri* strain RM4018 was resistant to 42 of the 65 antibiotics tested. Strain RM4018 showed resistance to all macrolides and sulfonamides tested and most of the β-lactam antibiotics, some quinolones, and also to chloramphenicol (Miller et al., 2007).

No plasmids were detected in strain RM4018; therefore all resistance mechanisms would be chromosomal in nature (Miller et al., 2007). So far, no genes coding to antimicrobial resistance were identified in plasmids (Douidah et al., 2014).

Virulence genes

The first genome sequence of *A. butzleri* strain RM4018 has been published by Miller et al. (2007) and derived from a human clinical isolate and is considered to be the second largest genome within the *Epsilonproteobacteria* (Miller et al., 2007).

In fact, the genome sequence possesses several putative virulence genes homologous to those of *C. jejuni* (*cad*F, *cj*1349, *cia*B, *mvi*N, *pld*A and *tly*A) and other pathogens (*hec*B, *irg*A, *hec*A, and *iro*E). In *Campylobacter*, *cadF* and *cj1349* encode fibronectin-binding proteins which promote the binding of bacteria to intestinal cells (Flanagan et al., 2009; Konkel et al., 1999) while CiaB (*Campylobacter* invasive antigen B) contributes to host cell invasion. *Mvi*N is an essential protein required for peptidoglycan biosynthesis in *E. coli* (Ruiz, 2008) but there is no direct evidence for the involvement of MviN in virulence of *Salmonella* Typhimurium or *E. coli* (Inoue et al., 2008). The outer membrane phospholipase PldA is associated with lysis of erythrocytes (Grant et al., 1997). HecA is a member of the filamentous hemagglutinin (FHA) family, and involved in attachment, aggregation and epidermal cell killing of *Erwinia chrysanthemi* (Rojas et al., 2002). The *hecB* encodes a hemolysin activation protein (Miller et al., 2007) and *tlyA* a hemolysin which is also present in *Mycobacterium tuberculosis* and *Serpulina hyodysenteriae* (Wren et al., 1998). The iron-regulated outer membrane protein Irg*A* and the periplasmic enzyme IroE are functional components for the iron acquisition in *E. coli* and therefore required for establishing and maintaining infections (Mey et al., 2002; Rashid et al., 2006; Zhu et al., 2005). Douidah et al. (2012) investigated 182 *A. butzleri* isolates originating from different sources and identified the presence of all nine virulence genes (*iroE* was not investigated) in 15 % (26/182) of analysed strains. In agreement with Douidah et al. (2012), Karadas et al. (2013) and Tabatabaei et al. (2014) also detected the presence of 6 genes (*cad*F, *cia*B, *c*j1349, *mvi*N, *pld*A and *tly*A) in all *A. butzleri* tested strains (Douidah et al., 2012; Karadas et al., 2013; Tabatabaei et al., 2014).

Ho et al. (2008a) have reported the existence of two flagellin genes (*flaA* and *flaB*) in *A. butzleri*. *FlaA* is essential for the bacterial motility. *FlaB* may not play an important role in motility, but might be involved in bacterial pathogenicity (adhesion and invasion) or/and serve for antigenic variation which is important in host-parasite interaction (Ho et al., 2008a).

It has not been determined yet whether all these putative virulence factors are functional or have similar function as their homologues in other bacteria. Furthermore other, so far unidentified, virulence factors might be encoded by *A. butzleri.*

1.1.3 Morphology and Physiology

Arcobacter spp. are gram negative, non-sporeforming, spiral-shaped rods (0.2-0.9 µm x 1-3 µm). They are highly motile by having a single, unsheathed polar flagellum at either one or both ends of the cell (with the exception of the recently described *A. anaerophilus* without a flagella) and exhibit a characteristic corkscrew-like movement (Vandamme and De Ley, 1991; Vandamme et al., 1992b; Sasi Jyothsna et al., 2013).

The key distinguishing features of the genus *Arcobacter* used to differentiate them from *Campylobacter* are: the ability to grow at lower temperatures ranging from 15 to 30°C (psychrophilic) and under aerobic conditions. Their optimal growth occurs under microaerobic conditions (3-10 % O_2) at 30°C (Vandamme and De Ley, 1991; Vandamme et al., 1991; Vandamme et al., 1992b). Only *A. anaerophilus* is obligate anaerob (Sasi Jyothsna et al., 2013). Growth at 37°C under microaerobic conditions was observed for all *Arcobacter* species, except for *A. nitrofigilis* and *A. thereius*. Compared to thermophilic *Campylobacter*, which can grow up to 42°C, only some *Arcobacter* strains are able to grow at high temperature (summarised by Collado and Figueras, 2011). After 48 h incubation at 30°C aerobically, the organism produces 2-4 mm grey or whitish colonies on blood agar. Swarming may occur on fresh agar (Vandamme et al., 1991; Vandamme et al., 1992b). The genus *Arcobacter* shares many phenotypic characteristics of the family *Campylobacteraceae* (Vandamme and De Ley, 1991). *Arcobacter* species show similar morphological characteristics to *Campylobacter*, and also share the same positive reactions to the standard biochemical tests. They are chemoorganotrophs. Carbohydrates are neither oxidized nor fermented. *Arcobacter* obtain their energy from organic acids and amino acids as carbon sources via citric acid/ tricarboxylic acid cycle (Vandamme and De Ley, 1991; Collado and Figueras, 2011).

1.2 Clinical relevance of *A. butzleri*

1.2.1 *A. butzleri* in humans and animals

Arcobacter spp. have become more important in veterinary and human public health due to their presence in different sources such as animals including various food, raw milk and water. *Arcobacter* have been considered to possess potential zoonotic implications (Ho et al., 2006a; Collado and Figueras, 2011).

Many studies described three species, *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* in association with human and sometimes animal diseases (such as diarrhea, reproduction disorders, abortion, mastitis) (Kiehlbauch et al., 1991; Vandamme et al., 1992a; Schroeder-Tucker et al., 1996; Engberg et al., 2000; Lehner et al., 2005). Among these species, *A. butzleri* is the most important species of the genus for human infections and has been classified as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002).

A. butzleri in humans

Clinical signs of an *A. butzleri* infection in humans are similar to those of *C. jejuni*. *A. butzleri* infections display symptoms such as diarrhea with abdominal pain, nausea and vomiting or rarely fever. Compared to *C. jejuni*, *A. butzleri* caused more persistent and watery, but less acute and bloody diarrhea (Vandenberg et al., 2004). *A. butzleri* have been associated with human enteritis and occasionally bacteraemia worldwide (e.g. UK, Chile, France, Turkey, Germany, Taiwan, China) (Lerner et al., 1994; On et al., 1995; Yan et al., 2000; Lau et al., 2002; Kayman et al., 2012). Most clinical infections are single cases with the source of infection rarely identified. The frequency of recovery in human stool samples ranged from 0.1 to 3 % for *A. butzleri* (Collado and Figueras, 2011). In two independent studies performed in Belgium and France apart from *Campylobacter* (currently the most frequently notified infectious agent in Europe) *A. butzleri* was the fourth most common *Campylobacterales* organism recovered from human stools of patients with diarrhea (Vandenberg et al., 2004; Prouzet-Mauleon et al., 2006). *A. butzleri* were detected in 8 % of cases in U.S. and European travellers with acute diarrhea in a study performed in Mexico, Guatemala and India (Jiang et al., 2010). *A. butzleri* was isolated from the stools of 0.4 % of patients suffering from infectious gastroenteritis in The Netherlands and 1.25 % of the stool samples of patients with gastroenteritis in Turkey (Kayman et al., 2012;

De Boer et al., 2013). In a South African study, 15 *A. butzleri* were identified among 3,877 *Campylobacteraceae* strains isolated from children's diarrheic stools (Samie et al., 2007). In addition, Collado et al. (2013) detected a prevalence of 1.4 % for *A. butzleri* in diarrheic samples in Chile (Collado et al., 2013). This species has also been implicated in extra-intestinal invasive diseases. *A. butzleri* was isolated from the blood of a neonate. An in utero sepsis was suggested for this bacteraemia (On et al., 1995). This species was isolated from blood cultures of an elderly man with liver cirrhosis and a woman with acute gangrenous appendicitis (Lehner et al., 2005).

Besides the reports of single cases of *A. butzleri* associated infections, three *A. butzleri*-related outbreak data on human infection have been described. Vandamme et al. (1992a) reported an outbreak that occurred in an Italian nursery and primary school. Ten children suffered from recurrent abdominal cramps without diarrhea. *A. butzleri* was identified as the only enteropathogen isolated from all stool samples. Although the isolates showed identical phenotypic characteristics and protein profiles, the infection source could not be determined (Vandamme et al., 1992a). In the second outbreak in a scout camp in the United States, 117 persons complained about nausea and vomiting followed by abdominal cramps and diarrhea. Among other fecal indicators, *A. butzleri* was present in the drinking water samples, but was not recovered from a patient stool sample (Rice et al., 1999). The recent outbreak occurred in USA at a wedding occasion, where 51 wedding guests in USA suffered from diarrhea, nausea as well as abdominal cramps. In four cases, *A. butzleri* were cultured from stool samples of infected guests (Lappi et al., 2013). Although only few case reports and some outbreak data on human infection with *A. butzleri* are available, it indicates, that *A. butzleri* is a truly human pathogen.

Some studies have also reported the isolation of *A. butzleri* from stool samples of people without clinical symptoms (Vandenberg et al., 2004; Houf and Stephan, 2007). A study on Belgians described human asymptomatic carriage of *A. butzleri* as being more frequent than for *C. jejuni* but without significant differences (Vandenberg et al., 2004). The low level of incidence reported in human clinical samples and the relatively scarce knowledge about their relevance in human infections are due to the lack of standardized isolation and detection methods (Fallas-Padilla et al., 2014)

A. butzleri in animals

Arcobacter spp. are isolated from animal and food products of animal origin, which has led to classify this bacteria as emerging foodborne pathogen. *Arcobacter* were identified in feces of clinically healthy animals and in animals with pathologies such as abortion, mastitis, gastritis, diarrhea and reproduction illnesses. *A. cryaerophilus* is the species predominantly associated with animal diseases while *A. butzleri* and *A. skirrowii* are less common (Kiehlbauch et al., 1991; Schroeder-Tucker et al., 1996; De Oliveira et al., 1997; On et al., 2003; Van Driessche et al., 2003; Van Driessche et al., 2004; Van Driessche, 2005; Ferreira et al., 2015). The occurrence of *A. butzleri* in the digestive tract of healthy farm animals may act as significant reservoir and infection source to humans (Hume, 2001). In farm animals *A. butzleri* are found more frequently in pigs than in cattle. In a slaughterhouse in Japan *Arcobacter* were isolated from 3.6 % and 10 % of the cattle and swine fecal samples, along with 14.5 % of chicken cloacal swabs. *A. butzleri* was the most prevalent species with 83.3, 60.0 and 47.1 % of the cattle, swine and chicken isolates, respectively (Kabeya, 2003). From fecal samples collected at slaughterhouse in Belgium, *Arcobacter* was isolated from 43.9 % of porcine, 39.2 % of bovine, 16.1 % of ovine and 15.4 % of equine samples (Van Driessche et al., 2003). In this study the most commonly detected bacterium was *A. butzleri* followed by *A. cryaerophilus*. All the animals were clinically healthy displaying none of the typical symptoms of *A. butzleri* infection. The literature also described *Arcobacter* as a part of the poultry intestinal flora. Fecal shedding of *Arcobacter* in poultry (chicken, ducks, turkeys, and domestic geese) without displaying clinical symptoms, indicate that poultry is a natural reservoir of *Arcobacter* species (On et al., 2004; Atabay et al., 2006; Atabay et al., 2008).

A. butzleri has been isolated from both ill and healthy non-human primate *Macaca mulatta.* The primates suffered from diarrheal disease with associated chronic, active colitis. These data suggest that *A. butzleri* may be endemic in this primate population (Anderson et al., 1993; Higgins et al., 1999). In addition, the presence of *A. butzleri* in pet cats and dogs suggests that contact with these animals can also be a potential route of human infection (Petersen et al., 2007; Houf et al., 2008). Only few studies reported *A. butzleri* in association with enteritis and diarrhea in pigs, cattle, horses and ostriches (Kiehlbauch et al., 1991).

1.2.2 *A. butzleri* infection through food and water

Several pathways of *A. butzleri* transmission have been identified to date. However, consumption of *A. butzleri* contaminated food or water has been considered the major transmission pathways. A growing number of studies reporting the isolation of *A. butzleri* from food and water (Vandenberg et al., 2004; Lehner et al., 2005; Ho et al., 2006b; Collado and Figueras, 2011; Sha et al., 2011). Water may play an important role in the transmission of this bacteria to humans and animals and it underscores the potential role of *A. butzleri* as a waterborne pathogen (Rice et al., 1999). Also the genome analysis of *A. butzleri* RM4018 shows that this species is most likely an environmental organism that may cause disease through either water-mediated food contamination or ingestions of *A. butzleri*-contaminated water (Miller et al., 2007). *A. butzleri* were isolated from drinking water in Germany and Turkey, canal water in Thailand, river water in Italy, Spain and Japan, sewage water, brackish lakes in Italy and seawater from coastal environment (Dhamabutra et al., 1992; Jacob et al., 1993; Musmanno et al., 1997; Fera et al., 2004; Morita et al., 2004; Collado et al., 2008; Ertas et al., 2010). *A. butzleri* have been linked to one waterborne outbreak. The outbreak was related with a breakdown of the automated water chlorinating system. *A. butzleri* was found to be sensitive to chlorine disinfection. These bacteria have become a public health concern, as these organisms are able to grow at low temperatures and may attach to inner surfaces (made of stainless steel, copper, plastic) of water-distribution pipes and form biofilms. This is a significant problem threatening public health in those food processing plants with poor hygienic standards (Assanta et al., 2002). These facts further support its potential as a waterborne pathogen. Collado et al. (2008) investigated the presence of *Arcobacter* spp. in 205 water samples of freshwater, seawater and sewage in Spain. The dominant species was *A. butzleri* (94 %) and was significantly associated for the first time with bacterial indicators of fecal pollution (*E. coli* and intestinal enterococci) (Collado et al., 2008). There are few studies reporting the presence of *Arcobacter* in shellfish such as oysters, mussels and clams (Romero et al., 2002; Collado et al., 2009). Collado et al. (2009) reported a significant prevalence of *Arcobacter* in mussels (41 %) detecting i.a. *A. butzleri* and *A. cryaerophilus*. The fecal contamination of environmental waters plays an important role on the presence and potential risk of *Arcobacter* in shellfish (Collado et al., 2008; Collado et al., 2009). Products of animal origin have also been suggested as an important potential transmission route of *Arcobacter*, which are common in the intestinal tract and fecal samples of healthy and diseased animals (Hume et al., 2001, Van Driessche et al., 2003; Ho et al., 2006b). The Literature reported a high prevalence of *Arcobacter* in foods of animal origin, with the highest found in chicken, followed by pork, beef

and also from other kind of meats such as lamb or rabbit (Rivas et al., 2004; Collado et al., 2009). Poultry meat is considered as the most important source of human infection. The most predominant species is *A. butzleri*, followed by *A. cryaerophilus* and *A. skirrowi* (to a lesser extent) and other *Arcobacter* species. Kabeya et al. (2004) examined the prevalence of *Arcobacter* in retail meats and isolated *Arcobacter* species from 2.2 %, 7.0 % and 23.0 % of beef, pork and chicken meat, respectively. Among the isolates the most dominant *Arcobacter* species was *A. butzleri* (Kabeya et al., 2004). *A. butzleri* has been linked to one foodborne outbreak. Undercooked chicken meat was probably the infection source. 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. Manipulation of raw meats, consumption of undercooked products and cross-contaminations during slaughter process are the most likely cause of *A. butzleri* infection to humans (Van Driessche and Houf, 2007a; Shah et al., 2011).

Many studies suggested that contamination of meat products by *Arcobacter* probably occurs when fecal material comes into contact with carcasses (cross-contamination) during slaughtering process (Atabay and Corry, 1997, Ohlendorf and Murano, 2002; Lehner et al., 2005; Van Driessche et al*.,* 2007). For example, Ho et al. (2008b) isolated *Arcobacter* spp. from 100 % of the chicken carcasses (n=95) and also from the intestinal content of more than 50 % of these samples, suggesting the fecal route as the main source of contamination of the meat (Ho et al., 2008b). Studies have also shown the presence of *A. butzleri* on slaughter equipment and cross contamination can occur from contact of raw ingredients with the processing equipment (Houf et al., 2003).

Some studies reporting other sources for a potential human infection with *A. butzleri*. In the study of Scullion et al. (2006) in Northern Ireland, *A. butzleri* was the only species isolated from raw milk samples (46 %, n=101), even with higher prevalence than in pork (35 %, n=101) and beef (37 %, n=108) (Scullion et al., 2006). In another study, *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* were isolated from 15 (42.8 %) fresh cow's milk samples, 12 (73.3 %) shellfish samples, 11 (55 %), chicken samples, 2 (10 %) pork samples, and 1 (5 %) beef sample. *A. butzleri* was detected as the predominantly species (Nieva-Echevarria et al., 2013). Recently, *Arcobacter* spp. contamination in fresh vegetables has also been reported, which is a considerable risk to human health, since most vegetables are consumed raw (Gonzalez and Ferrus, 2011; Ferreira et al., 2015). Further studies are needed to study the risks of *Arcobacter* contamination in these foods (Gonzalez and Ferrus, 2011).

A. butzleri has a wide distribution and more investigations are needed to fully understand their routes of transmission to humans. Table 1 and 2 summarizes the prevalence of *Arcobacter* spp. and *A. butzleri* in salad, foods of animal origin and water. To enhance recovery and proper identification of *A. butzleri*, a standardised isolation method must be established to determine the prevalence in food and water to understand the true role of this species in human disease.

Table 1: Prevalence of *Arcobacter* spp. *and A. butzleri* in foods of animal origin

Source	Number of samples	*Arcobacter* positive (%)	*A. butzleri* positive (%)	Country	References
Chicken	22	73	73	Australien	(Rivas et al., 2004)
	100	23	15	Japan	(Kabeya et al., 2004)
	125	96,8	96,8	Kanada	(Lammerding et al., 1996)
	100	68	68	Turkey	(Aydin et al., 2007)
	123	39	39	Malaysia	(Amare et al., 2011)
	82	85,4	40	Germany	(Bartholomä, 2006)
Beef	32	22	22	Australien	(Rivas et al., 2004)
	90	2.2	1.1	Japan	(Kabeya et al.,2004)
	108	34,3	59,5	GB	(Scullion et al., 2006)
	179	37,4	20,7	Belgium	(De Smet et al., 2010)
Minced Beef	100	9	6	Belgium	(De Smet et al., 2010)
	97	5,2	5,2	Turkey	(Ongor et al., 2004)
Pork	21	29	29	Australien	(Rivas et al., 2004)
	100	7	4	Japan	(Kabeya et al., 2004)
	101	34,7	21,8	GB	(Scuillon et al., 2006)
	75	16	16	India	(Patyal et al., 2011)
	169	96,5	3	Belgium	(Van Driessche and Houf, 2007a)
Lamb	13	15	15	Australien	(Rivas et al., 2004)
Dairy cattle milk	101	45,5	44,6	GB	(Scuillon et al., 2006)
	50	6	2	Turkey	(Ertas et al., 2010)
Milk	188	3,2	0,5	Brasil	(Pianta et al. 2007)
	177	15	x	Finland	(Revez et al., 2013)
Mussel	56	41,1	17,9	Spain	(Collado et al. 2009)
Fish	50	34	32	Germany	(Lehmann et al., 2015)

Table 2: Prevalence of *Arcobacter* spp. and *A. butzleri* in vegetables and water

Source	Number of samples	*Arcobacter* positive (%)	*A. butzleri* positive (%)	Country	References
Salad	50	14	14	Spain	(Gonzalez and Ferrus, 2011)
Water					
Drinking water	100	3	3	Turkey	(Ertas et al., 2010)
Well water	25	4	4	Turkey	(Ertas et al., 2010)
River water	17	23,5	23,5	Japan	(Morita et al., 2004)
	29	58,6	55,2	Spain	(Collado et al., 2008)
Lake Water	29	27,6	27,6	Spain	(Collado et al., 2008)
Sea water	101	42,6	35,6	Spain	(Collado et al., 2008)
	19	100	26,3	Spain	(Collado et al., 2008)
Sludge	27	96,3	44,4	Spain	(Collado et al., 2008)
Sewage	15	66,7	46,7	Spain	(Gonzalez et al., 2007)

1.3 Virulence factors and pathogenicity

In order to understand how *A. butzleri* causes disease in humans, more information on potential virulence factors and pathomechanisms are required. Over the past decades, a variety of *in vitro* studies were used to clarify the pathogenic mechanisms related to *A. butzleri*. However, up to now, only a limited number of *in vivo* studies has been performed. Investigations on the pathogenicity of *A. butzleri* primarily based on the knowledge on *Campylobacter* (Musmanno et al., 1997; Johnson and Murano, 2002). *Campylobacter* and *A. butzleri*-related illnesses shares similar clinical features. Therefore, it might be expected that some *C. jejuni* virulence factors would be present in *A. butzleri*.

In vitro studies: Adhesion, invasion and cytotoxicity

The intestinal colonization process involves a wide range of strategies: Pathogens in the intestinal lumen have to overcome the mucus barrier, adhere to mucosal surfaces, increase their population and detach (Carbone et al., 2003). The adhesive and invasive capacities on epithelial cell lines were assigned to investigate bacterial pathogenic potential *in vitro*, as both might contribute to colonization and infection of the host.

A number of *in vitro* studies reported adhesion, invasion and cytotoxicity of *A. butzleri* in various cell lines of human origin like HT-29 and HT-29/B6 (cryptic cells), Caco-2 (colonocytes), HeLa and INT407 (cervix cells) and Hep-2 (larynx cells). Also cell lines of

animal origin like CHO, IPI-2, IPEC-2 (intestinal epithelial cells) and Vero (kidney epithelial cells) were investigated (Musmanno et al., 1997; Villarruel-Lopez et al., 2003; Carbone et al., 2003; Gugliandolo et al., 2008; Karadas et al., 2013; Levican et al., 2013). The *in vitro* studies on pathogenicity of *A. butzleri* are summarized in Table 4. A significant variation in adherence and invasion have been observed, independent of the origin of strains and the cell lines used. While a majority of *A. butzleri* strains had adhesive capacities not all were able to invade the human cell lines (Collado and Figueras, 2011)

Two studies have been reported on the adhesive ability of *A. butzleri* strains isolated from environmental (sea water and river water) samples (Musmanno et al., 1997; Carbone et al., 2003). In one, only one of 18 strains adhered to Hela and INT 407 cells and none have been invasive (Musmanno et al., 1997). In the other, only one third of the strains (six of 17) tested were found to be adhesive to HEp-2 and Hela cells (Carbone et al., 2003). However, 12 *A. butzleri* strains isolated from human stool specimens were able to adhere to HEp-2 cells, of which four invaded the cells (Vandenberg et al., 2004). Similarly, Ho et al. (2007) showed that all eight strains of four *Arcobacter* species tested, among others *A. butzleri*, had the ability to adhere to both human Caco-2 and porcine IPI-2I cells, but none *A. butzleri* strain was invasive (Ho et al., 2007).

Karadas et al. (2013, 2015 Chapter 3) studied the effect of *A. butzleri* isolates on different humans (HT-29, HT-29/B6 and Caco-2) and porcine (IPEC-J2) intestinal epithelial cell lines. They concluded, that adhesion and invasion of *A. butzleri* occurs more prominent in cell lines derived from human colon cells compared to cells derived from porcine jejunal cells (Karadas et al. 2013; Karadas et al. 2015 Chapter 3).

Studies have shown that *A. butzleri* displayed cytotoxicity against Vero, HeLa and INT407 cells with rounding and nuclear pyknosis. Cell elongation (cytotonic effect) and/or cytoplasmal vacuole formation has been observed on Vero and CHO cells by certain *A. butzleri* strains (Musmanno et al., 1997; Johnson and Murano, 2002; Villarruel-Lopez et al., 2003; Carbone et al., 2003; Gugliandolo et al., 2008). This cytotoxic effect was similar to that observed by *C. jejuni* cytolethal distending toxin (CDT). Therefore the presence of *C. jejuni cdt* genes was studied in some *Arcobacter* species isolated from different sources, but no homologues were found. This agreed with the genome sequence of *Arcobacter butzleri* strain RM4018. Although some virulence factors identified in *C. jejuni* have homologs within the genome of *A. butzleri* strain RM4018, several *Campylobacter* virulence-associated genes were not identified including the genes encoding the cytolethal distending toxin *cdt*-ABC (Miller et al., 2007).

Bücker et al. (2009) suggested that the toxin is a heat-sensitive protein, cell-associated, rather than secreted with an apoptotic and necrotic effect (Bücker et al., 2009). Therefore, further research is still needed to better understand the mechanism of pathogenesis in *A. butzleri.*

Table 3: Pathogenesis of *A. butzleri* on different cell lines

Cell line	Strain origin	Number of positive/number of tested strains in regard to			
		Adhesion	Invasion	Cytotoxicity	References
Hep-2	Sea water	6/17 ǂ	ND	ND	(Carbone et al., 2003)
	Zooplankton	4/4	ND	ND	(Gugliandolo et al., 2008)
	Human feces	3/3	ND	ND	((Fernandez et al., 2010)
	Animal (mussels and feces from pelican, bovine, duck, dog, sparrow	33/33	ND	ND	(Fernandez et al., 2010)
	River water	15/15	ND	ND	(Fernandez et al., 2010)
	Meat (chicken giblets and carcass)	21/21	ND	ND	(Fernandez et al., 2010)
HeLa	River water	1/18	0/18	ND	Musmanno et al., 1997
	River water/Human (diarrhea)/ Animal (cattle and poultry)	ND	ND	7/7	(Johnson and Murano, 2002)
	Sea water	6/17 ǂ	ND	ND	(Carbone et al., 2003)
	Zooplankton	4/4	ND	ND	(Gugliandolo et al., 2008)
Vero	River water	ND	ND	17/18 ǂ	(Musmanno et al., 1997)
	Sea water	ND	ND	5/17	(Carbone et al., 2003)
	Meat (pork, chicken, beef)	ND	ND	76/80	(Villarruel-lopez et al., 2003)
	Zooplankton	ND	ND	3/4	(Gugliandolo et al., 2008)
INT407	River water	1/18	0/18	ND	(Musmanno et al., 1997)
	River water/ Human (diarrhea)/ Animal (cattle and poultry)	ND	ND	6/6	(Johnson and Murano, 2002)
CHO	River water	ND	ND	17/18	(Musmanno et al., 1997)
Caco-2	Human (blood)	1/1	0/1	ND	(Ho et al., 2007)
	Meat (turkey, duck, pig, chicken, beef)	6/6	6/6	ND	(Levican et al., 2013)
	Animal (clams, mussels)	4/4	3/4	ND	(Levican et al., 2013)
	Sewage	2/2	2/2	ND	(Levican et al., 2013)
	Human	3/3	3/3	ND	(Karadas et al., 2013)
	Meat (chicken)	3/3	3/3	ND	(Karadas et al., 2013)
IPI-2I	Human (blood)	1/1	0/1	ND	(Ho et al., 2007)
HT-29	Human	2/3	1/3	ND	(Karadas et al., 2013)
	Meat (chicken)	2/3	2/3	ND	(Karadas et al., 2013)
HT-29/B6	Human Meat (chicken)	2/3 3/3	2/3 3/3	1/3 2/3	(Karadas et al., 2015, Chapter 3)
IPEC-J2	Human Meat (chicken)	2/3 2/3	0/3 2/3	0/3 0/3	(Karadas et al., 2015, Chapter 3)

Adapted and updated from Ferreira et al. (2015).
*Caco-2, human colorectal adenocarcinoma cell line; CHO, Chinese hamster ovary cell line; HeLa, human cervix carcinoma cell line; Hep-2, human cervix carcinoma cell line (HeLa contamination); INT407, human embryonic intestine (might be HeLa derivative); HT-29, human colorectal adenocarcinoma cell line; IPI-2I, porcine intestinal

epithelioid cell line; Vero, kidney epithelial cell line; HT-29/B6, human colorectal adenocarcinoma cell line with mucus (clon of HT-29); IPEC-J2, porcine intestinal epithelium cell line
ND, not determined.
Ɨ The authors considered strains as non-adherent if the mean adhesion index were < 10 microorganism/cell.
ǂ The strain presenting no cytotoxic effects showed to induce cell morphological changes related with cytotoxic effect.

Barrier impairment

The intestinal epithelium is the largest mucosal surface providing an interface between the external environment and the mammalian host, and its permeability depends on the regulation of intercellular tight junctions (TJs) as well as on the activity of transcellular transport (Fasano et al., 2008). Tight junctions (TJ) are a multifunctional complex and seal the paracellular space between neighboring epithelial cells. TJs prevent paracellular diffusion of microorganisms and other antigens across the epithelium and regulate the entry of nutrients, ions, and water while restricting pathogen entry (Ulluwishewa et al., 2011). Enteric pathogens can cause barrier impairment by inducing TJ dysfunction, which is characterized either by expression changes or by redistribution or disruption of TJ proteins. All of this lead to a passive efflux of water and solutes from the circulation into the lumen of the intestine (leak flux diarrhea) (Bücker et al., 2009). Objectively, the function of TJs can be measured as a decrease in transepithelial electrical resistance (TER) and an increase in the paracellular flux of macromolecules.

The potential mechanism by which *A. butzleri* might induce enteritis have been published by Bücker et al. in 2009. *A. butzleri* strain CCUG 30485 was demonstrated to induce epithelial barrier dysfunction in cell culture assays. The infection of HT-29/B6 human colon cell monolayers by *A. butzleri* led to changes in tight junction protein composition and distribution. Expression of the tight junction proteins claudin-1, -5, and -8 was reduced and redistributed off the tight junctional strands forming intracellular aggregates. Moreover, an induction of necrosis and caspase-3-dependent epithelial apoptosis was observed. Epithelial cell death together with the tight junction changes might represent a leak flux type of barrier dysfunctions as pathomechanism for the *A. butzleri*-associated diarrhea (Bücker et al., 2009).

Immune response

The innate immune system serves as the first line of defence against a pathogen. A series of proinflammatory cytokines (IL-lα/p, IL-6, tumour necrosis factor-α (TNF-α), the chemokines such as IL-8, and the interferons) are synthesised *de novo* following bacterial or viral infections (Lunney, 1998). Little is known about the interaction of *A. butzleri* with the human immune system.

One study investigated host cell cytokine responses to four species of *Arcobacter* among others *A. butzleri*, using human (Caco-2) and porcine (IPI-2I) intestinal epithelial cell lines. They reported the ability of these *Arcobacter* strains to induce expression of the proinflammatory cytokine IL-8, which is also produced after *Helicobacter pylori* and *Campylobacter* spp. infections. An upregulation of IL-8 expression 2 h after infection was observed but no correlation between cell invasion or strong adhesion of the strains and the level of interleukin-8 induction was found (Ho et al., 2007).

The impact of *A. butzleri* on human macrophages using THP-1 derived macrophages as an *in vitro* infection model was studied by zur Bruegge et al. (2014). Pro-inflammatory response of macrophages (IL-1α, IL-1ß, IL-6, IL-8, IL-12ß, TNFα) infected with *A. butzleri* was induced. *A. butzleri* strains were able to survive and resist the hostile environment of phagocytic immune cells for up to 22 h. *A. butzleri* strains showed differences in invasion and survival capabilities THP-1 cells, which suggests isolate dependent phenotype variations and different virulence potentials (zur Bruegge et al., 2014).

***In vivo* studies**

So far it is still unknown whether *A. butzleri* adhere and invade human cells *in vivo* and which part of the human intestine might be affected. The colonization mechanisms and pathogenicity of *A. butzleri* to host tissues remain largely unknown. Few *in vivo* experiments have been performed with *A. butzleri* in different animal models.

Wesley et al. (1996) used cesarean-derived colostrum-deprived 1-day-old piglets to study the pathogenicity of *Arcobacter* spp. on the basis of the duration of fecal shedding and colonization of tissue. *A. butzleri* was isolated from feces and different organs (e.g. lung, brain, kidney, stomach and small intestine) up to 10 days after oral inoculation of piglets. The intestinal colonization and multiplication was demonstrated. In contrast, *A. cryaerophilus* and *A. skirrowii* showed only a short duration of fecal shedding with no isolation from the organs

(Wesley et al., 1996). Therefore *A. butzleri* indicates to be potentially more virulent. In another study, these *A. butzleri* were not able to colonize 3-days-old outbred chickens after oral infection with a high dosis of *A. butzleri*. In outbred turkeys *A. butzleri* showed a low susceptibility, but were able to colonize significantly (65 % overall) in inbred Beltsville White turkeys (Wesley and Baetz, 1999). The mortality rate varied between 17-54 % according to strains used for infection. Agglutination of *A. butzleri* strains with human, rabbit, and sheep erythrocytes revealed the presence of adhesion molecules in *Arcobacter*. A nonfibrial immunogenic hemagglutinin of 20 kDa was identified, which could interact with a D-Galactose-containing erythrocyte receptor on the surface of the blood cells.

In a different study, two strains of *A. butzleri* isolated from stool of healthy chickens were tested on albino rat by giving a single oral challenge to 65 healthy adult rats. All rats suffered from diarrheal illness from the fifth day and resolved from day 21 post infection. Histopathological lesion such as hepatic necrosis, villous erosion, desquamation, matting and necrosis of the segments of small intestine belonged to the observed clinical symptoms. The toxic ileitis necrosis pattern of pathology in the gut indicates persistent watery diarrhea, which display the clinical presentation of *Arcobacter* infection in humans (Adesiji et al., 2009).

A single oral challenge of healthy adult rats with *A. butzleri* and *A. cryaerophilus* produced infection in 100 % of the animals. Clinical symptoms such as diarrheal illness, weight loss, rough coat, and reduced activity and appetite were observed (Adesiji, 2010). Diarrhea was self-limiting after three weeks, although fecal shedding of *Arcobacter* was noted up to five weeks post infection. Experimental infection was dose-dependent with higher bacterial doses, which caused pyrexia (Adesiji et al., 2012). These studies showed the role of *A. butzleri* in diarrheal diseases. In a recent study, the possibility of enhancing the adherence capacity of *A. butzleri* was investigated by using a mouse intraperitoneal passage model. After several serial passages, low adherent *A. butzleri* strains showed an increased adhesive ability to Hep-2 cells. Furthermore, an increase *fla*A gene expression in strains was observed after increased number of intraperitoneal passages (Fernandez et al., 2013). Further research are needed to fully understand *A. butzleri* virulence mechanisms and determined host response upon *A. butzleri* infection.

In the following, the pathogenic potential and the pathogenicity of several *A. butzleri* strains by testing their abilities for adhesion, invasion and cytotoxicity in humans and porcine cell lines have been studied. We also investigated the potential of these *A. butzleri* strains to impair the transepithelial electrical resistance, as a measure of a pathomechanism that describes epithelial

barrier dysfunction. Further, the pathogenic potential of *A. butzleri in vivo* was studied in a gnotobiotic IL-10 deficient mouse model.

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Chapter 2: Presence of virulence genes, adhesion and invasion of *Arcobacter butzleri*

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Presence of virulence genes, adhesion and invasion of *Arcobacter butzleri*

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Running headline: Virulence genes, adhesion and invasion of *A. butzleri*

2.1 Abstract

Aims: The pathogenic potential of *Arcobacter butzleri* isolates was investigated by detecting the presence of putative virulence genes and analysing the adhesive and invasive capabilities in cell cultures of human cell lines.

Methods and Results: The presence of ten putative virulence genes in 52 *A. butzleri* isolates was determined by PCR. The genes *ciaB, mviN, pldA, tlyA, cj1349* and *cadF* were detected in all, whilst *irgA* (15 %), *iroE* (60 %), *hecB* (44 %) and *hecA* (13 %) were detected only in few *A. butzleri* isolates. On HT-29 cells, four of six isolates adhered to and three of them were able to invade, whilst all six isolates adhered to and invaded Caco-2 cells with higher degrees. The genes *ciaB*, *cadF* and *cj1349* of all six isolates were sequenced, but no considerable changes of the amino acids in putative functional domains were observed.

Conclusion: Selected *A. butzleri* isolates adhere to and invade HT-29 and Caco-2 cells, which emphasize their human pathogenic potential. The efficiency of invasion depends on the eukaryotic cell line and individual bacterial strain used. We could not show any functional correlation between the amino acid sequence of CadF, CiaB or Cj1349 and the adhesive and invasive phenotype.

Significance and Impact of the Study: We have shown that some *A. butzleri* strains invade various cell lines. This underlines their pathogenic potential and hints at their relevance in human disease.

Keywords: *A. butzleri*, adhesion, invasion, cell lines, virulence genes

2.2 Introduction

Arcobacter spp. are gram-negative, motile and spiralshaped bacteria belonging to the family of *Campylobacteraceae*. They are increasingly isolated from a wide range of food of animal origin and water. The highest prevalence in food is reported for poultry meat, followed by pork and beef (Rivas et al. 2004; Van Driessche and Houf 2007a, b). *Arcobacter* spp. have been rated a serious hazard to human health by the International Commission on Microbiological Specifications for Foods in 2002 (ICMSF, 2002). At present, 17 *Arcobacter* species have been characterized with *A. butzleri* as the most important and predominant species associated with human disease (Vandenberg et al. 2004). *A. butzleri* infections may result in abdominal pain with acute diarrhea or prolonged watery diarrhea for up to 2 months (Vandenberg et al. 2004). Little is known about the pathogenic mechanisms and putative virulence genes of *A. butzleri*.

The genomic sequence of *A. butzleri* RM 4018 revealed the presence of 10 putative virulence genes: *cadF, mviN, pldA, tlyA, cj1349, hecB, irgA, hecA, ciaB* and *iroE* (Miller et al. 2007), but it is still unknown whether these putative virulence factors have similar functions as described for their homologues in other bacterial species. MviN is an essential protein required for peptidoglycan biosynthesis in *E. coli* (Ruiz 2008), but there is no direct evidence for the involvement of MviN in virulence of *Salmonella* Typhimurium or *E. coli* (Inoue et al. 2008). In *Campylobacter*, *cadF* and *cj1349* encode fibronectin-binding proteins, which promote the binding of bacteria to intestinal cells (Konkel et al. 1999; Flanagan et al. 2009), whilst *Campylobacter* invasive antigen B (CiaB) contributes to host cell invasion. HecA is a member of the filamentous hemagglutinin family and involved in attachment, aggregation and epidermal cell killing of *Erwinia chrysanthemi* (Rojas et al. 2002). The *hecB* encodes a haemolysin activation protein (Miller et al. 2007) and *tlyA* a haemolysin, which is also present in *Mycobacterium tuberculosis* and *Serpulina hyodysenteriae* (Wren et al. 1998). The outer membrane phospholipase PldA is associated with lysis of erythrocytes (Grant et al. 1997). The iron-regulated outer membrane protein IrgA and the periplasmic enzyme IroE are functional components for the iron acquisition in *E. coli* and therefore required for establishing and maintaining infections (Mey et al. 2002; Zhu et al. 2005; Rashid et al. 2006).

The potential virulence of *A. butzleri* isolates on CHO, Vero, HeLa and INT 407 cell lines was investigated by several authors. *A. butzleri* isolates showed in vitro cytotoxic effects in CHO cells and Vero cells, but any toxin genes were not identified yet. Adhesion of *A. butzleri* was observed in several cell line models (Musmanno et al. 1997; Johnson and Murano 2002; Carbone et al. 2003; Ho et al. 2007; Gugliandolo et al. 2008), but data on its ability to invade

are sparse (Vandenberg et al. 2005). The aim of this study was to examine the presence of putative virulence genes in *A. butzleri* strains isolated from different sources, to investigate the capability of *A. butzleri* to adhere to and invade into the cell lines HT-29 and Caco-2 and to analyse the sequence of putative invasive and adhesion genes.

2.3 Material and Methods

Bacterial strains

All strains used in this study are listed in Table 1. *A. butzleri* were isolated from fresh meat (chicken meat, n = 23; pork, n = 12; mixed minced meat of pork and beef, n = 2), from water (n = 9) and from humans (n = 6). *A. butzleri* CCUG 30485 was included as reference strain. All isolates were grown on Mueller–Hinton blood agar (MHB; Oxoid, Wesel, Germany) or in Brucella Broth (BB; BD, Heidelberg, Germany) at 30°C in a micro-aerobic atmosphere (5 % O_2, 10 % CO_2) generated by the Anoxomat system (Mart, Drachten, The Netherlands). For the adhesion and invasion assays, the *A. butzleri* isolates were precultured overnight in BB at 30°C. The optical density of each preculture was measured at 600 nm and 10 ml BB inoculated obtaining an OD = 0.001. These cultures were further incubated for 24 h at 30°C. A total OD = 1 was centrifuged and resuspended in 450 µl PBS. Each well in the adhesion and invasion assay was inoculated with 50 µl of this bacterial suspension containing approx. 5 x 10^8 CFU.

Cell culture

The human colon adenocarcinoma cells HT-29 (DSMZ ACC 299) were grown in 25-cm^2 tissue culture flasks in RPMI1640 medium (Biochrom, Berlin, Germany) supplemented with 10 % foetal calf serum superior (Biochrom) at 37°C in a 5 % CO_2 humidified atmosphere until a confluence of approx. 80 % was reached. For adhesion and invasion assays, each well of a 24-well plate was seeded with 2 x 10^5 HT-29 cells and incubated for 24 h. The human colon adenocarcinoma cells Caco-2 (DSMZ ACC 169) were grown in 75-cm^2 tissue culture flasks in DMEM medium (Biochrom) supplemented with 10 % foetal calf serum superior (Biochrom), 1 % nonessential amino acids (Biochrom) and 5 µl ml^{-1} gentamicin (Roth, Karlsruhe, Germany) at 37°C in a 5 % CO2 humidified atmosphere until a confluence of approx. 90 % was reached. For adhesion and invasion assays, each well of a 24-well plate was seeded with 5 x 10^4 Caco-2 cells and incubated for 21 days with media changes every third day but without gentamicin.

DNA extraction

Bacteria were suspended in 500 µl 0.1 x TE buffer (1 mmol l^{-1} Tris/HCl, pH 8.0, 100 µmol l^{-1} EDTA; Roth) and centrifuged for 5 min at 16 000 g. The pellets were resuspended in 250 µl 5 % Chelex (Bio-Rad, Munich, Germany) and incubated for 1 h at 56°C followed by 15 min at 95°C. The supernatants obtained after centrifugation were stored at 4°C until used for PCR.

PCR assays

Detection of putative virulence genes was performed by PCR. Primers and PCR protocols for partial amplification of *cadF*, *pldA*, *irgA*, *hecA* and *hecB* were used according to Douidah et al. (2012). Primers for partial amplification of *cj1349*, *ciaB*, *mviN*, *tlyA* and *iroE* were designed with Primer3 version 0.4.0 (Rozen and Skaletsky, 2000) based on the published sequence of the whole-genome *A. butzleri* RM 4018 (ATCC 49616). All primers used in this study are listed in Table 3. PCRs were carried out in a total volume of 25 µl containing 1 x PCR buffer (Qiagen, Hilden, Germany), 200 µmol l^{-1} of each dNTP (Fermentas, St. Leon-Rot, Germany), 12.5 µmol l^{-1} of each primer, 0.5 U Taq-Polymerase (Qiagen) and 2 µl DNA. An initial denaturation step at 95°C for 4 min was followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and elongation at 72°C for 30 s and a final elongation step for 6 min at 72°C. To investigate whether adhesive and invasive phenotypes depend on alterations in the amino acid sequence of CadF, Cj1349 or CiaB, the genes of these proteins were sequenced and in silico-translated. The complete genes *cj1349*, *ciaB* and *cadF* were amplified in a total volume of 50 µl containing 1 x PCR buffer, 200 µmol l^{-1} of each dNTP, 12.5 µmol l^{-1} of each primer, 0.5 U Taq-Polymerase and 5 µl DNA. An initial denaturation step at 95°C for 5 min was followed by 36 cycles of denaturation (94°C, 60 s), primer annealing (the temperatures are indicated in Table 3) for 60 s and elongation at 72°C for 60 s followed by final elongation for 5 min at 72°C. After gel electrophoresis, PCR products were visualized with GR Green (Labgene, St. Ingbert, Germany) under UV light. PCR products were purified by GeneJet PCR Purification Kit (Fermentas) and sequenced (GATC Biotech, Konstanz, Germany) with primers shown in Table 3. Sequences were trimmed and cds-translated with standard genetic code by EditSeq (DNASTAR Lasergene version 7, Madison, WI, USA). Alignment of the amino acid (aa) sequences was performed with Multalin version 5.4.1 (Corpet 1988).

Adhesion and invasion assay

The adhesion and invasion assays with HT-29 and Caco-2 cells were performed with six randomly selected *A. butzleri* isolates (three chicken meat isolates, C1–C3; three human isolates, H1–H3). HT-29 cells and Caco-2 cells were infected with approx. 5 x 10^8 bacteria. This corresponds to an MOI 1000 for HT-29 cells. The MOI for Caco-2 cells could not be calculated as cells were only enumerated before seeding. For adhesion assay, infected monolayers were incubated for 1 h at 37°C and rinsed three times with phosphate-buffered saline to remove unbound *A. butzleri*. To count adherent *A. butzleri*, HT-29 and Caco-2 cells were lysed with 500 µl 1 % SDS for 10 min. To determine the invasive abilities of *A. butzleri*, monolayers were infected for 3 h, and extracellular bacteria were killed by 2-h incubation at 37°C with 300 µg ml^{-1} gentamicin (Roth). Cell monolayers were rinsed three times with PBS and lysed with 500 µl 1 % SDS for 10 min. As control, the *Campylobacter* (*Camp.*) *jejuni* 81-176 strain was included in the adhesion and invasion assays. For *Camp. jejuni* 81-176, cells were lysed with 1 % Triton X-100 (Roth). *Camp. jejuni* 81-176 was cultivated as described for *A. butzleri* except at 37°C. Total numbers of adherent or invasive bacteria were determined by plating serial dilutions of respective lysates on MHB agar, which were incubated for 48 h at 30°C. Each experiment was performed in triplicates, and average cell numbers were calculated out of three infected wells in each approach. Adhesion index and invasion index are calculated as ratio of adhering and invading bacteria of the inoculum, respectively.

Statistical analysis

P-values were calculated by nonparametric Mann–Whitney U-test (GraphPad Prism version 5, La Jolla, CA, USA). Results were considered significant at $P < 0.05$. Data were shown as median with interquartile range (IQR).

2.4 Results

Presence of putative virulence genes in Arcobacter butzleri

The presence of ten putative virulence genes in the 52 investigated *A. butzleri* strains is summarized in Table 2. Thirteen per cent (7 of 52) of the strains possessed all ten genes, whilst 31 % of isolates carried only six genes (*iroE*, *hecB*, *irgA* and *hecA* were not detected). In 25 % of the isolates, *hecB*, *irgA* and *hecA* were missing; in 10 %, *iroE*, *irgA* and *hecA* were absent; in 17 %, *irgA* and *hecA* were missing; and in 4 %, only *hecA* was missing (Fig. 3). All

investigated *A. butzleri* isolates carried the genes *mviN*, *cadF*, *cj1349*, *ciaB*, *tlyA* and *pldA* (Table 2). The gene *iroE* was detected in 50% of the human (3 of 6), pork (6 of 12) and minced meat (1 of 2) isolates and in 65–67 % of the chicken meat (10 of 23) and water (6 of 9) isolates. None of the two isolates from minced meat encoded *hecB*, but 33 % of the human isolates, 42–43% of the pork and chicken meat isolates and 67 % of the water isolates. The genes *irgA* and *hecA* were detectable in 17 % of the human isolates, 9 % of chicken meat isolates, 44 % of water isolates, but in none of the minced meat isolates. In isolates recovered from pork meat, only *irgA* (17 %) but not *hecA* was detected.

Adhesive and invasive abilities of *Arcobacter butzleri*

Three *A. butzleri* isolates recovered from chicken meat (C1-3) and three from humans (H1-3) were tested for their adhesive and invasive properties in HT-29 cells (Fig. 1). Two human and two chicken meat *A. butzleri* isolates adhered to HT-29 cells (Fig. 1a). Highest adherence was observed with the isolate C2 (1.14 x 100 fold [3.02×10^{0}] of the inoculum), followed by H1 (5.4×10^{-1} fold [5.76×10^{-1}]), H3 (1.49×10^{-1} fold [2.12×10^{0}]) and C1 (1.3×10^{-1} fold [9.22×10^{-1}]). Both chicken isolates showed invasion in HT-29 cells (C1 with 4.26×10^{-5} fold [1.54×10^{-4}] and C2 with 5.05×10^{-5} fold [2.73×10^{-4}] of the inoculum; Fig. 1b). Of the two adherent human isolates, only the human isolate H1 was invasive (2.86×10^{-5} fold [3.8×10^{-5}]). The chicken isolate C3 showed adhesion and invasion in one of three approaches only, but to a far lesser extent (1.0×10^{-5} fold [2.65×10^{-5}] and 5.0×10^{-7} fold [9.76×10^{-7}]). The differences between the adhesive and nonadhesive or between invasive and noninvasive *A. butzleri* isolates, respectively, were statistically significant. As control, the *Camp. jejuni* 81-176 strain was investigated. This strain showed 5.64×10^{-3} fold [3×10^{-3}] adhesion and 2.93×10^{-4} fold [2.7×10^{-4}] invasion on HT-29 cells. This result shows differences in the abilities of *A. butzleri* strains to adhere to and to invade on HT-29 cells, independent of their origin. All isolates investigated were adhesive on Caco-2 cells (Fig. 2a). Highest adherence was observed for the human isolate H1 (3.2×10^{0} fold [1.91×10^{0}]) followed by the chicken isolates C2 and C1 (1.37×10^{0} fold [2.18×10^{0}] and 1.07×10^{0} fold [1.35×10^{0}]). The isolates H3, C3 and H2 showed lower adherence with 7.55 9 10^{-2} fold [3×10^{-2}], 5.71×10^{-3} fold [1.2×10^{-3}] and 1.25×10^{-4} fold [2.45×10^{-3}]. These values were all significantly different ($P < 0.05$), except for C2 and C1. Further, all isolates were invasive on Caco-2 cells (Fig. 2b). The isolates H2, H3 and C3 showed lesser invasion (1.88×10^{-5} fold [6×10^{-5}], 1.43×10^{-4} fold [1×10^{-4}] and 6.47×10^{-4} fold [7×10^{-3}]) than the isolates C2 and H1 (1.52×10^{-3} and 1.47×10^{-3} fold

[2 x 10^{-3} and 2.5 9 10^{-3}]) and C1 (5.0 x 10^{-3} fold [1.5 9 10^{-2}]). On Caco-2 cells, 5.41 x 10^{-3} fold [2.5 x 10^{-3}] of the control strain *Camp. jejuni* 81-176 adhered to and 5.88 x 10^{-3} fold [1.5 x 10^{-2}] invaded.

Comparison of CadF, CiaB and Cj1349 amino acid (aa) sequences

To elucidate whether the different adhesive and invasive phenotypes depend on alterations in CadF, Cj1349 or CiaB, the genes of these proteins were sequenced and *in silico*-translated employing the standard genetic code in the six investigated *A. butzleri* isolates. The Cj1349 aa sequences of the H2, H3, C1, C2 and C3 isolates showed only 0.9-1.6 % substitutions compared with the reference strain *A. butzleri* CCUG 30485 (H1), but all isolates showed a conserved motif D/E-X-W/Y-X-H (Fig. 4A; positions 367-371). This motif is part of a conserved domain of the DUF814 super family, occurring in proteins annotated as fibrinogen-/fibronectin-binding proteins, and might be functionally important (Fig. 4A). When comparing the CadF sequences of isolates investigated with the reference strain *A. butzleri* CCUG 30485, aa alterations varied from 1.5 to 3.25 %. These alterations were often located at the same position (Fig. 4B). The fibronectin-binding site of *Camp. jejuni* (FRLS) described by Konkel et al. (2005) is substituted to YNLA in *A. butzleri*, but this site is conserved within the analysed isolates (box in Fig. S2B). Pronounced variations could be observed within the CiaB sequences (Fig. 4C). The aa sequences of H2 and H3 had the same lengths (630 aa) as the reference strain *A. butzleri* CCUG 30485 whilst CiaB in C2 is composed of 633 aa, in C3 of 623 aa and in C1 of 523 aa. The CiaB sequences of C1 and C2 showed a three-aa insert (AKS) in between positions 332 and 333. However, all isolates showed aa substitutions of 3-5 % compared with the reference strain. But they all shared the conserved zinc-binding domain TIGHEYGHIL at positions 441-450 (Onozato et al. 2009). In the isolates C1 and C3, isoleucine is substituted by valine I442V (box in Fig. 4C).

2.5 Discussion

Arcobacter butzleri is considered a potential foodborne pathogen (summarized by Collado and Figueras 2011), but little is known about the pathogenic mechanisms and putative virulence factors. Investigations on the pathogenicity of *A. butzleri* primarily based on the knowledge on *Campylobacter* (Musmanno et al. 1997; Johnson and Murano 2002). However, a study by Miller et al. (2007) indicated that the proteome of *A. butzleri* shares greater phylogenetic similarities with members of the *Helicobacteraceae* like *Sulfurimonas denitrificans* and

Wolinella succinogenes and those of the deep-sea vent Epsilonproteobacteria *Sulfurovum* and *Nitratiruptor* than with *Campylobacter*. In this study, we investigated the presence and distribution of putative virulence genes in *A. butzleri* isolates recovered from different sources (n = 52, including the reference strain *A. butzleri* CCUG 30485). Only the genes *mviN*, *cadF*, *cj1349*, *ciaB*, *pldA* and *tlyA*, which share homologies to genes in *Campylobacter*, were identified in all *A. butzleri* isolates. This is in agreement with Douidah et al. (2012) who detected these genes in all 182 investigated *A. butzleri* isolates originating from different sources. Furthermore, these authors identified the presence of nine virulence genes (*iroE* was not investigated) in 15 % of analysed strains, which is comparable to our results (13 %). In contrast to Douidah et al. (2012), we could confirm neither the similar occurrence of hecA in human, pork and chicken meat isolates nor the higher prevalence of hecB in human isolates compared with pork and chicken meat isolates. Furthermore, we could not detect a lower occurrence of irgA in pork isolates compared with human and chicken meat isolates. However, these conflicting results might be due to the lower number of isolates included in our study. Previous studies demonstrated the capacity of *A. butzleri* to adhere to different cell lines such as HeLa, Hep-2, INT 407, Caco-2 and IPI-2I (Musmanno et al. 1997; Ho et al. 2007). Only few studies examined the ability of *A. butzleri* to invade colon and larynx cells (Musmanno et al. 1997; Vandenberg et al. 2005; Ho et al. 2007). Amongst these studies, only Vandenberg et al. (2005) described an ability of *A. butzleri* to invade cell lines.

These authors showed that 33 % of the strains (4/12) were invasive in the human larynx carcinoma cell line Hep-2. We also investigated the adhesive and invasive properties of three human and three chicken meat *A. butzleri* isolates on HT-29 and Caco-2 cells. Whilst two human (H1 and H3) and two chicken meat (C1 and C2) isolates adhered to HT-29 cells, only one human (H1, CCUG 30485) but both chicken meat isolates also invaded. On Caco-2 cells, all investigated isolates adhered to and invaded. The two isolates H2 and C3, which showed no adhesion on HT-29 cells, showed also significantly lower adhesion on Caco-2 cells compared with the four isolates H1, H3, C1 and C3. Coincident with this, the three isolates H2, H3 and C3, non-invasive on HT-29 cells, showed lesser invasion in the Caco-2 cells. Therefore, it seems that the adhesive and invasive phenotype of *A. butzleri* depends on the isolate as well as the cell line used. That *A. butzleri* isolates investigated by others did not show invasive potential on the cell lines HeLa, INT 407, Caco-2 and IPI-2 could be explained by the different isolates, different cell lines or protocols used (Musmanno et al. 1997; Ho et al. 2007). So far, it is still unknown whether *A. butzleri* adhere to and invade *in vivo* and which part of the human intestine

might be affected. Based on the invasion data obtained by our assays, it can be speculated that *A. butzleri* invade poorly differentiated cryptic cells (HT-29: invasion index from 2.86 x 10^{-5} to 5.05 x 10^{-5} fold) with lower efficiency than the better differentiated colonocytes (Caco-2: invasion index from 1.88 x 10^{-5} fold to 5.0 x 10^{-3} fold). In addition, Bücker et al. (2009) demonstrated that *A. butzleri* CCUG 30485 is able to induce barrier dysfunctions in HT-29/B6 cells, thereby facilitating translocation of bacteria and probably inducing diarrhea. To elucidate which virulence genes might be responsible for adhesion and invasion, *A. butzleri* isolates with different virulence gene patterns were used for in vitro cell culture assays. The isolates H1 and C1 possessed all ten putative virulence genes, whilst in the isolates H2 and C2 the genes *irgA, hecA* and *hecB* and in H3 and C3 additionally *iroE* were missing. Based on these groupings, we could not detect any correlation between virulence gene patterns and adhesive or invasive capabilities. Because only two isolates per virulence gene pattern group were used, further strains need to be tested to confirm our observations. As all isolates possessed the adhesion genes *cadF* and *cj1349* and the invasion gene *ciaB* but had different adhesive and invasive phenotypes, we compared the amino acid sequences of these putative virulence factors. Although the amino acid sequences of the three genes showed alterations from 0.9 to 5 % when compared with the reference strain *A. butzleri* CCUG 30485, the putative functional domains were conserved for all three genes in the six isolates investigated. The CadF fibronectin-binding site of *Camp. jejuni* (FRLS) revealed substitution of similar amino acids within *A. butzleri* (YNLA). F/Y are both hydrophobic and aromatic, R/N are both polar, and S/A are both small and less hydrophobic amino acids. Therefore, this domain might represent also the fibronectin-binding site in *A. butzleri*. Similarly, both I and V (substitution in the zinc-binding domain of CiaB) belong to hydrophobic and aliphatic amino acids (Livingstone and Barton 1993). In addition, the C-terminus of CiaB does not seem to be important for efficient invasion of *A. butzleri* as isolate C1 showed high invasiveness in HT-29 and Caco-2 cells although the last 100 C-terminal amino acids of CiaB are missing. Also the insertion of the three-aa AKS in CiaB of the isolates C1 and C2 does not seem to be functionally relevant. In addition to these two strains showing invasion in HT-29 cells, strain H1 showed invasion on HT-29 cells, even though the three amino acids were absent in this isolate. The reasons for these differences in the amino acid sequence are not known. However, no correlation between adhesive and/or invasive phenotypes and the amino acid sequence of Cj1349, CiaB and CadF factors was shown.

We have shown here that *A. butzleri* isolates have the capability to adhere to and to invade cells as classical gastrointestinal pathogens do. However, further *in vitro* and *in vivo* studies are

required to clarify the role of these virulence factors in the pathogenesis of *A. butzleri* infections.

2.6 Acknowledgement

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2.8 List of Tables and Figures

Table 1: Bacterial isolates used in this study

strain	source	supplier	Strain designation
CCUG 30485	human	CCUG	H1
FR1	human	NRZ *Helicobacter*	H2
FR2	human	NRZ *Helicobacter*	H3
FR3	human	NRZ *Helicobacter*	
FR4	human	NRZ *Helicobacter*	
FR5	human	NRZ *Helicobacter*	
Ab 47	water	LGL Oberschleißheim	
Ab 48	water	LGL Oberschleißheim	
Ab 49	water	LGL Oberschleißheim	
Ab 50	water	LGL Oberschleißheim	
Ab 51	water	LGL Oberschleißheim	
Ab 52	water	LGL Oberschleißheim	
Ab 53	water	LGL Oberschleißheim	
Ab 54	water	LGL Oberschleißheim	
Ab 55	fresh poultry meat	LGL Oberschleißheim	
Ab 56	fresh poultry meat	LGL Oberschleißheim	
Ab 57	poultry	LGL Oberschleißheim	
Ab 60	poultry	LGL Oberschleißheim	
Ab 61	poultry	LGL Oberschleißheim	
Ab 62	poultry	LGL Oberschleißheim	
Ab 63	poultry	LGL Oberschleißheim	
Ab 64	poultry	LGL Oberschleißheim	
Ab 65	poultry	LGL Oberschleißheim	
Ab 66	poultry	LGL Oberschleißheim	
Ab 68	poultry	LGL Oberschleißheim	
Ab 69	poultry	LGL Oberschleißheim	
Ab 70	mixed minced meat	LGL Oberschleißheim	
Ab 73	mixed minced meat	LGL Oberschleißheim	
Ab 74	chicken	LGL Oberschleißheim	
Ab 75	pig	LGL Oberschleißheim	
Ab 84	water	LGL Oberschleißheim	
Ab 86	chicken	LGL Oberschleißheim	
Ab 87	chicken	LGL Oberschleißheim	
Ab 88	chicken	LGL Oberschleißheim	C1
Ab 89	chicken	LGL Oberschleißheim	C2
Ab 91	chicken	LGL Oberschleißheim	
Ab 92	chicken	LGL Oberschleißheim	

Ab 93	chicken	LGL Oberschleißheim	
Ab 94	chicken	LGL Oberschleißheim	C3
Ab 96	chicken	LGL Oberschleißheim	
Ab 97	pig	LGL Oberschleißheim	
Ab 98	pig	LGL Oberschleißheim	
Ab 99	pig	LGL Oberschleißheim	
Ab 100	pig	LGL Oberschleißheim	
Ab 101	pig	LGL Oberschleißheim	
Ab 102	pig	LGL Oberschleißheim	
Ab 103	pig	LGL Oberschleißheim	
Ab 104	pig	LGL Oberschleißheim	
Ab 106	pig	LGL Oberschleißheim	
Ab 109	pig	LGL Oberschleißheim	
Ab 110	pig	LGL Oberschleißheim	
Ab 111	chicken	LGL Oberschleißheim	

CCUG (Culture Collection University of Göteborg, Sweden); NRZ *Helicobacter* (National Reference Centre for *Helicobacter pylori*; University Medical Center Freiburg, Germany); LGL Oberschleißheim (Bavarian Health and Food Safety Authority, Oberschleißheim, Germany)

Table 2: Presence of putative virulence genes in *A. butzleri* isolates

Source	n	*mviN*	*cadF*	*cj1349*	*ciaB*	*hecA*	*hecB*	*iroE*	*irgA*	*tlyA*	*pldA*
Human	6	100	100	100	100	17	33	50	17	100	100
Chicken meat	23	100	100	100	100	9	43	65	9	100	100
Pork	12	100	100	100	100	0	42	50	17	100	100
Minced meat	2	100	100	100	100	0	0	50	0	100	100
Water	9	100	100	100	100	44	67	67	44	100	100

The presence of putative virulence genes is represented as percentage of analysed isolates for each source of origin.

Table 3: Primers used in this study

Target gene	Gene ID RM4018	Primer	Primer sequence	Amplicon (bp)	Annealing	Designed by
cadF	Abu_0481	cadF-F cadF-R	TTACTCCTACACCGTAGT AAACTATGCTAACGCTGGTT	283	50°C	Douidah et al. 2012
pldA	Abu_0861	pldA-F pldA-R	TTGACGAGACAATAAGTGCAGC CGTCTTTATCTTTGCTTTCAGGGA	293	50°C	Douidah et al. 2012
irgA	Abu_0726	irgA-F irgA-R	TGCAGAGGATACTTGGAGCGTAACT GTATAACCCCATTGATGAGGAGCA	437	50°C	Douidah et al. 2012
hecA	Abu_0940	hecA-F hecA-R	GTGGAAGTACAACGATAGCAGGCTC GTCTGTTTTAGTTGCTCTGCACTC	537	50°C	Douidah et al. 2012
hecB	Abu_0939	hecB-F hecB-R	CTAAACTCTACAAATCGTGC CTTTTGAGTGTTGACCTC	528	50°C	Douidah et al. 2012
cj1349	Abu_0067	AB0070F AB0070R	GAATTGTAAAAGTAGGGCATAA TTTGTTTGATTTCGCTCTTT	556	50°C	this study
ciaB	Abu_1549	AB1555F AB1555R	GGAATAAATAAAGAGTTGGTTGC ATTTACTCTATGGTCATTTTGTGC	498	50°C	this study
mviN	Abu_0878	AB0876F AB0876R	TTTTGCTGTTAGTAATTGTTGTTC TGATACTTGGTTAGCTTCTCTTTT	409	50°C	this study
tylA	Abu_1836	AB1846F AB1846R	AAATAAAGTTAAATGTGATGGTGA GTTGTCTCTTGCTTTTTGTATTG	529	50°C	this study
iroE	Abu_0727	AB0730F AB0730R	AATGGCTATGATGTTGTTTAC TTGCTGCTATGAAGTTTTG	415	50°C	this study
ciaB	Abu_1549	Abu_ciaBF Abu_ciaBR	TCGATAAAATACTCTCTTACAAAAA TAATGAACAAGATAAAAATAAAAGC	2019	63°C	this study
		Abu_ciaB,S1 Abu_ciaB,S2 Abu_ciaB,S3 Abu_ciaB,S4	CTGAAGTTGTAGATGCACTTGAAGA TTAAAACACCACTTTCAAAAAGTCC GAGTTCAATGGACTATTTTCAGCTC TTATTTTCATCCAAGCTCTATCCAC	for sequencing		
cj1349	Abu_0067	Abu_cj1349F Abu_cj1349R	AACAGAGTTGAAAATCTCTTAAAAA TTAAAGTTGATGTTGAAGCTGAAAA	1446	63°C	this study
		Abu_cj1349,S1 Abu_cj1349,S2	AGAGGATTTGGAAAAAGAATCAAATY ATGCACTTTGTTTTGCTTCTAAATC	for sequencing		
cadF	Abu_0481	Abu_cadF,S1 Abu_cadFR	CATTTAATTGAAGTTGATCATGGAG GAAGTTATCAAATAAAAATTAGGCAAT	616	60°C	this study
		Abu_cadF,F2 Abu_cadF,S3	TTTTAAAGAAAGGAAAGTTTATGA TTGGAGCAGCAACTGGAGTA	613	61,3°C	

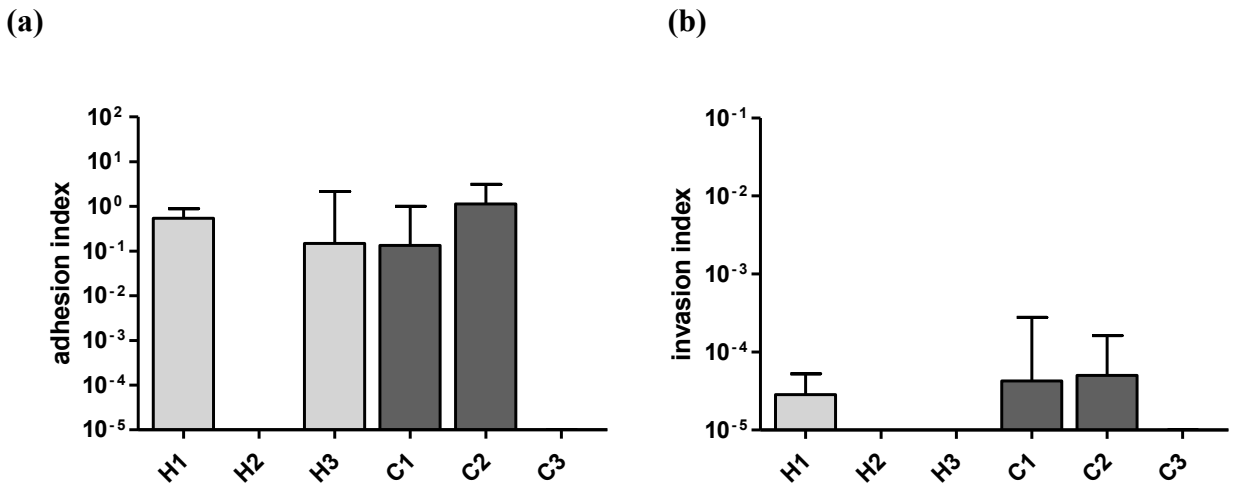


Figure 1: Adhesion index and invasion index of *Arcobacter butzleri* with the intestinal cell line HT-29. Adhesion of six *A. butzleri* isolates to HT-29 cells was detected after 1 h of incubation. To investigate the invasion ability, HT-29 cells were incubated for 3 h followed by 2-h gentamicin treatment, with six *A. butzleri* isolates. Adhesion index (a) and invasion (b) index are calculated as ratio of the inoculum. Expressed are the medians + interquartile range (IQR) (n = 3). H = human isolates, C = chicken meat isolates.

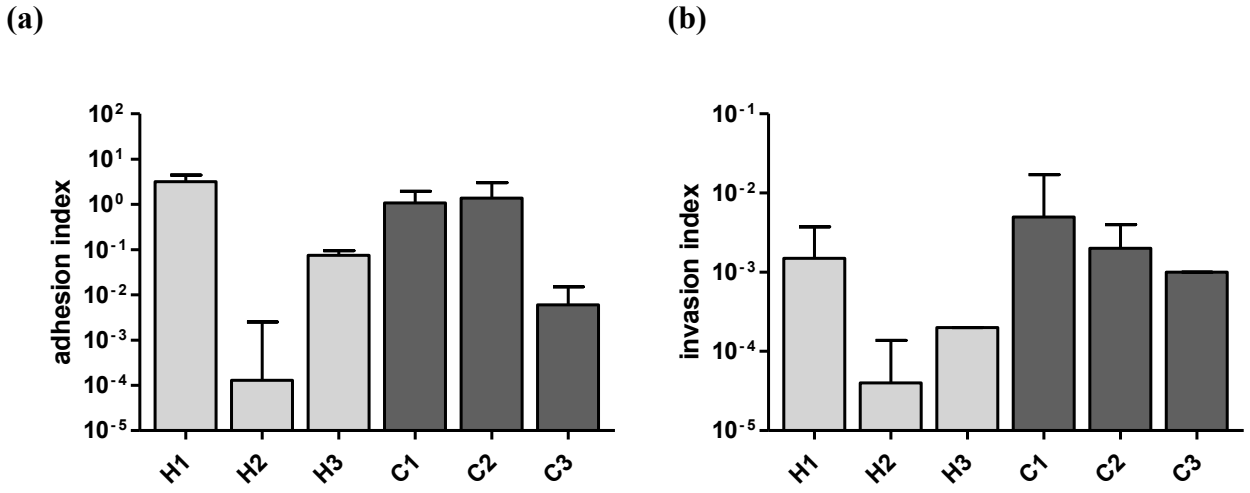


Figure 2: Adhesion index and invasion index of *Arcobacter butzleri* with the intestinal cell line Caco-2. Adhesion of six *A. butzleri* isolates to Caco-2 cells was detected after 1 h of incubation. To investigate the invasion ability, Caco-2 cells were incubated for 3 h followed by 2-h gentamicin treatment, with six *A. butzleri* isolates. Adhesion index (a) and invasion (b) index are calculated as ratio of the inoculum. Expressed are the medians + interquartile range (IQR) (n = 3). H = human isolates, C = chicken meat isolates.

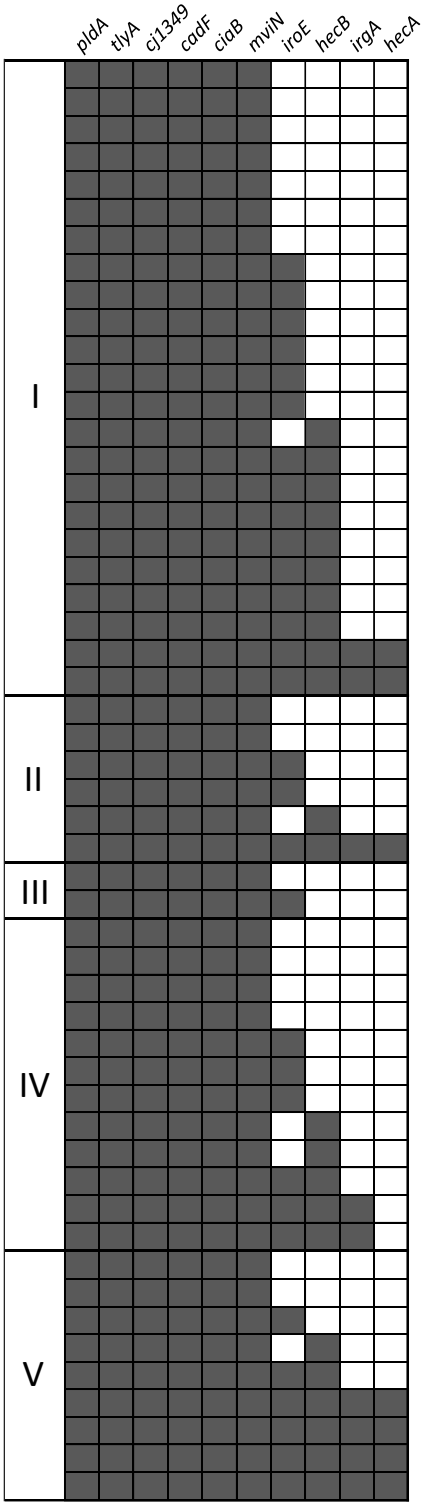
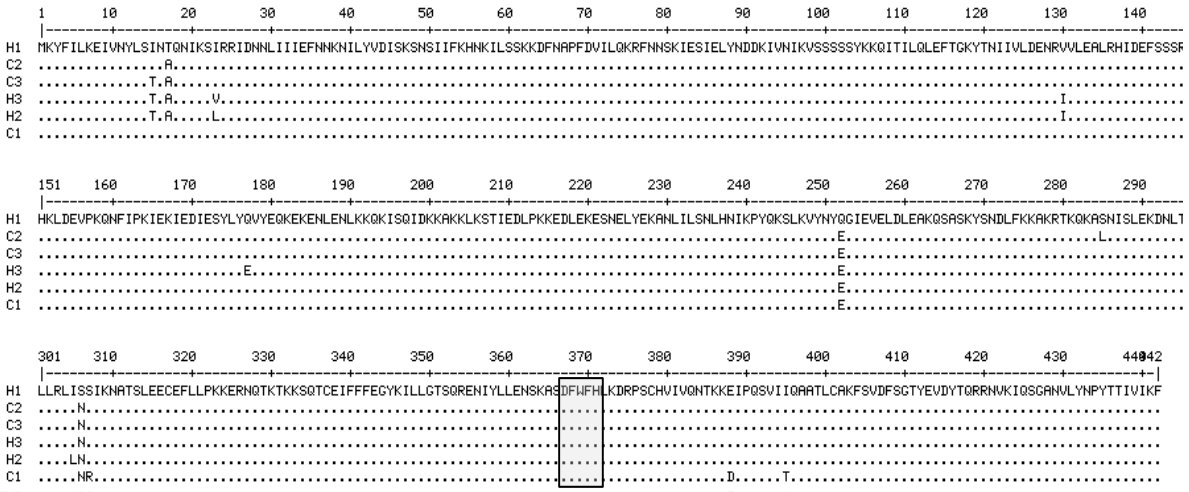


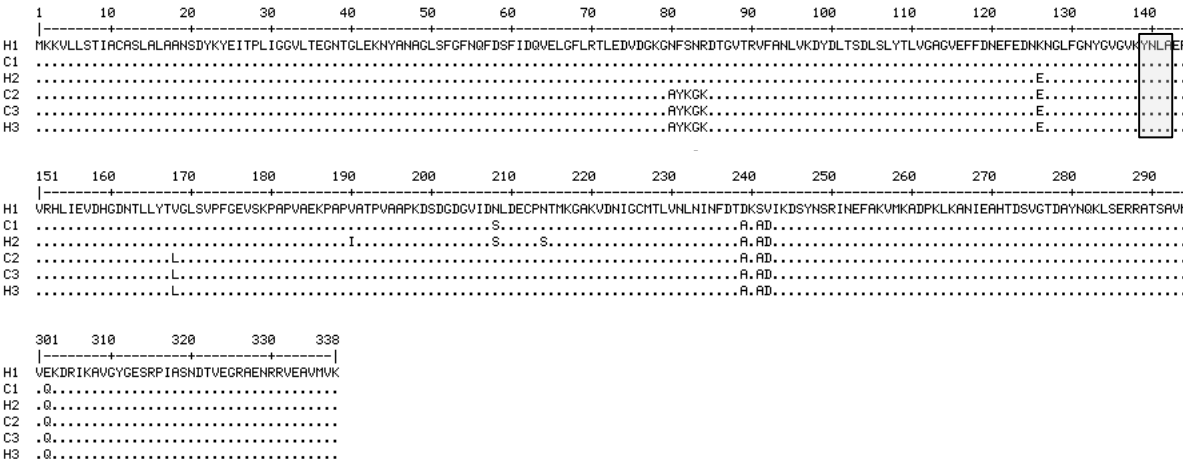
Figure 3: Occurrence of putative virulence genes in *A. butzleri* isolated from environment, food and human. The putative virulence genes were detected by PCR. Grey: PCR pos., white: PCR neg., source of isolates: chicken meat (I), human (II), minced meat (III); pork (IV), water (V)

A Cj1349



B

CadF



C CiaB

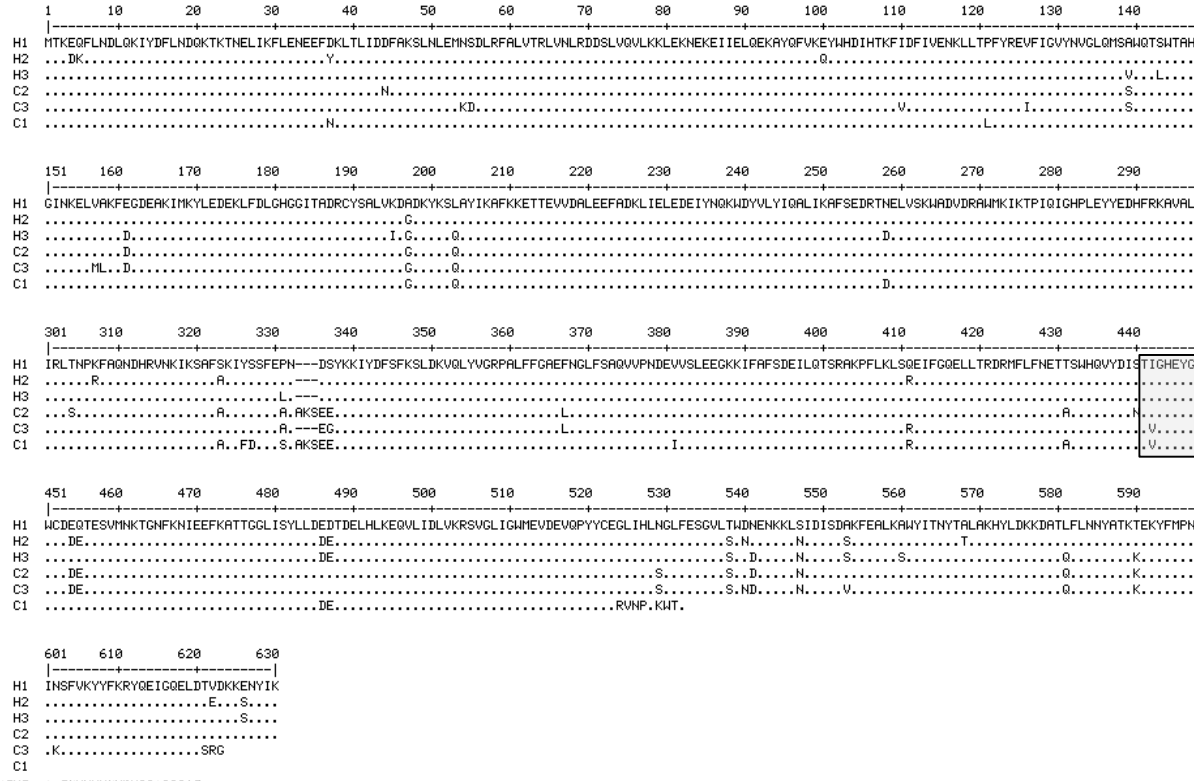


Figure 4: Alignment of Cj1349, CadF and CiaB sequence. The whole genes were sequenced and translated by standard bacterial code. The amino acid (aa) sequences were aligned with Multalin and conserved aa indicated as dots. The putative functional motif of Cj1349 (A), the putative fibronectin-binding domain of CadF (B) and the putative zinc-binding domain of CiaB (C) are highlighted in boxes.

Chapter 3: *Arcobacter butzleri* isolates exhibit pathogenic potentials in intestinal epithelial cell models.

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Chapter 4: General Discussion

In recent decades, *Arcobacter* (*A.) butzleri* has become more important in both veterinary and human public health due to their zoonotic potential (Collado et al., 2011; Ho et al., 2006a). An increasing number of reports on *A. butzleri* have highlighted the significance of this organism as emerging water and foodborne pathogen (summarized by Collado and Figueras 2011). The International Commission on Microbiological Specifications for Foods categorized *A. butzleri* as a serious hazard to human health (ICMSF, 2002). Patients suffering from an *A. butzleri* infection mostly presented symptoms such as persistent or watery diarrhea, abdominal pain, fever and vomiting (Vandenberg et al., 2004). Although *A. butzleri* are reported to be associated with human illness, there is rare information on the pathomechanisms and putative virulence factors of this disease. So far, investigations on the pathogenicity of *A. butzleri* primarily based on the knowledge on *Campylobacter* (Musmanno et al. 1997; Johnson and Murano 2002).

Miller et al. (2007) published the whole genome sequence of the *A. butzleri* strain RM 4018. Although, *A. butzleri* is a member of the *Campylobacteraceae*, the majority of its proteome is most similar to those of *Sulfurimonas (S.) denitrificans* and *Wolinella* (*W.*) *succinogenes*, both members of the *Helicobacteraceae*, and those of the deep-sea vent Epsilonproteobacteria *Sulfurovum* and *Nitratiruptor* (Miller et al., 2007).

This study focused on putative pathomechanisms and the pathogenic potential of *A. butzleri* isolates.

Presence of putative virulence genes

The genome sequence of *A. butzleri* RM 4018 revealed the presence of ten putative virulence genes: *cadF*, *mviN*, *pldA*, *tlyA*, *cj1349*, *hecB*, *irgA*, *hecA*, *ciaB* and *iroE* (Miller et al., 2007), but it is still unknown whether these putative virulence factors have similar functions as described for their homologs in other bacterial species. CiaB contributes to host cell invasion (Konkel et al., 1999; Rojas et al., 2002; Flanagan et al., 2009). MviN is an essential protein required for peptidoglycan biosynthesis in *E. coli* (Ruiz, 2008). HecA, Cj1349 and CadF promote adherence of bacteria to host cells. HecB, TlyA and PldA might be involved in lysis of erythrocytes (Grant et al., 1997; Wren et al., 1998; Miller et al., 2007). IrgA and IroE are functional components for the iron acquisition and required to promote and maintain infections (Mey et al., 2002; Zhu et al., 2005; Rashid et al., 2006).

We investigated the presence and distribution of putative virulence genes in *A. butzleri* isolates recovered from different sources (Chapter 2). Only the genes *mviN, cadF, cj1349, ciaB*, *pldA* and *tlyA*, which share homologies to genes in *Campylobacter*, were identified in all *A. butzleri* isolates. This is in agreement with other studies who detected the presence of *cadF*, *ciaB*, *cj1349*, *mviN*, *pldA* and *tlyA* in all strains and a lower presence of *hecA*, *hecB*, *irgA* and *iroE* (Douidah et al. 2012, Lehmann et al. 2015, Girbau et al. 2015). In contrast, Collado et al. (2014) showed a lower presence of these genes in *A. butzleri* isolated from shellfish. At least 73 % of *A. butzleri* isolates from shellfish harboured *cadF*, *ciaB*, *mviN*, *pldA*, and *tlyA genes*, although only 31 % of the isolates possessed *cj1349* gene (Collado et al., 2014). A high occurrence of these genes was also reported by Levican et al. (2014), where all *A. butzleri* isolates from meat, mussels, and sewage possessed *ciaB* gene (100 %), and 91.7 % harboured *cadF* and *cj1349* genes. In contrast to Douidah et al. (2012), we could confirm neither the similar occurrence of *hecA* in human, pork and chicken meat isolates nor the higher prevalence of *hecB* in human isolates compared with pork and chicken meat isolates. Furthermore, we could not detect a lower occurrence of *irgA* in pork isolates compared with human and chicken meat isolates. However, these conflicting results might be due to the lower number of isolates included in our study.

It has not been determined yet whether all these putative virulence factors are functional or have similar function as their homologues in other bacteria. Further studies are needed to elucidate the role of those genes in the pathogenesis of *A. butzleri* infections. Therefore, we have performed *in vitro* studies to investigate the pathogenic potential of *A. butzleri*.

Adhesive and invasive capacities

Bacteria must have the ability to colonize their host to cause infections. Adhesion and invasion of pathogenic bacteria represent the important initial step of infection. Pathogenic bacteria have evolved a wide range of molecular strategies to adhere to the epithelia and to proliferate at their surface despite the different defense mechanisms (Ribet and Cossart, 2015).

In vitro cell culture methods provide a useful alternative to investigate the interactions between *A. butzleri* and the host epithelial that occur during infection. Therefore, several *in vitro* studies have been performed to investigate adhesion and invasion of *A. butzleri* to host cells. Results regarding adhesion and invasion capabilities have shown a considerable variation, depending on the cell line studied and the origin of the isolates. Only few studies examined the ability of

A. butzleri to invade larynx cells (Musmanno et al. 1997; Vandenberg et al. 2005; Ho et al. 2007). These authors showed that 33 % of the strains (4/12) were invasive in the human larynx carcinoma cell line Hep-2.

To characterize and compare the interaction of *A. butzleri* with host cells, we investigated the adhesive and invasive properties of three human and three chicken meat *A. butzleri* isolates *in vitro*, which were chosen based on their virulence gene pattern (Chapter 2 and 3). The isolates H1 and C1 possessed all ten putative virulence genes, whilst in the isolates H2 and C2 the genes *irgA*, *hecA* and *hecB* and in H3 and C3 additionally *iroE* were missing. Adhesive and invasive abilities have been tested and compared on three human colon epithelial cell lines (HT-29, Caco-2 and HT-29/B6) and the porcine jejunal cell line IPEC-J2.

Overall, the human strains H1 and H3, as well as the chicken strains C1 and C2 showed adhesive abilities on all cell line used, of which only the both chicken strains C1 and C2 were invasive in all cell culture models. Whilst strain H1 invaded in all human cells, strain H3 could only invade on the human cells Caco-2 and HT-29/B6. The strains H2 and C3 showed no or only minimal adhesion or invasion on all cell lines investigated. Highest adhesion capacities were observed for the human cell line Caco-2 (6 of 6 strains) and HT-29/B6 (5/6), followed by HT-29 (4/6) and the porcine cell line IPEC-J2 (4/6). Highest invasion capacities were observed for the human cell line Caco-2 (6/6) and HT-29/B6 (5/6) followed by HT-29 (3/6) and lowest for the porcine cell line IPEC-J2 (2/6). Therefore, it might be that the adhesive and invasive phenotype of *A. butzleri* depends on the isolate as well as the cell line used. The invasion rates in HT-29/B6 cells were higher compared to the rates for HT-29 cells suggesting that the mucus layer produced by HT-29/B6 cells positively influences adhesion and invasion capacities of *A. butzleri*. This important point raises further questions concerning binding properties of the bacteria towards host surface targets and possible cellular receptors. Furthermore, it can be speculated that *A. butzleri* invade poorly differentiated cryptic cells (HT-29 and HT-29/B6) with lower efficiency than the better differentiated colonocytes (Caco-2). This is in agreement with the study of Levican et al. (2013), who showed that all *A. butzleri* strains used adhered to the human intestinal Caco-2 cells and most invaded the cell line. Taken together, these results suggest that adhesion and invasion of *A. butzleri* occurs more prominent in cell lines derived from human colon cells compared to cells derived from porcine jejunal cells. Whether this is due to the difference in intestinal tissue origin or due to differences between the host species has to be determined in further studies. However, variances in adhesion properties of several *Escherichia coli* isolates depending on investigated cell lines has been described recently (Frommel et al., 2013).

Furthermore, no correlation between virulence gene patterns and adhesive or invasive capabilities could be detect. This is in agreement with results described by Levican et al. (2013). As all isolates possessed the adhesion genes *cadF* and *cj1349* and the invasion gene *ciaB* but showed different adhesive and invasive phenotypes, we compared the putative functional domains of these putative virulence genes (Chapter 2). Although the amino acid sequences of the three genes showed alterations from 0.9 to 5 % when compared with the reference strain *A. butzleri* CCUG 30485, the putative functional domains were conserved for all three genes in the six isolates investigated. However, no correlation between adhesive and/or invasive phenotypes and the amino acid sequence of Cj1349, CiaB and CadF factors could be determined.

This let us hypothesise that more genes may be involved in the adhesion and invasion process. However, further *in vitro* and *in vivo* studies are required to clarify the role of these virulence factors in the pathogenesis of *A. butzleri* infections.

Cytotoxicity

The cytotoxicity of some *A. butzleri* strains has also been studied in cell culture systems. Those studies revealed that *A. butzleri* produce toxins, which have not been characterized yet. Nevertheless, these findings indicate that *A. butzleri* has the ability to induce cell death in mammalian epithelial cells, which can cause disease in humans. So far, the cytotoxic effects induced by *A. butzleri* was examined mostly morphologically (Musmanno et al., 1997; Johnson and Murano, 2002; Carbone et al., 2003; Villarruel-Lopez et al., 2003; Ho et al., 2007; Gugliandolo et al., 2008; Karadas et al., 2013; Levican et al., 2013). In our study, the cytotoxic effects of six *A. butzleri* strains, already used in adhesion and invasion assays (Chapter 2 and 3), on HT-29/B6 and IPEC-J2 cells were investigated by WST-1 assay (Chapter 3). This is a colorimetric assay, based on the enzymatic cleavage of the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases, which are only active in metabolically intact cells. No cytotoxicity was observed in IPEC-J2 cells by our *A. butzleri* strains in the WST-assay. In contrast to the IPEC-J2 cells, we could confirm cytotoxic effects of *A. butzleri* on HT-29/B6 cells. Enhanced cytotoxicity was firstly observed at 48 h after bacterial inoculation of HT-29/B6 cells but only if the HT-29/B6 cells were differentiated for seven days and not if cells were differentiated for two days only prior to inoculation. Therefore, a receptor-mediated interaction between a possible *A. butzleri* toxin and the HT-29/B6 epithelial cells could be hypothesized and that expression of this receptor might be dependent on the differentiation status of the

HT-29/B6 cells. Similar results have been reported for the Shiga toxin receptor globotriaosylceramide (Gb3), which is present in higher quantities in differentiated HT-29 cells compared to undifferentiated HT-29 cells (Jacewicz et al., 1995; Melton-Celsa, 2014). However, only the two *A. butzleri* strains H2 and C3 were highly cytotoxic, and to a lesser extend also strain C1. Interestingly, strains H2 and C3 were non- or only poorly adhesive and invasive in HT-29/B6 cells indicating strain-specific pathomechanisms of *A. butzleri*.

Epithelial barrier impairment

Studies have shown that the intestinal bacteria target various intracellular pathways, change the expression and distribution of TJ proteins, and regulate intestinal barrier function. The entrance of the enteric bacteria is mediated through disrupted tight junctions of epithelial cells (Ulluwishewa et al. 2011).

The mechanism by which *A. butzleri* may cause diarrhea was studied by Bücker et al. (2009), who showed that the adhesive and invasive strain CCUG 30485 was able to induce epithelial barrier impairment in the human colonic carcinoma cells (HT-29/B6) (Bücker et al., 2009). The infection of HT-29/B6 human colon cell monolayers by *A. butzleri* led to changes in tight junction protein composition and distribution. Expression of the tight junction proteins claudin-1, -5, and -8 was reduced and these proteins were redistributed off the tight junctional strands and formed intracellular aggregates. Moreover, an induction of necrosis and caspase-3-dependent epithelial apoptosis was observed. Epithelial cell death together with the tight junction changes represents a leak flux type of barrier dysfunction as pathomechanisms for the *A. butzleri*-associated diarrhea. We demonstrated, that not only the *A. butzleri* strain CCUG 30485, but also other *A. butzleri* strains of different origin, had the ability to impair the epithelial barrier impairment in human (HT-29/B6) and porcine (IPEC-J2) cell monolayers, irrespective of their invasive or cytotoxic phenotype (Chapter 3). These data suggest that also other processes than invasion could induce barrier impairment in HT-29/B6 monolayers after infection with *A. butzleri*. However, which of these mechanisms are more or less responsible for the reduced TER observed by the non-invasive *A. butzleri* strains has to be investigated in further experiments. The *in vitro* data presented in this work corroborate that *A. butzleri* has enteric pathogenic potential, characterized by defined interactions with human epithelial cells.

***In vivo* model**

The impact of the identified virulence factors of *A. butzleri* still remains unknown. This is mainly because of the lack of suitable infection models. Previous *C. jejuni* infection studies showed, that mice harbouring a conventional commensal gut microbiota can often not be stably colonized by *C. jejuni* due to their distinct host- and age-specific microbiota composition, which prevented the animals from infection (Bereswill et al., 2011). This studies underlined the important role of the murine microbiota in colonization resistance against *C. jejuni*. In contrast, in gnotobiotic IL-$10^{-/-}$ mice, *C. jejuni* colonized the entire gastrointestinal tract and developed infection-induced features of human campylobacteriosis. (Haag et al., 2012; Heimesaat and Lugert, 2014). Comparable to *C. jejuni* also the two *A. butzleri* isolates CCUG 30485 (H1) and C1 were not able to stable colonize in wildtype mice.

We therefore used the gnotobiotic IL-$10^{-/-}$ mouse model to determine the pathogenic potential of *A. butzleri* and to investigate its colonization ability in the murine intestinal tract. Gnotobiotic IL-$10^{-/-}$ mice were perorally infected with the two *A. butzleri* strains CCUG 30485 (H1) and C1 for which invasive capacities have been shown in *in vitro* assays (Chapter 2 and 3). Then, colonization properties alongside the intestinal tract, immune response, bacterial translocation and clinical symptoms were analysed. Following peroral infection either *A. butzleri* strain could stably colonize the intestinal tract with comparable loads in mice. *A. butzleri* could be cultured from the small and large intestinal lumen with highest loads of up to 10^8 CFU per g in the colon and approximately 10^4 CFU in the ileum of infected gnotobiotic IL-$10^{-/-}$ mice. Compared to the colon, *A. butzleri* could be cultured four orders of magnitude lower bacterial loads in the ileum (Heimesaat et al. 2015). Despite high intestinal *A. butzleri* loads, gnotobiotic IL-$10^{-/-}$ mice did not display any clinical symptoms such as diarrhea or occurrence of blood in feces. This is surprising, due to the fact that the two *A. butzleri* strains have been shown to have adhesive and invasive abilities *in vitro* (Chapter 2 and 3). *A. butzleri* could not be isolated from extra-intestinal organs such as kidney, spleen, liver or cardiac blood. However, *A. butzleri* infection induced not only intestinal but also extra-intestinal and systemic immune responses. A significant increase of distinct immune cell populations (T and B cells, regulatory T cells, macrophages and monocytes) which was accompanied by elevated colonic TNF, IFN-γ, nitric oxide (NO), IL-6, IL-12p70 and MCP-1 concentrations was observed. Inflammatory responses could be observed earlier in the course of infection by the CCUG 30485 (H1) as compared to the C1 strain. Although clinical symptoms of disease were not present, a stable colonization alongside the intestinal tract and immune response were observed. In conclusion, peroral

A. butzleri infections resulted not only in intestinal, but also in extra-intestinal and systemic immune responses in a strain dependent manner (Gölz et al., 2015).

Conclusion

Putative virulence genes, bacterial adherence to intestinal cells, invasive capability and the ability to induce cytotoxicity have been identified as pathogenic potential in *A. butzleri.* Furthermore, *A. butzleri* is able to influence the transepithelial resistance. *In vivo, A. butzleri* could stable colonize in gnotobiotic IL-10 deficient mice and induce an immune response of the host. Taken together, these *in vitro* and *in vivo* findings clearly indicate the enteric pathogenic potential of *A. butzleri* and strain-specific pathomechanisms.

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Chapter 5: Summary/Zusammenfassung

Summary

Arcobacter (A.) spp. is a gram-negative, motile and spiral-shaped bacterium belonging together with *Campylobacter (C.)* and *Sulfurospirillum* to the family of *Campylobacteraceae.* At present, 18 *Arcobacter* species have been characterized with *A. butzleri* as the most important and predominant species associated with human diseases, such as gastroenteritis, bacteremia or septicaemia. *A. butzleri* have been rated a serious hazard to human health by the International Commission on Microbiological Specifications for Foods in 2002. The bacteria have become more important in public health due to their presence in different sources such as animals including various food products of animal origin and water. However, the most frequent source of human *A. butzleri* infection is contaminated undercooked poultry meat and water. In few studies several authors demonstrated that *A. butzleri* is the fourth most common *Campylobacterales* species recovered from patients suffering from. Reported clinical signs of an *A. butzleri* infection are diarrhea associated with abdominal pain, nausea and vomiting or more rarely fever. Compared to *C. jejuni*, *A. butzleri* caused more watery and persistent, but less acute and bloody diarrhea. As human infections with *A. butzleri* are not routinely investigated, the relevance of this pathogen could not be determined so far. However, a few case reports and some outbreak data on human infection with *A. butzleri* are available indicating that *A. butzleri* is a truly human pathogen.

Although some progress has been made over the past decade, the knowledge about the pathogenic mechanisms and immune host responses of *A. butzleri* is still scarce. Several putative virulence determinants homologous to *C. jejuni*, were identified in the genome sequence of *A. butzleri* RM4018, while other virulence-associated genes of *C. jejuni* are missing. Results from phenotypic assays revealed adhesive, invasive and cytotoxic capabilities of *A. butzleri* on several cell lines *in vitro*. The barrier dysfunction caused by *A. butzleri* infection in monolayers of the human colon cell line HT-29/B6 highlights potential mechanisms by which diarrhea can be induced in human.

This work focused on the investigation of virulence mechanisms of *A. butzleri* including its interactions with intestinal epithelial cells *in vitro.*

Chapter 1 provides a literature review that emphasises the relevance of *A. butzleri* in human and veterinary public health. In the first study **(Chapter 2)**, the presence of virulence genes homologous to those of *C. jejuni* and other enteric pathogens was investigated in several

A. butzleri strains. Further the adhesive and invasive abilities of several *A. butzleri* strains on two different human intestinal epithelial cell lines (HT-29 and Caco-2) were demonstrated. No correlation was observed between putative virulence gene patterns and adhesive or invasive phenotypes with the tested cell lines; also the putative functional domains of CiaB, CadF and Cj1349 in the amino acid sequences showed no correlation with the different adhesive and invasive phenotypes.

The aim of the second study **(Chapter 3)** was to obtain additional information on the pathogenicity and the pathomechanisms of *A. butzleri* strains. *A. butzleri* was investigated on two further epithelial cell lines to characterize different epithelial cell interactions and strain-specific pathomechanisms. The ability of *A. butzleri* strains for adhesion, invasion and cytotoxicity in human (HT-29/B6) and porcine (IPEC-J2) intestinal epithelial cell lines could be demonstrated. These *A. butzleri* strains were able to influence the transepithelial electrical resistance. The *A. butzleri* strain specific pathomechanisms has been observed with the human colon cell line HT-29/B6. Furthermore, *A. butzleri* induced systemic immune response in gnotobiotic IL-10 deficient mice in a strain-dependent manner **(Chapter 4).**

Taken all data together, these findings emphasize the enteric pathogenic potential and strain-specific pathomechanisms of *A. butzleri.*

Zusammenfassung

Charakterisierung des Virulenzpotenzials von Arcobacter butzleri

Arcobacter (A.) sind gram-negative, bewegliche und spiralförmige Bakterien, die zusammen mit *Campylobacter* und *Sulfurospirillum* der Familie *Campylobacteraceae* angehören. Die Gattung umfasst 18 Spezies, wobei *A. butzleri* die wichtigste bzw. am häufigsten vorkommende Spezies darstellt, die mit Erkrankungen des Menschen wie Gastroenteritis, Bakteriämie oder Septikämie assoziiert wird. *A. butzleri* wurde bereits 2002 von der ICMSF zur erheblichen Gefahr für die menschliche Gesundheit eingestuft. Dieses Bakterium hat auf Grund seines Vorkommens in verschiedenen Quellen wie im Tier einschließlich zahlreicher Nahrungsmittel tierischen Ursprungs und Wasser erheblich an Bedeutung gewonnen. Die Hauptquelle für humane *A. butzleri*- Infektionen sind nicht ausreichend gegartes Geflügelfleisch und Wasser. Einige Studien zeigten, dass *A. butzleri* der vierthäufigste *Campylobacterales*-Erreger ist, der aus Fäzes von Patienten mit Durchfall isoliert wurde. Die Symptome einer *A. butzleri*-Infektion sind Durchfälle mit begleitenden Bauchkrämpfen, Übelkeit und Erbrechen oder seltener Fieber. Im Vergleich zu *C. jejuni* verursacht *A. butzleri* eher wässrigen und bis zu zwei Monaten anhaltenden, aber weniger akuten und blutigen Durchfall. Da *A. butzleri*-Infektionen nicht routinemäßig untersucht werden, kann die Relevanz dieses Erregers noch nicht eingeschätzt werden. Einige Fallberichte und *A. butzleri*-Ausbrüche belegen, dass *A. butzleri* ein echter Krankheitserreger des Menschen ist. Trotz gewisser Fortschritte im letzten Jahrzehnt existiert nur geringes Wissen über Pathogenitätsmechanismen von *A. butzleri* und die Immunantwort des Wirts. Miller et al. (2007) identifizierten in der Genomsequenz von *A. butzleri* RM 4018 mehrere putative Virulenzdeterminanten, die homolog zu *C. jejuni* sind, während andere Virulenz-assoziierten Gene von *C. jejuni* nicht existieren. Ergebnisse aus phänotypischen Untersuchungen zeigen adhäsive, invasive und zytotoxische Eigenschaften von *A. butzleri* auf mehreren Zelllinien *in vitro*. *A. butzleri* ist in der Lage Barriere-Funktionsstörungen in HT-29/B6 Monolayern hervorzurufen, welche potentielle Mechanismen sind, durch die Durchfall bei Menschen induziert werden könnte.

Diese Arbeit widmet sich der Untersuchung einzelner Virulenzmechanismen von *A. butzleri*, einschließlich ihrer Interaktion mit intestinalen Epithelzellen *in vitro*.

Kapitel 1 gibt eine Literaturübersicht, die die Relevanz von *A. butzleri* in der Human- und Veterinärmedizin unterstreicht. In der ersten Studie **(Kapitel 2)** wird die Verbreitung der 10 putativen Virulenzgene in *A. butzleri*-Stämmen beschrieben. Weiterhin werden die adhäsiven und invasiven Fähigkeiten ausgewählter *A. butzleri*-Stämme auf die zwei verschiedenen humanen Darmepithel-Zelllinien (HT-29 und Caco-2) gezeigt. Es wurde keine Korrelation zwischen dem putativen Virulenzgenmuster und den adhäsiven und invasiven Eigenschaften auf den getesteten Zelllinien beobachtet; auch die Aminosäuresequenzen der putativen funktionellen Domänen von CiaB, CadF und Cj1349 zeigten keine Korrelation zu den unterschiedlichen adhäsiven und invasiven Eigenschaften der untersuchten Stämme.

Das Ziel der zweiten Studie **(Kapitel 3)** war es, zusätzliche Informationen zur Virulenz und Pathogenitätsmechanismen der *A. butzleri*-Stämme zu erhalten. *A. butzleri* wurde auf zwei weiteren Epithel-Zelllinien getestet, um verschiedenen epitheliale Zell-Interaktionen und stammspezifische Pathogenitätsmechanismen zu charakterisieren.

Die Adhäsion, Invasion und Zytotoxizität von *A. butzleri* wurde bei humanen (HT-29/B6) und porzinen (IPEC-J2) Darmepithelzelllinien untersucht. Weiterhin waren diese *A. butzleri*-Stämme fähig, den transepithelialen elektrischen Widerstand zu beeinflussen. Eine stammabhängige Immunantwort konnte nach einer *A. butzleri*-Infektion in gnotobiotisch IL-10 defizienten Mäusen festgestellt werden **(Kapitel 4)**.

Zusammengefasst unterstreichen diese Daten das pathogene Potential und die stammspezifischen Pathogenitätsmechanismen von *A. butzleri.*

Publication list

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Eidesstattliche Erklärung

Ich erkläre hiermit, dass ich diese Dissertation selbstständig ohne Hilfe Dritter und ohne Benutzung anderer als der angegebenen Quellen und Hilfsmittel verfasst habe. Alle den benutzten Quellen wörtlich oder sinngemäß entnommenen Stellen sind als solche einzeln kenntlich gemacht.

Berlin, 29.01.2016

Gül Karadas