Investigation of virulence-associated factors in the pathogenesis of *Campylobacter jejuni,* and the anti-*Campylobacter* mode of action of clove essential oil

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Ph.D. Thesis

by

Judit Klára Kovács

Project Leader: Dr. György Schneider

Program Leaders: Professor Dr. Levente Emődy, Dr. Monika Kerényi

Head of the Doctoral School: Professor Dr. Júlia Szekeres

Department of Medical Microbiology and Immunology

Medical School

University of Pécs, Hungary

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"Humanity shares a common ancestry with all living things on Earth. We often share especially close intimacies with the microbial world. In fact, only a small percentage of the cells in the human body are human at all. Yet, the common biology and biochemistry that unites us also makes us susceptible to contracting and transmitting infectious disease."
― Brenda Wilmoth Lerner, *Infectious Diseases In Context*

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CHAPTER I. INTRODUCTION

1. Campylobacters

1.1. General features of campylobacters

Campylobacter is the most common causative agent of bacterial zoonotic food-borne diarrheal diseases all around the world. Most species belonging to the genus *Campylobacter* are adapted to the intestinal tract of warm-blooded animals. Certain campylobacters are commensal, but the majority of them are pathogens, infecting the human and animal gastrointestinal tracts (Evans and Brachman 1986; Van Vliet and Ketley, 2001). Based on our present knowledge, they are unique among pathogens associated with food-related illnesses by being microaerophilic (requiring decreased oxygen) and capnophilic (requiring increased carbon dioxide). The word "campylobacter" is derived from the Greek campylo ("curved"), and bacter ("rod"). The genus *Campylobacter* comprises small (0.2-0.9 μm wide and 0.5-5.0 μm long), spiral, none-spore forming Gram-negative bacteria (Figure 1) (Ketley et al., 1997). Coccoid forms usually are mentioned as viable, but non culturable (VNBC) cells, could only be found in older, or in oxygen-exposed cultures. They are highly motile using their polar flagellum at one or both ends to achieve their typical high-intensity "corkscrew" motility (Humphrey et al., 2007).

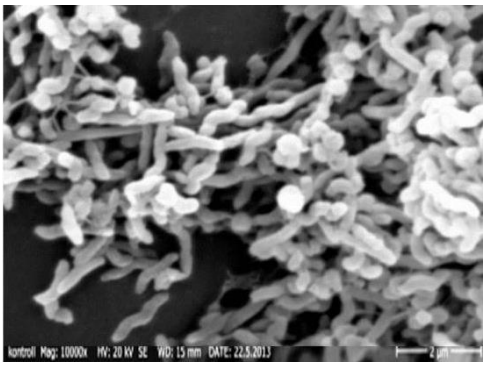

Figure 1. Scanning Electron micrograph of *Campylobacter jejuni* NCTC 11168 (10,000X)

1.2. Discovery and taxonomy of campylobacters

The first report of *Campylobacter* is believed to have been written in 1886 by Theodor Escherich, who observed non-culturable spiral-shaped bacteria isolated from the colon of a child, who died in "cholera infantum". Based on its morphological properties the organism, at that time, was misidentified, and published as a *Vibrio* (Silva et al., 2011; Epps et al., 2013). *Campylobacter* was identified in 1906 for the first time by two British veterinarians reporting „large number of peculiar organism" in the uterine mucus of a pregnant sheep (Skirrow, 2006). After this, in 1927 a *Vibrio*-

like bacterium was found in a faecal sample of a cattle with diarrhea, and named as *Vibrio jejuni* (today identified as *C. fetus*). The suspicion, that *Campylobacter* may be associated with diarrheal infections raised only in 1931 in the United States of America (USA), regarded in cattle, and in 1940, in pigs (today identified as *C. coli*). In 1950, King was the first who was able to segregate the "*Vibrio* related" bacteria from vibrios by culturing them on higher temperature. Due to their low DNA base composition, their non-fermentative metabolism, and their microaerophilic growth requirements an establishment of a new genus, *Campylobacter* was proposed by Sebald and Vernon, in 1963 (Oberhelman and Taylor, 2000). The first *Campylobacter* isolated from human diarrhea was described by Dekeyser et al. in 1972 (Butzler et al., 1973; Butzler, 2004).

Developing selective and filtration culture methods have led to isolation and identification of campylobacters more and more frequently in laboratories around the world. Since their first identification the taxonomic structure of the genus *Campylobacter* has been changed dramatically and it still has conflictions remained to be resolved. The genus *Campylobacter* belongs to the family *Campylobacteriaceae* in the epsilon division of the subdivision *Proteobacteria.* Based on 16S rRNA sequencing, the campylobacters belong to the rRNA superfamily VI (Vandamme et al., 1991). Two closely related genera, *Helicobacter* and *Archobacter*, include species previously classified as *Campylobacter* spp. Although, there are more than 20 *Campylobacter* spp. in the genus, the most human infections are caused by *Campylobacter jejuni* and *Campylobacter coli* in the developed countries, while in the developing regions *C. upsaliensis* is the most important agent (Table 1) (On et al., 2013).

Table 1. List of valid species and subspecies in the genus *Campylobacter* as of April 30, 2013, and their common hosts and disease associations in humans and animals. (On, 2001, 2013)

Taxon	Host animal species	Human disease association	Animal disease association
Campylobacter avium	Poultry	None as yet	None as yet
Campylobacter canadensis	Whooping cranes	None as yet	None as yet
Campylobacter coli	Pigs, poultry, ostriches, cattle, sheep	Gastroenteritis	Gastroenteritis, infectious hepatitis
Campylobacter concisus	Humans, domestic pets	Gastroenteritis, periodontitis	None as yet
Campylobacter cuniculorum	Rabbits	None as yet	None as yet
Campylobacter curvus	Humans	Periodontitis, gastroenteritis	None as yet
Campylobacter fetus subsp. *fetus*	Cattle, sheep, reptiles	Gastroenteritis, septicaemia	Spontaneous abortion
Campylobacter fetus subsp. *venerealis*	Cattle, sheep	Septicaemia	Infectious infertility
Campylobacter gracilis	Humans	Periodontitis	None as yet
Campylobacter helveticus	Dogs, cats	Periodontitis	Gastroenteritis
Campylobacter hominis	Humans	None as yet	None as yet
Campylobacter hyointestinalis subsp. *hyointestinalis*	Cattle, deer, pigs, hamsters	Gastroenteritis	Gastroenteritis
Campylobacter hyointestinalis subsp. *lawsonii*	Pigs	None as yet	None as yet
Campylobacter insulaenigrae	Seals, porpoises	None as yet	None as yet
Campylobacter jejuni subsp. *doylei*	Humans	Septicaemia, gastroenteritis	None as yet
Campylobacter jejuni subsp. *jejuni*	Poultry, cattle, pigs, ostriches, wild birds	Gastroenteritis, Guillain-Barré syndrome	Spontaneous abortion, avian hepatitis
Campylobacter lanienae	Cattle	None as yet	None as yet
Campylobacter lari subsp. *concheus*	Shellfish	Gastroenteritis	None as yet
Campylobacter lari subsp. *lari*	Wild birds, dogs, poultry, shellfish, horses	Gastroenteritis, septicemia	Avian gastroenteritis
Campylobacter mucosalis	Pigs	None as yet	None as yet
Campylobacter peloridis	Shellfish	Gastroenteritis	None as yet
Campylobacter rectus	Humans	Periodontitis	None as yet
Campylobacter showae	Humans	Periodontitis	None as yet
Campylobacter sputorum	Humans, cattle, pigs, sheep	Gastroenteritis, abscesses	Spontaneous abortion
Campylobacter subantarcticus	Birds in the subantarctic	None as yet	None as yet
Campylobacter upsaliensis	Dogs, cats	Gastroenteritis	Gastroenteritis
Campylobacter ureolyticus	Humans	Gastroenteritis, Crohn's disease	None as yet
Campylobacter volucris	Black-headed gulls	None as yet	None as yet

1.3. Isolation and identification of campylobacters

The importance of *Campylobacter* in diarrheal diseases has been clarified by the breakthrough of the appearance of adequate culture and identification methods. Special conditions are required for the cultivation of campylobacters. Being essentially microaerophilic, they grow best at an atmosphere with lower oxygen tension (5% O_2, 10% CO_2, and 85% N_2) (Garénaux et al., 2008; Gharst et al., 2013). Because of their sensitivity to oxygen and oxidizing radicals, numerous selective media have been developed for their cultivation, containing oxygen scavengers such as blood, ferrous iron, and pyruvate (Silva et al., 2011). The most commonly used selective medium for direct plating of campylobacters is the charcoal cefoperazone deoxycholate agar (CCDA). Since campylobacters lack many of genes crucial for degradation of carbohydrates and amino acids, the use of specific complex media is essential for their cultivation (Cody et al., 2010). Applying selective agents, mainly antibiotics in the media is recommended to inhibit the growth of other presenting faecal bacteria and fungi.

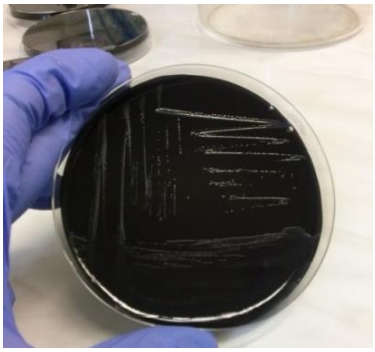

Figure 2. The morphology of the *C. jejuni* NCTC 11168 colonies on CCDA

Depending on the type of medium, *Campylobacter* colonies may differ in their appearance. Usually, they are round in shape, flat and grey-white in colour on the CCDA (Figure 2). *Campylobacter* species are able to grow between 37 and 42 ^{o}C. Being thermotolerant, their optimum temperature is 41.5 ^{o}C, probable as a consequence of their association with avian species. In the absence of cold-shock proteins, they are incapable to grow under 30 ^{o}C (Levin, 2007). The growth of *Campylobacter* spp. can be limited by the sodium chloride concentration greater than 2% w/v, and by frozen storage at -15 ^{o}C, nevertheless, campylobacters cannot be eliminated by freezing. They are extremely sensitive to heat and desiccation, furthermore, they are unable to survive at the condition of pH under 4.9 and above pH 9.0 (Bhunia et al., 2007; Silva et al., 2011). *Campylobacter* species are more sensible to ionizing radiation than *Salmonella* and *Listeria* spp. (Curtis et al., 2003). Detection, identification, and separation of the various species within the genus of *Campylobacter* the routine

diagnostic laboratory is based on cultivation, staining, biochemical tests (oxidase, catalase, nitrate), serological (latex agglutination), and molecular biological methods (*in situ* fluorescence hybridisation and polymerase chain reaction (PCR)). They are Gram-negative, show positive reactions in catalase, oxidase, and nitrate reduction (with the only exception of *C. jejuni* subsp. *doylei*) tests and reduce fumarate to succinate, while they are negative in the methyl red, acetoin tests, and indole forming (Table 2) (Wassenaar and Newell, 2000). To differentiate *C. jejuni* and *C. coli*, the most frequently isolated species, hippurate hydrolysis is suggested to be applied. *Campylobacter coli* is unable to hydrolyze hippurate (Epps et al., 2013).

Table 2. Biochemical tests used to identify campylobacters (Steinbrueckner et al.,1999)

Characteristic	*C. jejuni*	*C. coli*	*C. lari*	*C. upsaliensis*
oxidase	+	+	+	+
catalase	+	+	+	+/-
hippurate hydrolysis	+	-	-	-

1.4. Epidemiology of *Campylobacter jejuni*

The World Health Organisation (WHO) reported 4.5 billion human diarrheal incidence causing 1.8 million deaths annually around the world (WHO, 2004). According to Centers for Disease Control and Prevention (CDC), the reported incidence of infections caused by *Campylobacter* has been increased by 14% compared within the period 2006-2008 (CDC, 2013). Similar increasing trends have been observed from 2008 till 2012 in numerous countries of the European Union (EU), just as well in Hungary (EU/EFTA, 2012) (Figure 3). Species information shows, that from the reported campylobacteriosis cases approximately 90% is caused by *C. jejuni*, and *C. coli* accounting for much of the rest (Friedman et al., 2000; Gillespie et al., 2002; Moore et al., 2005). Campylobacters are crucially important pathogens from a socio-economic perspective as well, affecting 1% of the human population in Europe in every year (Denny et al, 2007), and infecting 13 of every 100,000 persons in the United States (Ailes et al., 2008). Based on the report of the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), in the last 5 years, campylobacteriosis has become the most commonly reported zoonotic disease in the EU (45 cases per 100,000) followed by salmonellosis and shigellosis (Figure 4). It is estimated, that campylobacteriosis is an underreported event, the reported cases are 200,000/ year, and the estimated ones are 9 million/ year (an estimated 2.1% of all cases are currently reported) (EFSA, 2011; Havelaar et al., 2013).

Campylobacteriosis cases have been increased progressively in other parts of the word just like in New Zealand in 2010 with incidence of 400 per 100,000 populations (Silva et al., 2011).

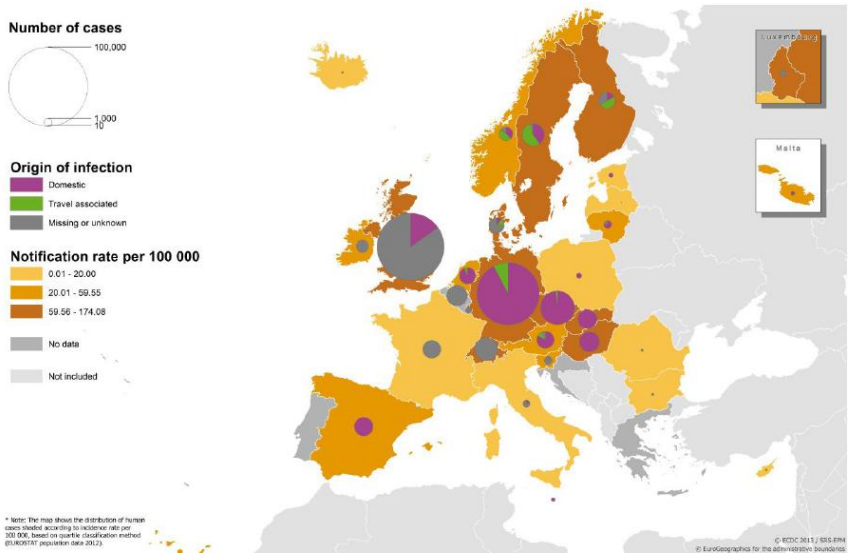

Figure 3. Notification rates and origin of infection in human campylobacteriosis in EU/EFTA, 2012

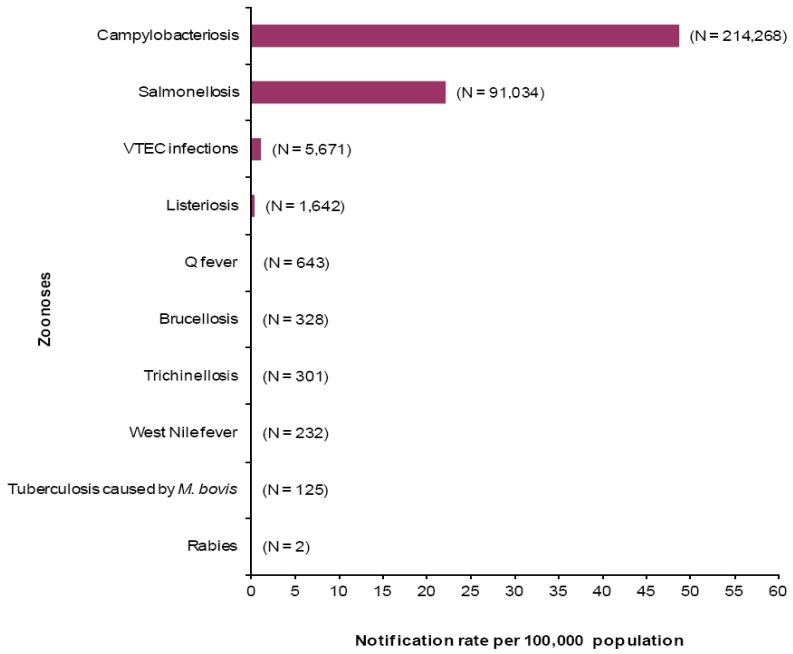

Figure 4. Reported notification rates of zoonosis of confirmed human cases in the EU, EU/EFTA 2012

In Hungary, a great majority, 98.8% of the enteric bacterial diseases are campylobacteriosis and salmonellosis, and the first one is continuously rising over the years with short pauses. In the year of 2013, the prevalence raised with 1,000 cases (13.6%) compared with the previous year (Epinfo, 2014).

In spite of the limited surveillance data in the developing countries, it is evident that *C. jejuni* infections are hyperendemic in these regions as well, especially among children under the age of five, sometimes resulting in death (Coker et al., 2002; Zilbauer et al., 2008). Children, who are recurrently infected with this pathogen in the hyperexposed developing countries, become asymptomatic with *C. jejuni*. Evolvement of similar immunity against *Campylobacter* is observable in industrialized countries, among people working with poultries and drinking raw milk (Moore et al., 2005).

In the western world, the majority of *Campylobacter* infections are sporadic, and unlike for other food-borne pathogens, huge outbreaks are not typical. Nevertheless it is likely, that outbreaks or small case clusters occur far more frequently than previously suggested (Taboada et al., 2013). The incidence of reported cases is slightly higher in males than females. Although *C. jejuni* infections represent seasonal variation (marked peak in the summer), it may occur in any time of the year (Tauxe, 1992). Based on earlier surveys, cost of treatments and missing working hours due to campylobacteriosis creates a huge burden also on the economy, namely 2.4 billion EUR/ year in the EU (EFSA, 2011) and 6.2 billion dollars in the USA per year (Forsythe, 2000).

1.5. Epidemiological typing of *Campylobacter jejuni*

In order to detect and track food-borne pathogens a number of typing systems has been improved. Since *C. jejuni* is present everywhere in the nature, and most of the cases are sporadic, detection of the sources of infection is troublesome (Eberle and Kiess, 2012). Numerous studies have been reported, where several genotypes of *C. jejuni* were isolated from a single clinical sample complicating further the matters. Typing systems can be divided into phenotypic methods detecting characteristics expressed by the isolates, and genotypic tests analyzing the genetic elements like DNA and RNA of the bacteria. Several essential criteria have to be realized: stability, reproducibility, sensitivity, typeability, discriminatory power, and the ease of interpretation (Nielsen et al., 2000). By these epidemiological typing methods, we are able to apply effective surveillance programs to prevent and reduce the constantly growing number of food-borne cases around the world (Taboada et al., 2013).

1.5.1. Phenotyping assays

Biotyping is used to differentiate isolates based on their metabolic activities detected by biochemical reactions (see under section 1.1.1.3).

Serotyping tests use specific antibodies to detect different cellular surface structures of microorganisms as antigens. The *Penner scheme* differentiates strains on the basis of their cell surface located heat-stable (HS) antigens (Moran and Penner, 1999). Primarily it is based on capsular polysaccharides (CPSs) structures, but lipooligosaccharide (LOS) can also contribute to serotype scheme involving altogether 47 *C. jejuni* serotypes (Karlyshev et al., 2000). The *Lior scheme* detects heat labile (HL) antigens using a slide agglutination technique with live bacteria (Lior et al., 1982). Both serotyping methods are reproducible and typeable, but the best differentiation can be achieved by performing them together (Eberle and Kiess, 2012).

1.5.2. Genotyping assays (Phylogenotyping assays)

Within a species different subtypes can be determined by using the following DNA based methods: i) methods based on restriction sites in the genome, ii) methods based on PCR, and iii) methods based on single nucleotide polymorphism (SNPs).

PulseNet system using *pulsed-field gel electrophoresis (PFGE)* is one of the most important surveillance programs to assist CDC in detection of case-clusters of food-borne disease outbreaks as well as sporadic cases. PFGE developed by Schwartz and Cantor (1984) analyses the DNA fingerprints of bacteria creating clonal groups among them. This technique has emerged as one of the best choice for molecular epidemiological studies. In a nutshell, the bacterial cells are embedded in agarose plugs, and treated with enzymes in order to disclose the cells and to access the genomic DNA for digestion with restriction enzymes with infrequent recognition sites. Using periodically changing electric field, the separation of large DNA molecules (macrorestriction pattern) is feasible. Although PFGE is relatively expensive, and the analysis takes 4-5 days, it is conceived, that this technique is a widespread discriminatory typing method for *C. jejuni* (Steele et al., 1998).

Flagellin typing, performed by restriction fragment length polymorphism (*RFLP),* predicts clonal groups by detecting fingerprints of the highly variable flagellin gene. The flagella of *Campylobacter* are encoded by a major flagellin gene, *flaA*, and a minor gene, *flaB*. During the procedure, the *flaA* gene is amplified by PCR, and the product is digested with restriction endonucleases. Digested fragments are separated by agarose gel electrophoresis resulting in a characteristic microrestriction pattern. Although it is easy, cheap and fast, this technique alone is not suitable for epidemiological surveys, because the intra- and intergenomic recombination within the flagellin genes. According to

Fitzgerald et al. (2001), this technique with PFGE is suitable to differentiate among *C. jejuni* isolates of different origins. A modified method is based on sequencing the 321bp short variable region of *flaA* gene (*flaA*-SVR) possessing higher discriminatory power even when it is used alone (Eberle and Kiess, 2012).

Multilocus sequence typing (MLST) has become the most widely used molecular typing method for *Campylobacter* spp. based on *multilocus enzyme electrophoresis (MEE)* (Wiedmann, 2002). MEE is suitable for distinguishing bacterial isolates by variation in the electrophoretic mobility of genes for different enzymes resulting in different electrophoretic types (ETs) depending on mutations in their gene locus. In the case of MLST, short sequenced fragments are required within housekeeping genes (7-11) and upon their match to allelic profiles generated in the sequence to a global PubMLST (http://pubmlst.org/) database. According to the database, sequence types (STs) are rendered to allele numbers and STs that share four or more alleles belong to the same clonal complex (CC). MLST results are easy to reproduce, and the method overcomes the problem of comparison of typing schemes between laboratories (Maiden et al., 1998).

Ribotyping is a hybridisation technique, which is based on the comparison of the variable regions located around the conserved rRNA sequences. Despite of the improvements in its automatisation (riboprinting) the procedure is time consuming, and it has low discriminatory power therefore, its application is limited to epidemiological surveys (Bouchet et al., 2008).

By the use of *amplified fragment length polymorphism (AFLP)* the total chromosomal DNA is digested and the fragments are amplified by PCR, using primers labeled with radioactive or fluorescent dyes. The products are analyzed in denaturing polyacrylamide gel (Vos et al., 1995). Though AFLP is a broadly applicable genotypic method, its analysis is complex (Eberle and Kiess, 2012).

Since *C. jejuni* isolates are not routinely typed, epidemiologic studies based on the above described methods are important to follow up emerged cases, and by this, they are crucial in controlling this pathogen.

1.6. *Campylobacter jejuni* infections

1.6.1. Sources of infections

Campylobacteriosis is considered to be a food-borne disease rather than food poisoning associated with a range of foods (Ketley et al., 1997). *Campylobacter jejuni* is a member of the normal intestinal microbiota of both wild and domesticated animals, mainly used for food production (e.g. poultry, cattle, and sheep). Although the majority of the cases (50-70%) are associated with the

consumption of raw or undercooked poultry, unpasteurized milk, raw vegetables, and environmental water sources are all potential reservoirs (Figure 5). Because *Campylobacter* is widespread in the environment, poultries can be infected and colonized easily. Because of their higher (40-42 ^{o}C) metabolic temperature, they are prominent reservoirs for the thermotolerant *C. jejuni* (Horrocks et al., 2009).

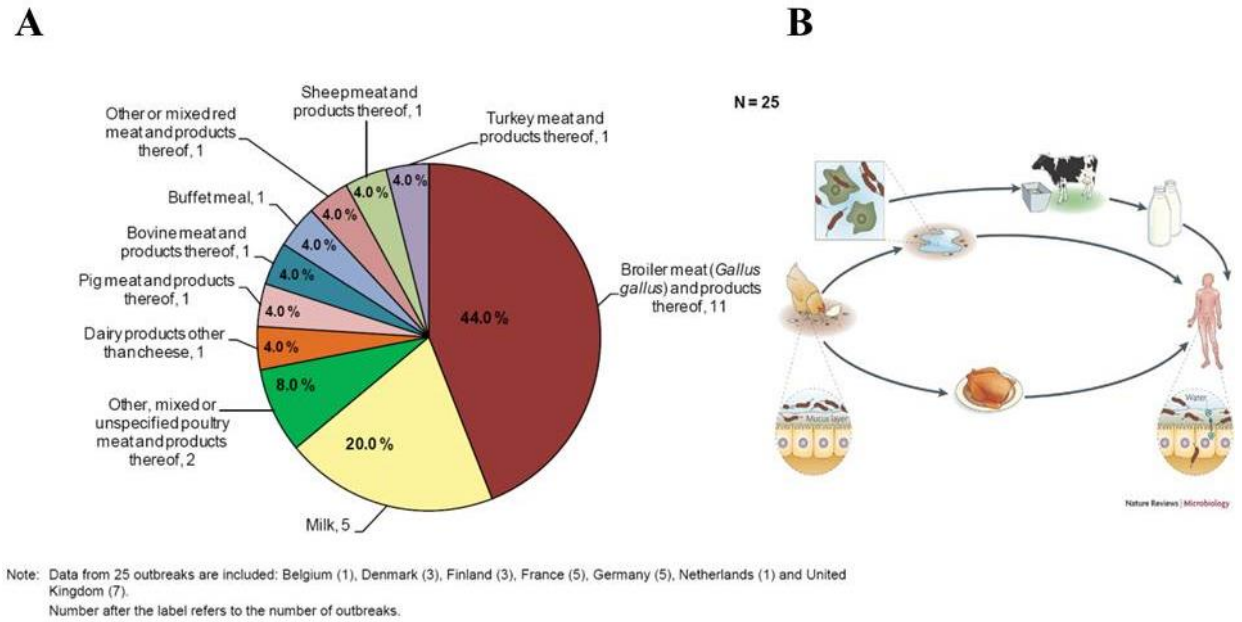

Figure 5. Distribution of food vehicles in strong-evidence outbreaks caused by *Campylobacter* in the EU, 2012 (A); Environmental routes and reservoirs of *Campylobacter jejuni* infection. (Young et al., 2007) (B)

In poultries the colonisation of the intestine does not cause any symptoms, thus detection, tracking and control of this pathogen is very difficult. European Union-wide baseline survey on the prevalence of *Campylobacter* in broiler chicken and carcasses revealed that *Campylobacte*r was present in 71.2% of live chickens, and 75.8% of carcasses was *Campylobacter*-contaminated. Nevertheless, the prevalence of *Campylobacter*-colonized broiler varied widely between countries: it ranged from the minimum 2% (Estonia) to maximum 100% (Luxembourg), and in Hungary it was 50% (EFSA, 2010). It is estimated that up to 98% of the retail meat is contaminated in the USA. Previous data also shows that the high numbers of *Campylobacter* in the intestinal tract of birds probably contaminate the meat during processing at slaughterhouses. In one study, up to 1,000-fold increase in bacterial count on carcasses was observed during transportation (Stern and Robach, 1995). It can be stated, that proper cooking of meat, the pasteurisation of milk, and chlorination of water can eliminate *Campylobacter* cells (Friis, 2007). Increased attention has to be taken to the

kitchen hygiene during food preparation (Hoffmann and Taylor, 2005). Human to human (faecal-oral way) transmission can occur, but it is very rare.

In contrast to salmonellosis, campylobacteriosis is a growing public health issue calling for changes for more effective food safety strategies. Decreased gastric acidity increases the chance to be infected with *Campylobacter*, furthermore, people with decreased immune response have greater risk to have more severe symptoms with campylobacteriosis (Sorvillo et al., 1991; Neal et al., 1996).

1.6.2. Clinical manifestations

The infectious dose of *C. jejuni* for humans is very low, as few as 500-800 organisms. After oral ingestion of *Campylobacter* the usual incubation period is 24-72 h, but it can also take 1 week or more, especially in the case of an infection with lower inoculum number. Prodromal symptoms are generally nonspecific like headache, myalgia, chills, and fever in the first 24 h. The major typical symptom appearing in the next days of the illness is the cramping abdominal pain, sometimes so heavy, that it is indistinguishable from appendicitis. The *C. jejuni*-associated diarrhea can vary from mild to severe bloody dysentery depending on individuals and the stadium of the infection (Blaser, 1997). In the first part of the illness the diarrhea is watery with one day more than eight bowel movements, usually followed by bloody diarrhea causing dehydration. The fever and the presence of blood and leukocytes in the stool are indications of diffuse inflammation in the bowel. The disease is self- limiting, usually after a peak, which lasts for two days, the illness resolves gradually over a week. In the absence of treatment the relapse is 20%. Death in association with *C. jejuni* enteritis is very rare; in the UK approximately 76 persons die each year due to campylobacteriosis (Silva et al., 2011), and are mainly confined to very young or elderly patients and to those, who suffer from immunocompromising diseases like Acquired Immune Deficiency Syndrome (AIDS).

Interestingly, in the developing countries watery diarrhea is dominant, likely because of the high level of exposure to the organism early in life, resulting in a protective immunity developing gradually over the years. There is a relevant morbidity and mortality among children in these regions. Bloody diarrhea is more common in the industrialized countries. Intriguingly, patients present symptoms depending on in which part of the world they are living, emphasizing the important role of host immune status on the course of infection. Furthermore, disease outcome is suggested to be dependent on the virulence of *C. jejuni* strains (Oberhelman and Taylor, 2000).

Extraintestinal infection can occur, such as cholecystitis, pancreatitis, cystitis, and bacteraemia is likely to appear in immunocompromised hosts (Zilbauer et al., 2008; Louwen et al., 2012). Guillain-Barré syndrome (GBS) is a serious, but rare sequel of *Campylobacter* infections, it occurs in 1 out of

1,000 cases. GBS is an immune-mediated polyneuropathy of the peripheral nervous system (PNS) resulting in neuromuscular paralysis (Van den Berg et al., 2014).

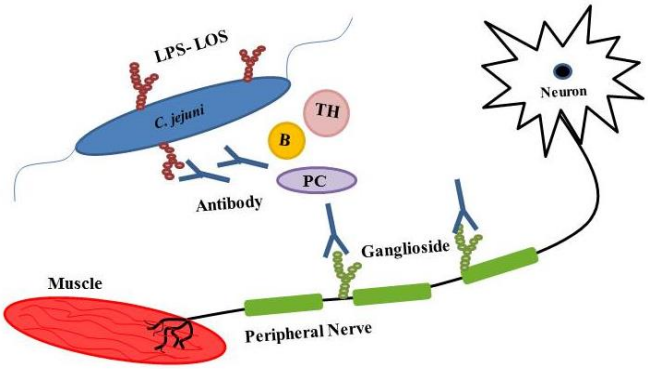

Figure 6. The molecular background of the *C. jejuni* induced autoimmune GBS. TH: T helper lymphocyte, B: B lymphocyte; PC: Plasma cell

The nerve damage can be so serious that can lead to muscle paralysis and death in 5% of GBS cases. Approximately 20% of patients with GBS are left with some disabilities. It is published that GBS "has become the most frequent cause of acute flaccid paralysis, since the near elimination of poliomyelitis in the world". Three sub-forms of GBS are described, which can be differentiated by electrodiagnostic techniques: (1) acute inflammatory demyelinating polyneuropathy (AIDP) causing the demyelination of peripheral nerves, (2) acute motor axonal neuropathy (AMAN) characterised by degeneration of axonal components of peripheral nerves, and (3) Miller Fischer syndrome (MFS) resulting in areflexia, ataxia, and ophthalmoplegia. Campylobacteriosis is the most common cause of GBS; about two-thirds of GBS patients have evidence of recent *Campylobacter* infection. GBS develops 1 to 3 weeks after the infection. The syndrome results from a case of molecular mimicry: during infection the host produces anti-*Campylobacter* antibodies, which recognize the surface polysaccharides of bacteria. These LOS structures of the pathogen resemble neural glycolipids (gangliosides), and crossreact with the antibodies inducing an attack on the peripheral nervous system (Figure 6) (Nyati KK and Nyati R, 2013).

1.6.3. Therapy and antibiotic resistance

Since campylobacteriosis is self-limiting, the replacement of lost fluids and electrolytes is generally sufficient. Antibiotics are recommended in the case of prolonged or severe enteritis, septicaemia, extraintestinal complications or for immunocompromised patients. Antibiotic resistance among campylobacters is an emerging public health problem around the world recognized by WHO

(McDermott et al., 2006). There is a growing resistance of campylobacters against: macrolides (erythromycin), tetracycline, and fluoroquinolones, the most frequently prescribed antibiotics against *Campylobacter*. The only alternative treatment is gentamicin (Aarestrup and Engberg, 2001). Antibiotics have been used for decades as food additives in livestock in order to prevent and control infections and enhance growth rates (Igimi et al., 2008; Rozynek et al., 2009). This unregulated use of antimicrobial agents has contributed to an increased resistance against multiple antibiotics also in microbes with human importance. In Europe and in USA the administration of fluoroquinolones to poultry caused an increased resistance in *C. jejuni* against these agents, isolated from animals and humans (Smith and Fratamico, 2010). The indiscriminately use of antibiotics as a supplemented animal feed is estimated to constitute for more than a half of the total antimicrobial use around the world (Moore et al., 2005).

1.7. Pathogenesis of *Campylobacter jejuni* infection

The pathogenic mechanism by which *C. jejuni* causes disease is enigmatic, the virulence factors leading to infection thought to be unique compared to other enteric pathogens. In the year 2000, the completion of the total genome sequencing of *C. jejuni* NCTC11168 (1.6 megabases) was a remarkably progress in *Campylobacter* research (Parkhill et al., 2000), but still, the molecular background of virulence mechanism contributing to campylobacteriosis is not well understood (Bouwman et al., 2013). Furthermore, it is still an open question which particular host or bacterial factors may contribute to the extensive variation in the clinical manifestation. Numerous potential virulence properties have been reported such as motility, chemotaxis, adhesion, invasion, intracellular survival, and production of toxin (Ketley et al., 1997). *Campylobacter jejuni* 81-176 possess a plasmid pVir proposed to have an important role in virulence. *VirB11* can be found on this plasmid encoding type IV secretion system (Bacon et al. 2002).

To further complicate matters, it has been described that strains possessing different affinity to invade cells *in vitro* and *in vivo* (e.g. strains isolated from patients with non-inflammatory diarrhea show the same invasion capacity *in vitro* compared to strains originated from severe colitis) (Everest et a., 1992). Besides pathogenic factors, the role of host dependent factors should also be considered (Havelaar et al., 2013).

Simple sequence repeats (SSR) in the *Campylobacter* genome provide rapid adaptation to the changing environments and greater advantage during colonisation of intestinal cells evading the host immune system. This adaptive process mediated by high mutation rates and reversible mutation of SSR is called phase-variation (PV), and it is widespread among pathogens and commensals as well.

Most of the hypervariable sequences are found in genes coding flagellum and cell surface carbohydrate structures like capsule and LOS. The presence of hypervariable sequences is partly due to the lack of DNA-repair genes. *Campylobacter jejuni* and *Neisseria meningitidis* are common in PV mediated by homopolymeric G/C repeats. These phenomena greatly contribute to differences in virulence properties between strains, and in the survival of different environmental circumstances (Bayliss et al., 2012).

1.7.1. Virulence factors of *Campylobacter jejuni*

1.7.1.1. Chemotaxis

The colonisation of the mucus is an essential early step for establishing infection. Mucin glycoproteins harboring serine and threonine repeats responsible for variable numbers of tandem repeats (VNTR), are the major components of mucus toward which *C. jejuni* displays chemotaxis. Although *C. jejuni* avidly colonizes the mucus layer resulting in heavy contamination rates in poultries, it does not adhere or invade their intestinal epithelial cells and does not cause any disease in them. Recent studies demonstrate, that mucin modulate the pathogenicity of *C. jejuni* in a species-species manner, which might explain why the same *C. jejuni* strain is pathogenic in humans and commensal in chickens (Alemka et al., 2012).

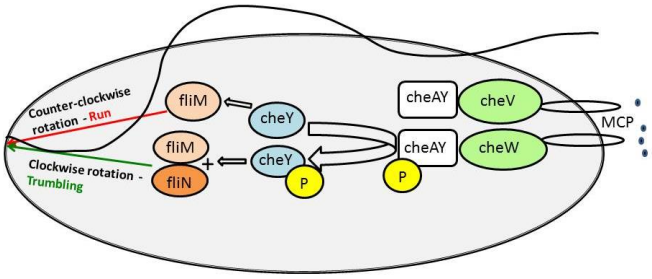

Figure 7. A model of chemotaxis signal transduction pathways in *C. jejuni*

By clockwise and counter-clockwise rotation of flagella *C. jejuni* is able to change directions depending on the extracellular signals (sugars and amino acids) sensed by chemoreceptors named methyl-accepting chemotaxis proteins (MACPs/MCPs) (there are 10 in total). MACP relays the bound signal to CheA, which autophosphorylates and phosphorylates CheY promoting a clockwise rotation of the flagella. The more receptors are occupied by chemoattractant ligands, the more of non-phosphorylated CheY are present inducing a counterclockwise rotation of the flagella resulting in a fast straightforward movement of the cell, a so-called “run”. When the concentration of the ligands is decreasing, the phosphorylated CheY is increasing being able to bind strongly the FliM,

resulting in a clockwise flagellar rotation, a "tumbling" bacterial movement (Figure 7) (Zautner et al., 2012). By using non-chemotactic mutants of *C. jejuni* the association of chemotaxis and colonisation could be revealed (Takata et al., 1992). Reduction was found in the colonisation of chicken's gut after the mutation of MACPs/MCPs such as *docB* and *docC,* which through methylation provides proper chemotaxis to an appropriate environmental component. Two genes, *cetA* and *cetB* proved to be important in energy metabolism directing bacteria towards higher energy-producing capabilities (Hendrixson et al., 2001).

1.7.1.2. Motility

The single unsheathed flagellum is necessary for the peristalsis and the entry into the mucus layer, which process is enhanced by the corkscrew morphology of *C. jejuni*.

Campylobacter jejuni flagellum consists of a basal body, a hook and a filament (Figure 8). The flagellin filament components (FlaA, FlaB) are O-linked glycosylated proteins. Mutation in the major flagellin *flaA* gene results in a truncated flagellum, and leads to the loss of colonisation ability both in human and animals (Wassenaar et al., 1991). It paralyzes the bacterium, which is able to adhere, but unable to invade intestinal cells *in vitro* (Yao et al., 1994). Bacteria with minor flagellin gene *flhB* mutation do not assemble flagella (Matz et al., 2002), are impaired in cell binding, and cell invasion. The filament cap is coded by *fliD* gene. The *flgE* and the hook length control genes (*flgD* and *fliK*) are responsible for the hook subunit. The *flgK* and *flgL* genes enable the hook-filament junction. The basal body comprises: the distal and the proximal rod subunits; the anchor rings (L-ring, P-ring, MS-ring); C-ring (motor switch proteins connected with the chemosensory system). Previous work has demonstrated, that *C. jejuni* polar flagellum is involved not only in motility, but in the secretion of flagellar proteins and invasion antigens (*ciaB*) through its type III Secretion System (T3SS) (*flhA, flhB, flip, fliR, fliI, fliQ,* and *fliH*) (Konkel et al., 2004). FlhB has been reported to have an important role in virulence (Matz et al., 2002). The stator elements (*motA, motB*) are responsible for gaining energy for motility.

To regulate *C. jejuni* flagellum, a phase-variable two-component system comprising the sensor FlgS and the regulator FlgR is essential.

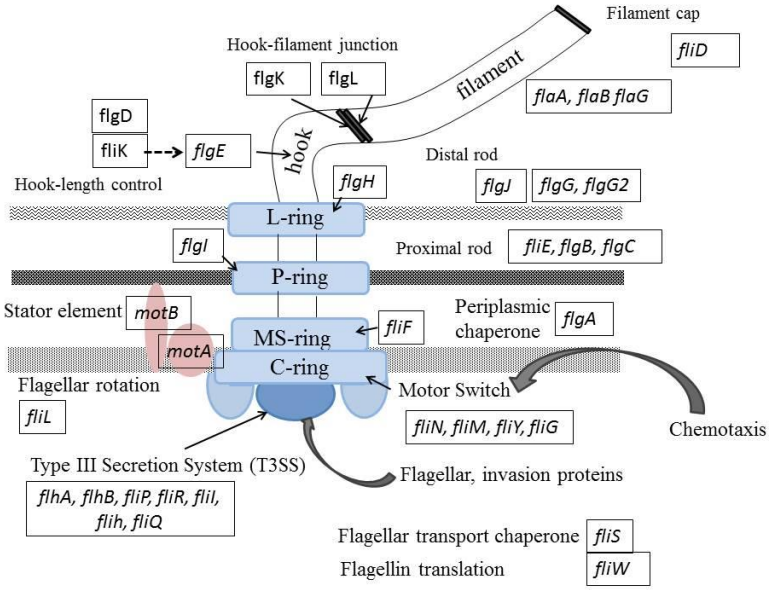

Figure 8. Flagellar structures and regulatory cascades of *C. jejuni*

To produce functional flagella, bacteria must coordinate both the temporal expression of over 40 flagellar genes and the ordered production and secretion of the encoded proteins. *Campylobacter jejuni* uses two alternative sigma factors, 28 (FliA) and 54 (RpoN), to mediate transcriptional regulation of specific flagellar genes. Sigma28 is involved in the transcription of a small subset of genes, including the major flagellin gene (*flaA)*, while sigma54 is required for transcription of many genes encoding the flagellar rod, basal body, and hook components and a minor flagellin (*flaB*) (Jagannathan et al., 2001).

1.7.1.3. Adhesion

Although the exact mechanism of the pathogenic process of *C. jejuni* has not yet been revealed in detail, one of the first important steps is colonisation. After the penetration through the mucus layer, *C. jejuni* can directly interact with the epithelial cell layer. It has been revealed that a close connection exist between the severity of symptoms and the adherence ability of the strains (Fauchere et al., 1986).

The precise molecular mechanism of the attachment of *C. jejuni* to eukaryotic cells is still undiscovered, but is revealed to be a multifactorial event. According to certain signals, originated from human or chicken intestinal environment, *C. jejuni* will be induced the over or under expression of its adhesion factors. Early studies have revealed adhesion of *C. jejuni* to the intestinal colonic cells of human (van Spreeuwel et al., 1985), *Macaca mulata* monkey (Russel et al., 1994) and newborn piglet (Babakhani et al., 1993). In contrast, no adhered *C. jejuni* cells were observed in the intestine

of mice (Lee et al., 1986) and chicken (Beery et al., 1988), although the bacteria were present in the mucus in a high number. This could mean that adhesion to the epithelial cells is not necessary for the colonisation of gut (Backert and Hofreuter, 2013). Unlike *Salmonella* and *Escherichia coli*, *Campylobacter* adhesion is not mediated by appendages like fimbria or pilus (Nougayrede et al., 2003). Two outer membrane proteins of *C. jejuni*, CadF and FlpA specifically bind fibronectin (Fn), a glycoprotein found in the extracellular matrix (EM), and located basolaterally on epithelial cells *in situ*. *Campylobacter jejuni* lacking of CadF and FlpA revealed to have reduced ability to adhere chicken intestine (Monteville et al., 2003; Flanagan et al., 2009), the same result could be observed in experiments with human and chicken cell lines (Monteville et al., 2003; Flanagan et al., 2009). Another characterised adhesin, JlpA is a loosely surface-exposed lipoprotein, which binds to the heat-shock protein 90 (Hsp90) found in Hep-2 cells. Several other factors have been described but their role in adhesion is only hypothetic. CapA is an autotransporter lipoprotein, in the lack of this protein, reduction was reported in adherence to Caco-2 cells (Ashgar et al., 2007). Peb1 was identified as a major antigenic protein of *C. jejuni*, and it was described to adhere to HeLa cells (Kervella et al., 1993). Peb1 is located on the cell and/or in the periplasm, it is a component of an aspartate/glutamate ABC transporter. Its indirect role in the adhesion or as a bifunctional protein remains to be discovered (Pei et al., 1998). Peb3 protein is highly immunogenic, its possible direct role in adhesion has not been proved yet (Linton et al., 2002). Peb4 deficient bacteria were less successful in adhesion of INT407 cells and in biofilm formation. Its suggested role in adherence has not been confirmed yet (Yanagawa et al., 1994; Asakura et al., 2007; Rubinchik et al, 2012;). The major outer membrane protein (MOMP) is implicated in adherence of *C. jejuni* to INT407 cells (Moser et al., 1997). This is a pore-forming protein consisting of 16-18 membrane strands driven through the membrane in antigenically variable loops and turns having a role in ion transport. Having a crucial role in cell metabolism, to draw a final conclusion regarding its role in the adhesion is problematic (Goulhen et al., 2004). CPS may facilitate bacterial interaction with the host cell, but this outcome was not supported by complementation tests. CPS may be necessary in the beginning of bacterial interaction with the mucus layer (Rubinchik et al, 2012).

From the host side, extracellular matrix proteins (ECMPs) may play an important role in bacterial adhesion. Type IV collagen and laminin is an important part of the basal lamina associated with fibronectin. Fibronectin exists as a protein dimer, binds to membrane-spanning receptor proteins called integrins. In vertebrates two types of fibronectin are present: soluble plasma and insoluble cellular fibronectin. Fibronectin similarly to laminin and collagen IV, is a high-molecular-weight glycoprotein found in the basement membranes of intestinal epithelial cells, having the crucial role to

establish contacts with other macromolecules, let it be eukaryotic or prokaryotic (Kuusela et al., 1989).

1.7.1.4. Invasion

Genome analysis has revealed that *C. jejuni* lacks the typical invasion genes observed in many other intestinal pathogens making the prediction, that the mechanism in *C. jejuni* could be characterised by individual traits. A wide variety of *in vitro* cell culture models have been used to investigate the factors involved in the adhesion and invasion mechanism. Unfortunately, the use of different cell cultures with diverse *C. jejuni* strains led to further confusion and controversies in the literature. However, for host cell invasion, the binding to eukaryotic cells of bacteria is required. (Rubinchik et al, 2012).

To maintain a successful infection, microbial gut pathogens have to develop various strategies to invade tissues, avoid or withstand the offence of the immune system, disturb the normal gut flora, damage the cells, and multiply in high number. In campylobacteriosis, invasion is the key step of the infection to develop disease. The healthy intestinal cells have apical-basal polarity, apical microvilli structures, and proper junctional complexes. Similarly to other intestinal pathogens, *C. jejuni* is also able to adhere and invade into polarised (e.g. Caco-2) (Russell and Blake, 1994) and non-polarised cells (e.g. INT407) (Monteville et al., 2003).

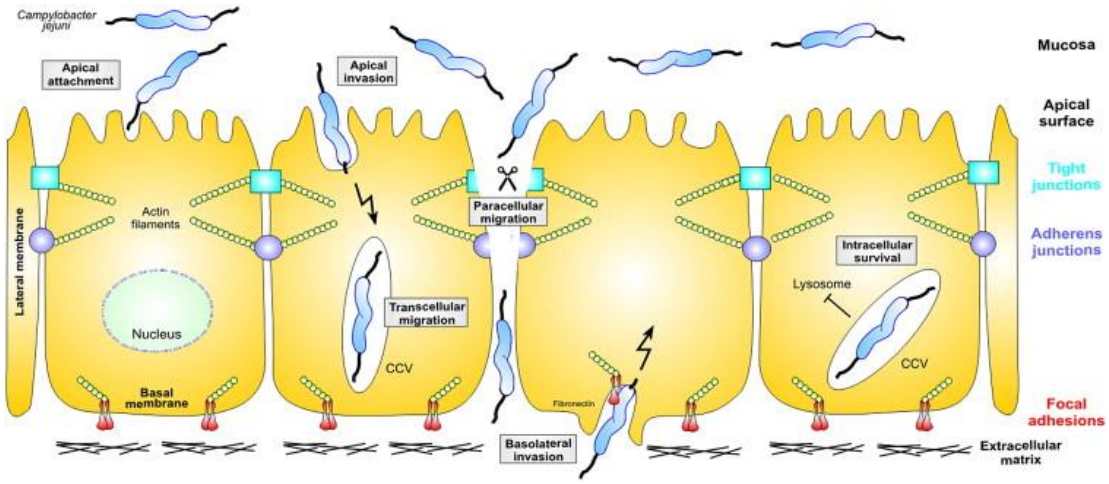

Figure 9. Hypothetical models for *C. jejuni* invasion mechanism (Backert and Hofreuter, 2013)

Enteropathogens use several mechanisms to transmigrate across the intestinal cells; however, our knowledge is still limited concerning the *C. jejuni* invasion process. Two considered routes of transmigration of *C. jejuni* through the polarized intestinal cells are suggested (Figure 9). In the case of transcellular route, bacteria enter at the apical side and exit at the basal aspect of the cells. The

paracellular route is taken by bacteria crossing through the tight and adherence junctions between epithelial cells, and entering at the basal side of the intestinal cells or continuing their way through the lamina propria (Konkel et al., 1992; Bouwman et al., 2013). Several studies have shown that *C. jejuni* is able to enter into the intestinal epithelium and travel to different parts of the human body (liver, spleen, mesenteric lymph nodes and vessel) via lamina propria. For initiating their own uptake into the eukaryotic cells, they subverse the signaling pathways of the hosts using (i) "zipper" (*Yersinia, Listeria*) or (ii) "trigger" (*Salmonella, Shigella*) routes (Cossart and Sansonetti, 2004). By the "zipper" invasion mechanism the pathogen uses its bacterial surface proteins (adhesins, invasins) to bind by high affinity to the receptors of the host cell (Figure 10). Via receptors cytoskeletal rearrangement is induced resulting zippering and engulfing the plasma membrane around the pathogen. On the other hand, the "trigger" mechanism includes type-III and type-IV secretion systems injecting bacterial effector proteins to trigger bacterial uptake by inducing signaling events like cytoskeletal reorganisation. Internalised bacteria can survive within the vacuole or escape from it.

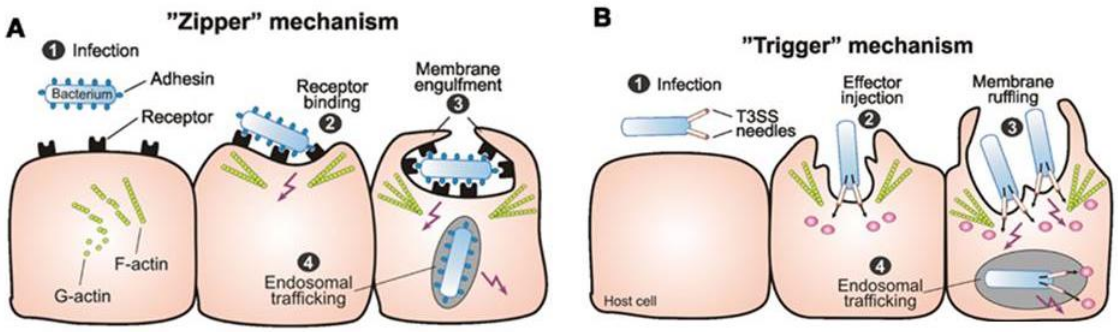

Figure 10. Mechanism of bacterial invasion into non-phagocytic host-cells (Ó Cróinín and Backert, 2012)

Membrane ruffling was observed by high resolution electron microscopy (EM), induced by *C. jejuni* in a contact-dependent way on non-polarised INT407 cells, followed by the internalisation into the cells (Krause-Gruszczynska et al., 2011). The entry begins with the flagellar tip of the bacteria.

Recent studies demonstrate the importance of eukaryotic plasma membrane lipid rafts (caveolae) interacting with receptor molecules (Watson and Galán, 2008). Pharmacological inhibitor studies revealed that heterotrimeric G proteins could be involved in the uptake process. Although there are suggestions and evidences for the involvement of numerous receptor protein kinases: EGF and PDGF (Boehm et al., 2011), phosphatidylinositol 3-kinases (PI3-kinase), protein kinase C (PKC), and mitogen-activated protein kinase (MAPKs) (Hu et al., 2005) in internalisation process, till now the only receptor pathway, which was verified by independent research groups is the: CadF

→fibronectin →integrin signaling cascade (Monteville et al., 2003; Eucker and Konkel, 2012), where integrin α5β1 (specific for fibronectin) facilitates thirosine phosphorylation of focal adhesion kinase (FAK) and paxillin (Monteville et al., 2003). Activation of Rho GTPases (Rac1, cdc42) stimulates microtubules formation (Krause-Gruszczynska et al., 2011). Dynamics of MTs and MFs contribute to cytoskeletal rearrangements and bacterial uptake, but after about 20 years of research the whole triggering pathways are not clear (Ó Cróinín and Backert, 2012), and only a hypothetical model is available for the probable signaling events generated during internalisation of *C jejuni* into the host cell (Figure 11).

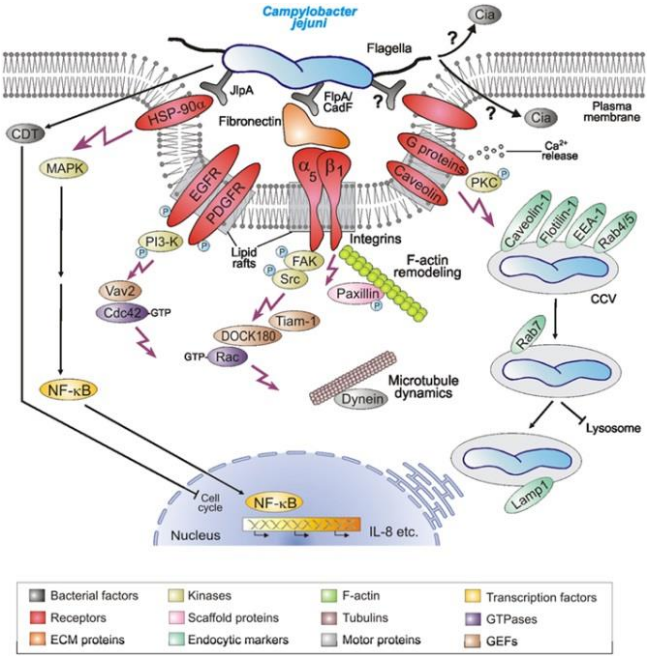

Figure 11. Hypothetical model for *C. jejuni*-induced signaling events (Ó Cróinín and Backert, 2012)

After internalisation into intestinal epithelial cells, a *C. jejuni*-containing vacuole (CCV) is developed, avoiding the delivery into lysosomes as it deviates from the canonical endocytosis (Watson and Galán, 2008). In the shelter of the endocytic vacuole with the help of the late endosomal marker (Lamp-1) *C. jejuni* can evade the host immune response for a considerable time, and hence it is able to cause a long-term persistent infection. The *Campylobacter* gene, *ciaI* is important in the intracellular trafficking and survival (Buelow et al., 2011).

1.7.1.5. Toxin

The only verified toxin of *C. jejuni* is the cytolethal distending toxin (CDT). CDT consisting of three subunits (CDTA, -B, and -C) is expressed during colonisation and causes distension of the epithelial cells, which manifests in bloody diarrhea (Figure 12) (Dasti et al., 2010). The enzymatically active

subunit encoded by *cdtB*, shares homology with *DNase I* of *E. coli* (Lara-Tejero and Galán, 2000). This toxin arrests cell cycle at the G_1/S or G_2/M phase depending on the type of the cell induces cellular distension and cell death. CdtA and CdtC are thought to have a role in binding the bacteria to host cell (Whitehouse et al., 1998).

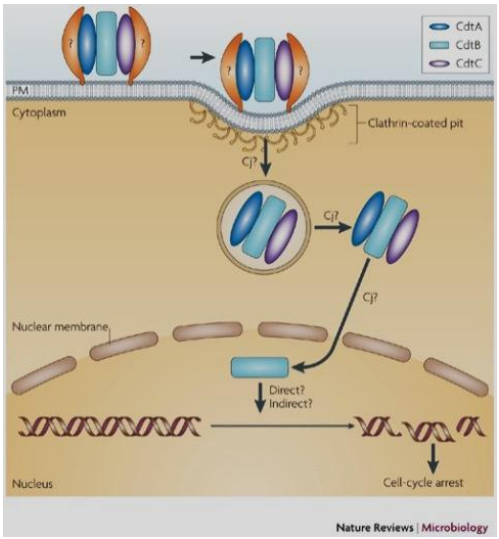

Figure 12. Uptake and activity of cytolethal distending toxin (Young et al., 2007)

1.7.1.6. Further factors important for host colonisation

Lipooligosaccharide

LOS is thought to trigger GBS by molecular mimicry between gangliosides GM_1, GD_1 and LOS. The LOS biosynthesis genes (*wlaN, cgtB*) in *C. jejuni* are localised in a hypervariable locus, both are coding for β-1,3-galactosyltransferases to synthetise gangliosides that mimic host cell tissues, and trigger the production of autoreactive antibodies leading to GBS (Linton et al, 2000). *CstII* and *cstIII* take a part in sialyltransferation leading to the synthesis of multiple mimic types (Houliston et al., 2011). Mutation in LOS biosynthesis genes affect adherence to INT407 cells (Fry et al., 2000). LOS also contributes to serotype specificity (Penner and Hennessy, 1980).

Capsule

CPSs cover the bacterial surfaces playing an essential role in survival, persistence in various environmental circumstances, and generally contribute to pathogenesis. CPS provides resistance for *C. jejuni* in avoiding phagocytosis and killing by complement immune system. Its existence remained unnoticed until the genome-sequencing project (Parkhill et al., 2000). Having a pivotal role in serum resistance, these molecules are major antigens in the Penner serotyping scheme. It was published by Bacon et al. (2001), that a *cps* mutant shows reduced invasion capacity. An extensive variation in the capsule structure was revealed due to phase-variation of structural genes and

modification of O-methyl phosphoramide (Guerry et al., 2012). Altogether 8 CPS types of *C. jejuni* have been described (Chen et al., 2008).

Glycosylation system

Two protein glycosylation systems have been revealed in *C. jejuni*: (i) O-linked glycosylation modifies serine or threonine residues in flagellin; (ii) N-linked glycosylation alters asparagine residues on several proteins. Interestingly, N-linked glycosylation can only be found in eukaryotes and archaea with the only exception of *C. jejuni* (Young et al., 2002). O-linked glycosylation is essential for the proper assembly of the flagellar filament (Guerry et al., 2006). The locus of the O-linked system is heterogeneous, while the N-linked glycan structure (encoded by *pgl* genes) is conserved unlike other surface carbohydrate structures suggesting an important role in the biology of *C. jejuni.* Mutation in *pgl* genes results in reduction of the adhesion and invasion capacity (Hendrixson and DiRita, 2004).

2. Essential Oils

2.1. History of essential oils

Essential Oils (EOs) are aromatic, volatile (ethereal) oily liquids extracted from plant organs (buds, flowers, leaves etc.). The term "essential oil" is proposed to derive from a Swiss reformer of medicine Paracelsus von Hohenheim in the 16th century who named the effective component of a drug as "Quinta essential" (Guenther, 1948). Several methods are available to obtain EOs from plant material, but the most common and simplest method for producing EO (as it used in the commercial production) is the steam and hydro distillation. This method was first used 2000 years ago in East, and improved in the 9th century by the Arabs.

By the 13th century, EOs were being made by pharmacies, but only the 16th century brought the widespread use of them (traded in London). In the 17th century 15-20 different EOs were sticking by pharmacies (Bauer et al., 2001). In the 19th and 20th centuries the medical use of EOs became secondary to their use for flavour and aroma. Presently, approximately 3000 EOs are known, and 300 are in commerce.

Biological activities of EOs depend on numerous factors: species, climate, soil composition, age, vegetative cycle stage, season, plant organ, and the extraction method itself (Angioni et al., 2006). Liquid carbon dioxide, applying low temperature and high pressure is more expensive, but the produced EO has greater antimicrobial activity. It also can be enhanced if herbs are harvested during or immediately after flowering (Burt, 2004).

Commercialized EOs are chemotyped and analysed by gas chromatography (GC) and mass spectrometry (MS) (Smith et al., 2005). EOs are complex mixtures, containing 20-60 compounds (in some cases more than 60) at quite different concentrations. Generally one or limited number of more major component(s) are responsible for the biological effects, but in some cases, minor components may also have critical part in antimicrobial activity. Major components can take up to 85% of the EO, while other minor components can be found only in trace amounts (Bakkali et al., 2008). Synergistic effects can be also observed, if an EO as a whole material has greater biological activity, than the components of EO separately.

Three major groups can be differentiated among EO components: terpenes (limonene, p-cymene), terpenoids (menthol, piperitone), phenilpropenes (eugenol, cynnmanaldehyde), and "other" (Burt, 2004).

2.2. Antibacterial action of essential oils

Essential oils are secondary metabolites playing a crucial role in the protection of plants as antimicrobials, antifungals, antivirals, insecticides, and against herbivores (Bakkali et al., 2008). Some of them are always present, and some are produced as a response to physical injury or invasion by microbes and insects (Hyldgaard et al, 2012). The antimicrobial properties were first evaluated by De la Croix (Burt, 2004). Essential oils generally act on distinct target sites by different mode of action therefore it is suggested, that no bacterial resistance can be developed against EOs (Carson et al., 2002).

The following antibacterial mechanisms of essential oils have been described (Figure13):

- Due to their hydrophobicity they can cause partition of the lipids in the bacterial cell membrane causing increased permeability, depolymerisation, and decreased membrane potential disturbing the ion flow of the membrane.
- Appearance of radicals evoking oxidative stress as a concomitant of the increased membrane permeability usually leads to cell death.
- The permeable membrane causes the loss of essential molecules like adenosine triphosphate (ATP), depleting the intracellular ATP pool. Disturbing the proton motive force (PMF) can cause the loss of motility (Turina et al., 2006).
- Essential oils components are also able of interfering with proteins in the wall usually involved in the transport of essential molecules into the cell.
- Essential oils alter membrane fluidity by decreasing the proportion of the unsaturated fatty acids (UFAs) by direct effect on the fatty acids or by modifying enzymes involved in fatty acid synthesis. This may cause morphological changes of the cell (Di Pasqua et al., 2007). Rod shaped bacteria were found to be more sensitive to EOs than coccoids (Hafedh et al., 2009).
- Essential oils can act on proteins taking part in cell division (FtsZ). Generally the upregulation of thioredoxin (cell division), DnaK, GroEL (protecting proteins by folding them), and enolase (energy metabolism) can be observed as a result of EO treatment (Kumar and Berwal, 1998; Di Pasqua et al., 2013).
- Essential oils can influence the ATP production in the cell wall, or in the cytosol. They can inhibit membrane bound ATPase activity (F1F0 ATPase), and by disrupting membranes they cause ATP loss (Dorman and Deans, 2000).
- Essential oils can act on metabolome (glucose accumulation) (Carneiro et al., 2011).

- Essential oils also act on quorum sensing (QS) - on the intercellular communication system of bacteria - affecting the expression of virulence factors, and biofilm production and so on (Zaki et al., 2013).

Generally Gram-negative bacteria are more resistant to EOs than Gram-positive ones (Trombetta et al., 2005). One explanation could be, that the hydrophilic external part of lipopolysaccharide (LPS) on the outer membrane of Gram-negatives, create a defensive barrier against macromolecules and hydrophobic molecules like the components of EOs (Nikaido, 2003).

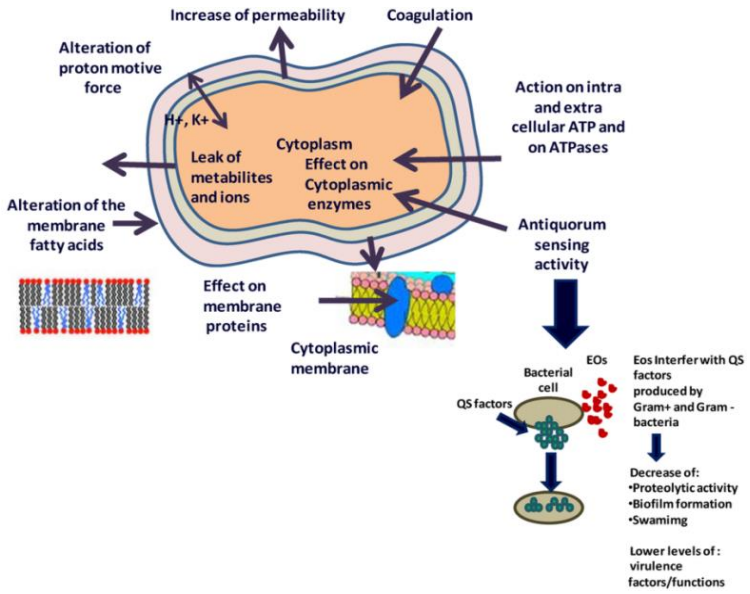

Figure 13. Mechanism of action and target sites of essential oils on microbial cells (Nazzaro et al., 2013)

The sub-lethal concentration of EO is the most suitable to elucidate those molecular changes that are behind the scene, and reveal the antibacterial mode of actions of EOs. Different techniques are available to study the changes on and in the bacterial cells contributing to the antibacterial activity. EOs are not only able to influence basic cellular functions, but also could have an effect on those factors of a pathogenic bacterium that play a key role in the pathogenesis (Derakhshan et al., 2010).

2.3. Clove essential oil

Clove EO can be obtained from aromatic dried flower buds of a tree, *Syzigium caryophillatum* (L.) Alston, *Syzigium aromaticum* (L.) Merr and Perry, belonging to the family *Myrtaceae* (Srivastava and Malhotra, 1991; Chaieb et al., 2007). Besides, that clove is an important aromatic spice, it is used in Ayurveda, chinese medicine, and western herbalism. It is commercially cultivated among others in India, Madagascar, Sri Lanka and Indonesia. Clove is widely used in flavour and fragrance industry (Chaieb et al., 2007), and has a prominent role in folk medicine as diuretic, odontalgic, and

stomachic (Boulos, 1983). Cloves are used in the in dentistry and the medicine as a carminative, to increase hydrochloric acid production in the stomach and to improve peristalsis (Prashar et al., 2006). In addition, clove is anti-mutagenic (Miyazawa and Hisama, 2003), anti-inflammatory (Kim et al., 1998), and antioxidant (Saeed and Tariq, 2008).

Clove EO is one of the most powerful antimicrobial agents. It is used for acne, warts, scars, and parasites. Furthermore, it reliefs the pain, used as antiseptic in oral infections. Its bacteriostatic effect has been described on *Escherichia coli*, *Salmonella enteritidis*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Campylobacter jejuni* (Smith-Palmer et al, 1998). Beside its positive effects, clove EO can be toxic to human cells in a certain concentration, causing acute respiratory distress syndrome, fulminant hepatic failure, and central nervous system disorder. The lethal oral dose of clove EO is 3.752 g/kg body weight (Hartnoll et al., 1993).

Several constituents of clove EO have been identified: eugenol, eugenyl acetate, β-caryophyllene, 2-heptanone (Chaieb et al., 2007), acetyleugenol, alpha-humulene, methyl salicylate, isoeugenol, and methyleugenol (Figure 14) (Yang et al., 2003). Eugenol, its main component is thought to be responsible for the strong biological and antimicrobial properties of the clove EO (Srivastava and Malhota, 1991).

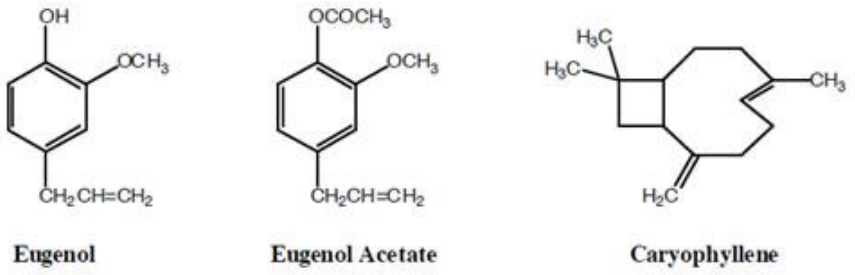

Figure 14. Chemical structure of three major components of clove essential oil (Yang et al., 2003)

Eugenol was found to permeabilize the cell membrane by non-specific ways such as inducing increased transport of potassium and ATP out of the cells. In addition, it contributes to changes in the fatty acid profile. By the interaction with proteins, eugenol is suggested to bind to and affect the properties of proteins at sublethal concentration. The following enzymes thought to be concerned: ATPase, histidine decarboxylase, amylase, and protease (Hyldgaard et al., 2012). Clove EO was found to inhibit the QS as well (Zaki et al., 2013).

CHAPER II. AIMS AND OUTLINE

Campylobacter jejuni is a major food-borne pathogen with strain-dependent pathogenic potential. A total of 400 *Campylobacter jejuni* isolates were collected from diarrheal stool samples of individual patients in the Department of Microbiology of the South Transdanubian Regional Public Health Institute in the year of 2006, and altogether 190 strains were stored. In this period the strain collection represents 19.7% of all campylobacteriosis cases in this region of Hungary.

We aimed to answer the following questions paving the way of the Ph.D. dissertation:

- What is the clonal relationship among the characterised 190 isolates, and what is the phylogenetic relationship between the clonal groups and the severity of the symptoms?
- Is there any correlation between the virulence traits and the severity of the clinical symptoms of the investigated isolates?
- By using whole transcriptomic analysis is it possible to identify novel candidate genes that could play roles in the so far not completely understood invasion process of *C. jejuni*?
- Is the antibacterial effect of clove essential oil general for *Campylobacter jejuni* and by this does this essential oil have a potential to control *C. jejuni*?
- Which are the attendant molecular and phenotypic changes on and in the *C. jejuni* cell, as a result of antimicrobial effect of clove EO?
- Is it possible to apply a simple detection method by which the presence of high molecular weight lipopolysaccharide structures of different *Campylobacter jejuni* isolates could be detected? Elucidation of this question could solve a long existing debate.

CHAPTER III. MOLECULAR AND EPIDEMIOLOGICAL CHARACTERISATION OF *CAMPYLOBACTER JEJUNI* STRAINS ISOLATED FROM HOSPITALISED PATIENTS

1. Aims of the study

In the year 2006, 190 *C. jejuni* strains were available from hospitalised patients with gastrointestinal symptoms in the South-West Hungarian region. Based on this strain collection our aims were the followings:

- to reveal the genetic relatedness among the isolates,
- to characterise their virulence potentials by phenotypic and genotypic testing, and confer them to the severity of the symptoms.

2. Materials and methods

2.1. Bacterial strains

Campylobacter jejuni strains were cultured at thermophilic condition and subsequently identified and tested for species by Gram stain and microscopic morphology, colony morphology, oxidase activity, hippurate hydrolysis, and indole acetate activity. *C. jejuni* strains were stored in phosphate-buffered saline (PBS) containing 20% glycerol at -80 ^{o}C. Isolates were grown on CCDA at 42 ^{o}C under microaerophilic condition for 24 h.

2.2. Genotyping methods

2.2.1. *FlaA*- Restriction fragment length polymorphism (*flaA*-RFLP)

FlaA-RFLP was performed as described by Wassenaar and Newell (2000). Total DNA of the isolates was gained by boiling 1 ml bacterial suspension set to optical density (OD) of 1.0 at 650 nm for 10 min and used for the PCR reaction. The amplified *flaA* gene product was digested with a single restriction enzyme *DdeI* (Thermo Scientific, USA). The digested products were run in a 2% agarose gel. Photographs of gels stained with 0.1 ‰ ethidium bromide solution were analysed by the GelCompar II software (Applied Maths). According to the patterns, chladograms could be drawn and sorted into molecular types (MTs) as subtypes.

2.2.2. Pulsed-field gel electrophoresis (PFGE)

PFGE was performed by the standardised method of Campynet PFGE Subtyping Group (2000). To perform PFGE method, altogether it takes five days of preparation. On the first day the bacteria were harvested and collected in tubes containing Pett IV buffer (1 M NaCl, 10 mM Tris, 10 mM ethylene diamine tetra acetic acid (EDTA) pH 8.0), and cell densities were adjusted to MacFarland 6-7. 100 µl of 37% formaldehyde was added to 1 ml bacterial suspension, and incubated at room temperature for 1 h in order to inhibit bacterial *DNase* (Thermo Scientific, USA) activity, and to prevent DNA degradation. The suspension was centrifuged at 10,000 x g, for 10 min, and washed three times with 1 ml Pett IV buffer to remove any trail of formaldehyde, which may inhibit the enzymatic digestion further on. The pellet was suspended in 600 µl Pett IV buffer. 1% plug agarose gel was prepared and cooled back to 56 ^{o}C in waterbath (Julaba SW22). 700 µl agarose was dispersed with 300 µl bacterial suspension and the mixture was poured into a 12-well chamber. Agar blocks were removed by inserting a blunt-tipped glass pipette and placed each set of 12 agar plugs into a 15 ml plastic tube containing 5 ml ESP buffer (0.5 M EDTA, 1% Sarcosyl) followed by an overnight digestion with *proteinase K* (1 mg/ml) (Sigma-Aldrich, USA), warmed to 56 ^{o}C just before use, and incubated the reactions at the same temperature. Next day the ESP buffer was decanted and the plugs were washed six times, each for at least 20 min periods in 2 ml of 10:1 TE (10 mM Tris, 1 mM EDTA) buffer. The plugs can be stored for several weeks in fresh TE buffer in a fridge. On the third day, before digestion with restriction endonuclease, *SmaI* (20,000 U/ml) (New England BioLabs, UK), the plugs (0.25 cm) were preincubated in 100 µl equilibration buffer (10 µl 10X T buffer, 10 µl 100X bovine serum albumin (BSA)) for 1 h. The digestion was performed by 20 U/sample overnight, at room temperature. On the fourth day, the digested whole genomic DNA was electrophoresed in a 1.2% special agarose gel. 2.5 l of 0.5X TBE buffer was prepared by adding 125 ml 10X TBE (0.9 M Tris, 0.9 M boric acid, 0.02 M pH 8.0 EDTA) to 2,375 ml milli Q water (Bio-Rad equipment). The well-forming comb must be inserted into the assembly before the agarose poured in. The digested DNA slices were placed into the wells, followed by overlaying molten 1% agarose on the wells. Before starting the run, 0.5X TBE buffer was poured into the electrophoresis chamber and circulated through a pump to cool the unit (12 ^{o}C) ca. 2 h before use. The run was performed on PFGE equipment (Amersham Bioscience, Gene Navigator System) by the program presented in Table 3.

Table 3. Program for PFGE electrophoresis

	north-west	east-west	phases (h)
phase 1/2	0.5	0.5	24: 00
phase 2/2	40.0	40.0	02: 30

After running for 26.5 h, the gel was stained with ethidium bromide for 7 min, differentiated in water for 20 min, viewed under UV light, and then photographed. The macrorestriction patterns of the isolates were compared according to the Dice similarity index (1-1% tolerance interval) using the GelCompar II software (Applied Maths).

2.2.3. Polymerase chain reaction (PCR)

C. jejuni total DNA was achieved as described above (see 2.2.1.). Tested genes, primers and annealing temperatures are listed in A/4. PCR was performed in a DNA Thermal Cycler (Eppendorf Mastercycle, Germany) using standardised amplification parameters: 95 ^{o}C for 1 min for initial denaturation followed by 30 cycles of denaturation at 95 ^{o}C for 30 sec, various annealing for 2 min, elongation step at 72 ^{o}C for 2 min. DNA bands were obtained by electrophoresis on 1% agarose gel, stained by ethidium bromide, and visualised using BioCapt Imaging System.

2.3. Phenotypic methods

2.3.1. Solid-phase binding assay

The following ECMPs were applied in our experiments: i) fibronectin from human foreskin fibroblast (F2518, 0.5 mg, Sigma-Aldrich, USA), ii) laminin (from Engelbreth-Holm-Swarm murine sarcoma basement membrane, L2020, 1 mg, Sigma-Aldrich, USA) and iii) collagen type IV (from human placenta, C7521, 5.0 mg, Sigma-Aldrich, USA). The wells of microtitre plate (96-well plate, Sarstedt) were filled with 100 µl aliquots of 10 µg/ml fibronectin, laminin and collagen type IV in PBS, respectively, and incubated overnight at 4 ^{o}C. Next day, the wells were washed three times with 200 µl PBST (PBS, 0.5% Tween 20) and blocked with 100 µl 2% BSA for 2 h at room temperature. BSA was removed by washing the plates three times in PBST. Bacterial cells were grown as defined above, harvested in PBS, and suspended to an OD of 1.0 at 650 nm. To each well of a 96-well enzyme-linked immunosorbent assay plate, 100 µl of bacterial cell suspension was added, and incubated at 37 ^{o}C, for 3 h. Plates were then washed three times with PBST, and 1 ml 1% PBS-TritonX-100 (Calbiochem, USA) was added to the protein-bacterium complex, incubated at 37 ^{o}C, for 10 min. After the incubation the resuspended protein-bacterium complex (10 µl) was bled on

CCDA. After 48 h incubation under microaerophilic condition at 42 ^{o}C, colony forming units (CFUs) were counted, and calculated the ratio of protein-binding ability.

2.3.2. INT407 cell adhesion and internalisation assay

Adhesion and invasion analysis of the clinical strains were performed on semi confluent monolayer ATCC CCL 6, INT407 human embryonic intestine (jejunum and ileum) cell line, on 24-well culture plates. For experimental assays, semiconfluent cell monolayers were prepared (3×10^5 cells per well). The intestinal cells were cultured in RPMI 1640 medium (BioWhittaker, Lonza, Switzerland) supplemented with 10% heat-inactivated (30 min for 56 °C) calf bovine serum (Sigma-Aldrich, USA)), 10,000 U of penicillin per ml, 10 μg/ml of streptomycin and 0.5 mg/ml of neomycin, and incubated overnight at 37 °C in a humidified, 5% CO_2 incubator. On the next day, bacterial suspensions with different optical densities (OD 1.0, OD 0.1, OD 0.01) were added to INT407 cells to reach a multiplicity of infection (MOI) ranging from 10 to 500 (Backert and Hofreuter, 2013). Plates were centrifuged at 100 x g for 10 min at room temperature, and incubated at 37 °C in a humidified, 5% CO_2 incubator for 3 h. For the adhesion and invasion assay, two separate plates were used.

After incubation, the "adhesion" plates were washed three times with PBS followed by adding 1 ml Triton X-100 (Calbiochem) solution (0.1% (v/v) in order to lysate the INT407 cells on the plastic plate. To assess the total number of adhered and internalised bacterial count, 10 μl of the 1 ml suspended cells were plated on CCDA, and incubated under microaerophilic condition for 48 h at 42 ^{o}C.

The gentamicin protection assay (GPA) was used to quantify the number of internalised bacteria. In the case of the "invasion" plates, after a three-time washing step with PBS, an additional incubation (30 min) with 1 ml RPMI-gentamicin (Sanofi-Aventis), at bactericidal concentration (20 μg/ml) was performed to get the invaded/ intracellular bacterial count. After washing the plates three times with PBS, 1 ml Triton X-100 (0,1%) was added, and 10 μl volumes of the samples were plated as in the case of the "adhesion" plates.

To assess the accurate number of adhered bacteria, the total numbers of adhered and invaded bacteria (counted from the "adhesion" plates") have to be subtracted with the number of the invaded bacteria (counted from the "invasion" plates") (Backert and Hofreuter, 2013).

3. Results

3.1. *flaA*-RFLP and PFGE

By *fla*-RFLP, 183 strains could be typed, and at the 90% homology, 69 groups could be identified with the distribution detailed in A/2.

By evaluation of PFGE, the analysis of chladograms was performed. Out of 190 clinical isolates 164 proved to be typable, and 122 PFGE profiles could be distinguished. The minimal rate of homology was 31% for all the typable isolates (Figure 15). PFGE patterns revealed one large and 15 smaller groups including isolates with 100% homology within the respective groups. Altogether 28% of the typable isolates could be ranged into these 16 groups (A/3). At the level of 40% homology, three major groups, a four-member group (2006-80, 2006-79, 2006-47, and 2006-21), and a strain with a completely individual pattern (2006-170) could be differentiated. 24 strains fullfield the terms of the 90% Dice's coefficient (similarity coefficient), and comprised 12 PFGE groups.

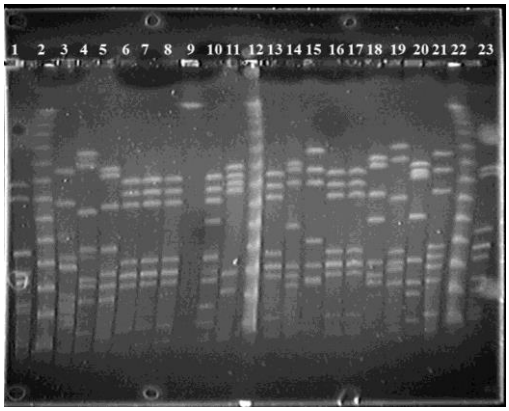

Figure 15. Analysed human *C. jejuni* isolates by PFGE. Lane 1, 2006-126; lane 2, Lambda PFG marker; lane 3, 2006-108; lane 4, 2006-109; lane 5, 2006-110; lane 6, 2006-111; lane 8, 2006-112; lane 9, 2006-113; lane 10, 2006-114; lane 11, 2006-115; lane 12, 2006-116; lane 13, Lambda PFG marker; lane 14, 2006-117; lane 15, 2006-118; lane 16, 2006-119; lane 17, 2006-120; lane 18, 2006-121; lane 19, 2006-122; lane 20, 2006-123; lane 21, 2006-124; lane 22, 2006-125; lane 23, Lambda PFG marker; lane 24, 2006-127. Strain 2006-113 was not typeable by PFGE.

Using the arbitrary value of ≥90% similarity of the banding patterns 69 RFLP groups and 122 PFGE groups were established (A/2 and A/3). However, 10 strains (6% of the collected isolates) exhibited identical PFGE but could be further divided based on their by RFLP patterns. According to our results most of the isolates are related to sporadic cases. However, 6% of them, still a considerable number, belonged to a single molecular type suggesting possible common source of infection unrevealed by routine investigations.

3.2. PCR

PCRs were performed to determine the presence of 16 putative virulence-associated genes among 190 clinical and 4 reference *C. jejuni* isolates. The results are summarized in Figure 16 and Table 4. All strains possess the toxin gene: *cdtB;* the adhesion gene: *cadF*, and the gene for flagellar hook: *flgE2*. Genes involved in i) flagellar biosynthesis (*flaB, flhB,* and *flgB*); ii) colonisation (*docA*, *docB*); and iii) invasion (*iamA*) were detected in ~90% of the clinical isolates. The *ciaB*, another gene which is proposed to take part in invasion process, appeared with an incidence of 87%. *VirB11* gene could be revealed in 2 isolates, implicating that these strains may carry the pVir plasmid. Both strains proved to be positive in possessing pVir plasmid as tested with a positive control, *C. jejuni* 81-176. The gene *docC* was present in about half of the strains. Genes, *wlaN* and *cgtB* involved in LOS synthesis were present in 44% and 63%, of the strains, respectively.

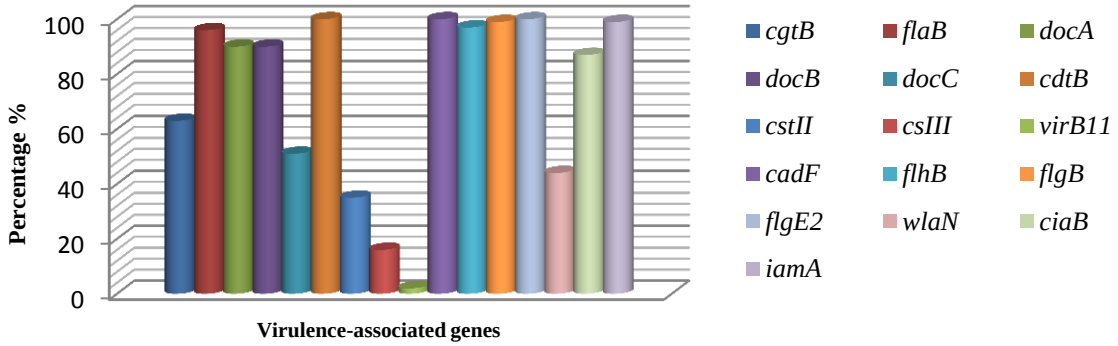

Figure 16. Occurrences of the 16 investigated virulence associated genes in the 190 *C. jejuni* strains. $p \leq 0.05$.

Table 4. Distribution of virulence associated genes in the 190 *C. jejuni* strains.

Virulence determinants	Positivity (%)	Virulence determinants	Positivity (%)
cgtB	63	*virB11*	3
flaB	96	*cadF*	100
docA	90	*flhB*	97
docB	90	*flgB*	99
docC	51	*flgE2*	100
cdtB	100	*wlaN*	44
cstII	35	*ciaB*	87
csIII	16	*iamA*	99

Additionally, in order to separate isolates harboring sialylated LOS from isolates with non-sialylated LOS, we looked for the presence of *cstII* and *cstIII*. The majority of isolates were *cstII*-positive

(35%), and only 16% of the strains were *csIII*-positive. 49% of isolates were negative for both, *cstII* and *cstIII*, which indicated, that these strains possess a non-sialylated LOS.

3.3. Quantitation of matrix protein binding ability of *C. jejuni* strains

All the *C. jejuni* strains showed high level of variability in their ability to bind ECM proteins. Taking the binding values 0.1% as positive, 16% of the strains were found to be able to adhere each of the three ECM proteins tested in solid-phase assay. The binding to extracellular basement membrane proteins revealed to be strain specific. Strains were able to bind: only collagen type IV (5%), fibronectin (2.5%), and laminin (4%). 26% of isolates were found to bind collagen type IV at the highest level (average binding 1.9%) followed by fibronectin with 24% (average binding 1.8%) and laminin with 21% (average binding 12%). A medium group with 29% was unable to bind any of the ECMP. *Pseudomonas aeruginosa* was used as a positive control, binding collagen type IV with 0.16%, fibronectin with 0.16%, and laminin with 0.1%.

3.4. Adhesion and invasion abilities of *C. jejuni* strains

Adhesion and invasion ability varied considerably among the members of the human isolate strain collection (Table 5). We have grouped the isolates into (i) high adhesion but low invasion (e.g. *C. jejuni* 2006-3, 120, and 148), (ii) low adhesion but high invasion potential (e.g. *C. jejuni* 2006-48, 64, and 94), (iii) high adhesion and high invasion potential (e.g. *C. jejuni* 2006-18, 101, and 119), and (iv) low adhesion and low invasion (e.g. *C. jejuni* 2006-16, 58, and 154) (Figure 17) categories. Adhesion ability was determined from the adhered bacteria number, which was divided with the number of invaded bacteria. 10% of the tested strains were found to adhere to INT407 at a very high level, but they could not enter into the cells. It was shown that 14% of the isolates could neither adhere to nor invade into the cells. We found, that approximately 10% of the strains were able to adhere and invade into INT407 at a very high level. Among this 10%, only three strains (2006-119, 2006-134, and 2006-165) were isolated from patients with bloody diarrhea. Interestingly, in group 2 isolates the relatively low adhesion capacity was coupled with high rate of invasiveness that is a high percentage of the adherent bacteria invaded the eukaryotic cells. The invasive patterns of the strains proved to be reproducible in repeated assays.

Table 5. Four groups were identified depending on the ability of *C. jejuni* isolates to adhere to and invade into INT407 human intestinal epithelial cells.

	Different adhesion/ invasion potential	Distribution (%)
Group 1	High adhesion- low invasion	10.0
Group 2	Low adhesion-high invasion	63.0
Group 3	High adhesion-high invasion	9.5
Group 4	Low adhesion-low invasion	14.0

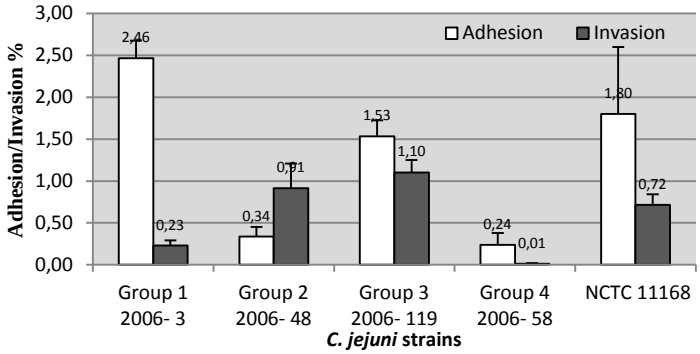

Figure 17. Comparison the adhesion and invasion potentials of 1-1 representatives from the four different basic groups. Groups and (representatives): 1, high adhesion-low invasion (*C. jejuni* 2006-120); 2, low adhesion-high invasion (2006-49); 3, high adhesion-high invasion (2006-119); 4, low adhesion-low invasion (2006-58).

4. Discussion

Campylobacter jejuni is the most frequently reported bacterial cause of sporadic food-borne infection around the world. Despite intensive studies, it is still not clear, what bacterial factors and bacterial-host interactions influence the severity of symptoms during the infection.

The 31% homology among the strains gained by *FlaA*-RFLP and PFGE analysis is a clear demonstration that our studies were performed on a very diverse strain collection. Although 6% of the strains proved to be identical by PFGE, but by using *flaA*-RFLP the members of these 6% could be further divided. Results of the two methods did not match completely as it is reported in the literature (Fitzgerald et al., 2001), but because of their different resolution the gained information is complementary. No relationship could be found between the clonality of the 190 strains and the severity of the symptoms.

High level of presence of *flaB*, *docA*, *docB*, *cdtB*, *cadF*, *flhB*, *flgB*, *flgE*, and *iamA* demonstrates the importance of these genes in *C. jejuni* pathogenesis although only *cadF*, *cdtB* and *flgE* were present in all isolates. Louwen et al. (2008) found, that strains possessing sialylated LOS structures showed significantly higher invasiveness than mutants impaired in sialysation. In contrast, we found that

strains harboring non-sialylated LOS had the same ability to invade into INT407 cells than strains possessing the *csII/csIII* genes for sialylated LOS.

Although we have only tested a small segment of virulence-associated genes, diversity in the results previewed the differences among the strains revealed *in vitro* tests. Categorizing the strains into four groups based on their adhesion-invasion potentials was subjective, but results of this comparative study have clearly demonstrated extant phenotypes in this experimental setup. Several proteins are dedicated to the involvement in invasion (A/5-J). FlhB is one of them reported to be necessary for the proper flagellar function, and also serves as an export apparatus of several invasion associated protein such as CiaB (Konkel et al., 2004). Although we could show the presence of flhB in 97% of the isolates, some strains lacking of this gene but having outstanding invasion and adhesion abilities suggest that at least in certain strains this gene is not crucial for successful invasion *in vitro*. Alteration of the *flhB* (could not be detected by the used primers (Müller et al., 2006)) gene or presence of alternative invasion mechanism could only be hypothesized.

In addition, the hyperinvasive *C. jejuni* isolates could not be distinguished from the low invasive ones of *C. jejuni* strains based on the presence of any of the virulence-associated genes tested, probably because: (i) not all of the virulence genes we have investigated might be essential in the pathogenic process; (ii) different combinations of the virulence genes might affect the mechanisms involved; (iii) new, hitherto undisclosed genes might also influence the pathogenic processes.

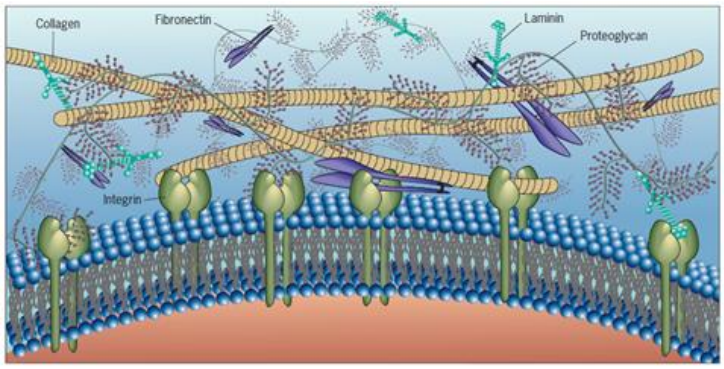

Figure 18. Composition and orientation the extracellular matrix proteins (ECMPs) on the eukaryotic epithelial cells (Karp, 2009)

Different abilities of the tested strains to bind different ECMPs further confirm the diversity of the strains isolated from hospitalised patients from the South-Transdanubian area. Extra cellular matrix (ECM) is a complex structural tissue of the outer surface of epithelial cells while also present in the interstitial matrix below and between the basement membranes (Figure 18). Obviously, the ability to bind widespread molecules found in the ECM is advantageous for an invasive organism, such as *C.*

jejuni. Approximately 100 known fibronectin binding bacterial outer surface adherence proteins (adhesins) exist, conferring specific benefits to pathogens (Larson et al., 2013). Fibronectin could only be important in adhesion, since there was no significant correlation found between high rate fibronectin binding and the invasiveness of the isolates. In contradiction, our data support that collagen IV binding could contribute to greater invasive potential since 76% of the collagen IV binding strains showed more marked invasion capacity. Adhesion to collagen type IV may activate the FAK and extracellular signal-regulated kinase (ERK) signal transduction routes (Sanders et al., 2000), and by this could contribute to evoke cell-internalisation.

In addition, we have also found, that collagen type IV protein binding itself does not mean increased ability to bind to INT407. It might suggest that fibronectin is important for anchoring, while collagen IV binding for internalisation. Nonetheless, other strains with higher invasiveness were not found to bind collagen type IV more successfully. It means that collagen might promote internalisation in only some cases, while other strains might use other signal transduction doors to enter into the target cell. It is reported, that the ability of individual strains to bind both collagen and laminin is not common (Trust et al., 1991).

The general lack of correspondence between pathogen genotype and clinical presentation is due to extensive genetic variation driven by multiple mutagenic mechanisms and lateral gene transfer among *C. jejuni* strains (Bayliss et al., 2012). Till now no concrete virulence factor has been described that could be responsible for effective invasion. Based on the data of the literature, and knowing the high heterogenicity of *C. jejuni* isolates it is more likely that different processes are responsible to accomplish such pathogenic steps like invasion and could influence the outcome of the whole pathogenic process itself.

Keeping the previous sentences in mind, it is not astonishing that no clear indication could be revealed among the results of phenotypic and genotypic tests and the symptoms of hospitalised patients. Three strains were isolated (2006-119, 2006-134, and 2006-165) with macroscopic presence of blood in stool of the patient. These strains all possess all the tested virulence genes, and showed also higher abilities to bind ECMPs, adhere and invade the INT407 cell line. Much deeper information can only be received from functional analyses, and therefore the next part of my work aimed to carry out a transcriptomic analysis with strain 2006-119.

CHAPTER IV. IDENTIFICATION OF INVASION-ASSOCIATED FACTORS OF *CAMPYLOBACTER JEJUNI*

1. Aims of the study

In order to broaden our view about the factors involved and might be crucial during invasion of *C. jejuni*, a whole genome transcriptomic analysis was carried out. For this purpose the *C. jejuni* strain 2006-119 was used, isolated from a patient with bloody diarrhea, exhibiting outstanding levels of invasion of INT-407 tissue culture cells, and possessing with a high level of adhesion to ECMPs.

2. Materials and methods

2.1. Isolation of RNA from the cultured and host-cell invaded *C. jejuni*

A three-hour long GPA invasion assay was carried out with *C. jejuni* strain 2006-119 on the same cell line and by the same technique as described before (Chapter III). The same bacterium strain (24 ml OD_{600}=1) was used for control in RPMI (without the presence of the INT407 eukaryotic cell-line) incubated at 37°C in a humidified, 5% CO_2 incubator for three hours. For RNA isolation, the bacterial cells of the control and the trypsine-harvested (Life Technologies, USA) INT407 cells from the invasion assay were centrifuged at 8,000 x g for 15 min. Collected cells were homogenized in RNazol (Molecular Research Center, USA) in an 1,5 ml reagent tube. In the case of the eukaryotic cells, the tubes were dropped three times into liquid nitrogen for more effective exploration of the cells, to access the RNA of intracellular bacteria. The total RNA concentration and purity was measured using the ND-1000 Spectrophotometer (Nanodrop, Thermo Scientific, USA). MICROB*Enrich*^{TM} kit was used to reduce the eukaryotic RNA in the sample from the invasion assay to be able to detect prokaryotic RNA by the RNA-Seq experiment.

2.2. Whole transcriptome analysis (RNA-Seq)

RNA qualitative and quantitative measurements were performed on Bioanalyzer (Agilent Technologies, USA) and Qubit (Life Technologies, USA). High quality (RIN >8.5) total RNA samples from three biological replicates were pooled and processed using the SOLiD total RNA-Seq Kit (Life Technologies, USA), according to the manufacturer's instructions. Briefly, 3 µg of pooled RNA was *DNaseI* treated, and the ribosomal RNA was depleted using RiboZero Prokaryotic rRNA Removal Kit (Epicentre, USA). The leftover was fragmented using *RNaseIII*, the 50–200 nt fraction

size-selected, sequencing adaptors ligated and the templates reverse transcribed using ArrayScript RT. The cDNA library was purified with Qiagen MinElute PCR Purification Kit (Qiagen, Germany), and size-selected on a 6% TBE-Urea denaturing polyacrylamide gel. The 150–250 nt cDNA fraction was amplified using AmpliTaq polymerase, and purified by AmPureXP Beads (Agencourt). Concentration of each library was determined using the SOLiD Library TaqMan Quantitation Kit (Life Technologies, USA). Each library was clonally amplified on SOLiD P1 DNA Beads by emulsion PCR (ePCR). Emulsions were broken with butanol, and ePCR beads enriched for template-positive beads by hybridisation with magnetic enrichment beads. Template-enriched beads were extended at the 3′ end in the presence of terminal transferase and 3′ bead linker. Beads with the clonally amplified DNA were deposited onto sequencing slide and sequenced on a SOLiD 5500XL Instrument using the 50-base sequencing chemistry.

Bioinformatics

Bioinformatic analysis of the whole transcriptome sequencing was performed in color space using Genomics Workbench (CLC Bio). Raw sequencing data were trimmed by removal of low quality, short sequences so that only 45-50 nucleotide long sequences were used in further analysis. Sequences were mapped onto the *C. jejuni NCTC 11168* reference genome, using default parameters. Results were manually curated to remove false positive hits, which showed highly skewed mapping of reads. Only genes with at least 1.5 fold up- and downregulation detected after normalisation were considered for further analysis.

3. Results

3.1. Expression of virulence determinants during invasion

The total gene expression profiles of intracellular and control bacteria were compared. Altogether 1668 open reading frames (ORFs) were detected with different fold changes and unique gene reads. At the third hour of the invasion, 963 genes were significantly upregulated. Among them 59 ***membrane proteins*** (A/5-A), 39 ***periplasmic*** (A/5-S), and 134 ***hypothetical proteins*** (A/5-W) were detected to be elevated. The expression of several hypothetical proteins was overexpressed, they were published to have putative role in adhesion, invasion and colonisation but many of them remain to be discovered. Only the pathogenically important and the most significantly elevated ORFs are presented in A/5. Genes with significantly elevated expressions during invasion were identified and sorted into various categories. In some cases, we have also presented unchanged or downregulated genes (A/5) in order to settle the actions in a wider context. Here we present our main findings by

categorizing the upregulated genes based on their functions, and by presenting their expression changes (fold changes) in brackets.

Transmembrane proteins with known and unknown functions

Sec proteins control the translocation of proteins synthetised in the cytosol into the membrane itself or across the inner membrane into the periplasmic area. We found that *secY* (5.655), *secE* (10.713), and *secG* (1.950) were upregulated (A/5-A). The products of these genes form a channel called translocon, located in the membrane. SecA, another ATPase protein, is located at the inner surface of the translocon in the membrane, but bound to SecY. Two genes found to be elevated encoding two subunits located on the periplasmic side of the translocon (*secD* (3.209), *secF* (3.067)). Next to the SecY another subunit is associated, coded by *yidC* (2.139), and acting as an insertase in cooperation with the Sec Translocon (du Plessis et al., 2011).

Colicin is a toxic protein, produced by bacteria like *E. coli* and other closely related bacteria to reduce the competition for nutrients in the environment. The group A colicins use the ***tol-dependent translocation system*** including a protein coded by *tolB*, which was found to be upregulated (1.749) (Hands et al., 2005).

The *ompA* gene was overexpressed (3.622) coding for a monomeric ***pore-forming channel protein***. Porins allow exchanges of hydrophilic compounds through the OM of Gram-negative bacteria. It is reported, that OmpA protein has an important role in the pathogenic process (adhesion, invasion, and intracellular survival) of Gram-negative bacteria. Furthermore, OmpA can serve as potential vaccine candidate (Confer and Ayalew; 2013). In addition, upregulation of *omp50* was also detected (3.564). However, its function is till unknown, it seems to be a species specific outer membrane porin, only found in *C. jejuni* and *C. lari*, therefore it can be used as a species marker (Dedieu et al., 2004).

Cme genes (e.g. *cmeB* (1.567) and *cmeA*) coding for a ***Cme efflux pump proteins*** were upregulated with the exception of *cmeC*. They are repressed by CmeR regulator (see later). The CmeABC proteins extrude a variety of substances such as antibiotics, bile salts, and they proved to be important in the colonisation of the chicken gut (Malik-Kale et al., 2008; Oakland et al., 2011).

A transmembrane protein coding *cj0268c* was elevated (2.881). It has been proved to be required for adhesion and colonisation *in vitro* (Tareen et al., 2013).

The LspA was revealed to be elevated (5.328), its function in *C. jejuni* has not been revealed yet, but its possible importance in the virulence of *Mycobacterium tuberculosis* has been implicated (Sander et al., 2004).

Adhesion proteins

Beside their various functions bacterial lipoproteins are involved in the adhesion to the host cells (A/5-G). At present, only four ***lipoproteins*** proved to have a role in the adhesion process of *C. jejuni*: JlpA, CapA, CjaA, and FlpA. The two ***autotransporter proteins*** coded by *capA* (12.809) and *capB* (5.124) were found to be elevated, but because of their low unique gene reads, their expression values are only indicative. They have been implicated in the host-pathogen relationship. Furthermore, a study confirmed the role of CapA as a putative adhesin on chicken intestinal cells (Flanagan et al., 2009). The ***fibronectin binding adhesion proteins*** coding genes could be identified with elevated expression during the invasion: *flpA* (2.909) and *Cj1349c* (2.160). They are reported to contain Fn type III domains, and to act as a Fn and fibrinogen-binding protein, respectively. They have been found to have an important role in colonisation process *in vitro* (*Cj1349c*) and *in vivo* (*flpA*) as well (Flanagan et al., 2009). The *jlpA* (1.803) was elevated, which codes an antigenic adhesion protein taking part in the immune response. The surface-exposed JlpA is involved in the interaction with the Hsp90 of the host cell, triggering the signal transduction pathway of NF-κB and p38 MAP kinase (Kawai et al., 2012). The overexpression of two additional lipoprotein-coding genes was revealed: *cj0090* (1.879) and *cj0091* (2.132), probably taking a part in adhesion, modulated (repressed) by CmeR regulator (Oakland et al., 2011).

Gene *cj0588* was elevated, it is found to be involved in adhesion (Salamaszynska-Guz and Klimuszko, 2008). We detected the upregulation of *peb3* (4.861) (Kervella et al., 1993) and *peb2* (2.443), they are coding two well-known adhesion proteins (Pei et al., 1991). In addition, the expression level of *cadF* (1,535) was elevated coding a highly characterised adhesion protein (Monteville et al., 2003). The Peb1 (Pei et al., 1998) and the Peb4 (Asakura et al., 2007) proteins coding genes were not increased significantly during the invasion.

Bacterial shape determinant genes

We could detect all of the ***Mre-based bacterial cytoskeleton proteins*** responsible for the determination of bacterial cell shape, to be increased during the invasion: *pbpC* (4.455), *mreC* (2.831), *pbpB* (2.661), *rodA* (2.242), *mreB* (1.533) (A/5-H). Beside that these proteins are found to be responsible to maintain polarity, division, and rod-shape morphology of bacteria, they proved to have a role in the virulence in *Salmonella* species (Doble et al., 2012). This virulence modulating function has not yet been described in *C. jejuni* (Ikeda and Karlyshev, 2012).

Surface associated saccharides

A group of ***capsular polysaccharide (CPS)*** genes could be detected with increased expression level (A/5-B). The capsule was found to be essential for the successful colonisation of the epithelial cells.

The gene *kpsM* and *kpsE* were overexpressed with 4.240 and 1.882, respectively. In the lacks of these genes the colonisation of the chicken gut were not detected. The enormous antigenic diversity among isolates (serotypes) is mainly due to phase variation of the capsule. The gene *kpsT* (3.897) coding for transport of capsule, and *kpsC* (3.447) responsible for capsule modification were elevated at the third hour of the infection of epithelial cells. The most radically elevated gene among the capsule biosynthesis genes was *hddD* (33.303) (Hermans et al., 2011). However, because of low gene reads of *hddD*, we should evaluate this value with care (Bacon et al., 2001).

Analysis of the ***lipooligosaccharide*** (***LOS)*** related ORFs revealed that several genes were upregulated including genes responsible for LOS biosynthesis like *galE* (4.054), *waaF* (3.357), *Cj1135* (2.417), *gmhE* (1.549), and *lpxB* (lipid A biosynthesis) (3.718) (Karlyshev et al., 2000) (A/5-D). Because of the low read number, the upregulation of the following genes are important, but only indicative: *cstIII* (20.494), *neuB1* (15.371), *cj1136* (11.528), *cj1137c* (8.966), *wlaN* (6.148) and *neuA* (4.099). The gene *cj1136* is reported to take a part in the hyperinvasion ability (Javed et al., 2012).

Invasion proteins

Two protein coding genes (*cipA* and *ciaB*) were increased with 2.616 and 2.036 respectively (A/5-J). CipA was reported to interact with cdc42, causing cytoskeletal rearrangement, possibly contributing to the internalisation of *C. jejuni* (Harriff et al., 2009; Javed et al., 2010). The CiaB has been shown to be involved in invasion by demonstrating that a null mutation in *ciaB* results in a non-invasive phenotype (Konkel et al., 1999).

The expression of invasion associated marker genes (*iamA*, *iamB*) were not significantly increased in our experiment (Carvalho et al., 2001).

The recently discovered lipoprotein coding *cj0497* was elevated (2.242), indicating its possible role in the invasion (Javed et al., 2010).

The two ***ATP-dependent protease*** coding genes, *lon* (3.057) and *clpP* (1.931) were elevated, found to be involved in invasion and colonisation at higher temperature (Cohn et al., 2007).

During the invasion the ***serine protease*** coding *htrA* was elevated slightly, which has a role in adherence and invasion (Rathbun et al., 2009). *Cj1171c* was also elevated (2.515) coding a peptidyl-prolyl cis-trans isomerase.

The bipartite ***energy taxis receptors*** coded by *cetA* (2.329) and *cetB* (2.350) were elevated (Elliott et al., 2009). Inactivation of chemoreceptor, CetB (Cj1189) in *C. jejuni* completely eliminated their ability to invade cultured human epithelial cell lines (Golden and Acheson, 2002).

Iron acquisition

Several members of various iron uptake systems were found to be elevated during the invasion including the ***iron-uptake ABC transporter ATP-binding proteins***, the ***ferrous iron transport proteins***, and the ***hemin uptake system proteins*** (A/5-L).

The ***siderophore-mediated iron uptake system*** plays a central role in the pathogenesis of many Gram-negative bacteria. A The gene *ceuE*, a member of the *ceuBCDE* operon was found to be upregulated (2.292); it serves as a differentiating factor between *C. coli* and *C. jejuni* (Gonzalez et al., 1997; Bang et al., 2003, Palyada et al., 2004). The overexpression value of the other members: *ceuC* (6.587) and *ceuD* (2.846) are only informative because of their low gene reads (van Vliet et al., 2002).

Colonisation

A number of colonisation-associated genes were upregulated during the invasion (A/5-I). Certain genes of the ***Liv-system*** (*livJ* (4.207) and *livK* (4.639)) were upregulated, they are reported to be involved in the colonisation of the chicken epithelial cells (Ribardo and Hendrixson, 2011).

Cj0561c is repressed by the CmeR regulator, encoding a putative periplasmic protein with 3.052 raise in the invasion process (Guo et al., 2008). Furthermore, expression of the following genes was found to be increased: *dnaJ* (3.705), *pldA* (Ziprin et al., 2001), *Cj0379c* (2.885) (Hitchcock et al., 2010) and *docA* (1.770) (Bingham-Ramos and Hendrixson, 2008), thought to have a crucial role in the colonisation of the chicken gut.

Regulatory systems

Transcriptional regulators of *C. jejuni* have not yet been discovered in detail. The ***two- component regulatory (TCM) systems*** (e.g. FlgSR, CbrR, DccSR, RacRS, and CheY) have gained more attention recently. The expression of dccS was elevated ((2.638), it is a member of the ***DccRS system***, which has been found to be important for *in vivo* colonisation and controlling the expression of several genes encoding probable membrane-associated proteins (MacKichan et al., 2004). The overwexpression of *racS* (1.733) was detected, a member of the ***RacR-RacS system***, which is involved in a temperature-dependent signaling pathway (Apel at al., 2012; Brás et al., 1999). We could reveal that in the majority the sensor part was activated of the TCM systems with the only exception of the ***FlgSR system*** (the flgR was upregulated), controlling the flagellar regulation (see later) (A/5-P).

On the contrary, ***non TCM-system regulators*** were found to be decreased *cmeR* (-1.644) (repressor of *cmeABC*) (A/5-F), *hspR* (-1.441) (negative regulator for heat shock) with the exception of spoT

(2.204) (survival in various environmental stress) and Fur proteins (iron homeostasis) (Guo et al., 2008).

Toxin genes

In the case of the toxin genes, *cdtA* were slightly increased, while *cdtB* and *cdtC* (-1.561) were decreased (A/5-C).

Chemotaxis genes

The chemotaxis genes *cheB* (1.868), *cheW* (1.616), *cheV, cheR,* and *cheA* were increased while the expression of *cheY* and *tlp3* did not change significantly (A/5-E). The expression level of *docA* and *docB* (coding MCP-domain signal transduction protein) were found to be slightly decreased (Hendrixson et al., 2001).

Flagellar machinery

By the end of the third hour of the invasion experiment, the following flagellar genes were upregulated (Figure 19): (i) the ***stator proteins*** coding *motA* (2.586), *motB* (4.534), (ii) certain genes of ***flagellar transport T3SS system*** (T3SS) proteins, and (iii) genes coding the ***motor switch proteins*** (with the exception of *fliR* (-1.981) (A/5-V). The overexpressed *cheW* chemotaxis gene may induce flagellar rotation changes via FliM and FliM. Through the activated flagellar secretion system the upregulated invasion protein CiaB may be transported.

We found, that the transcriptional factor coding *rpoN* (sigma54) (2.021) is activated, which requires multifactorial activation of certain genes like the *FlgS* and *flgR* coding for sensor histidine kinase and an Nitric-like response regulator, respectively. At the third hour of the infection the expression level of *flgR* was elevated (4.262), while its activating factor was not changed, implying that its upregulation and kinase phosphorylating activity has been completed.

Additional activating factors of RpoN are the T3SS system *(f*lhA (3.697), *flhB* (6.904), *fliP* (1.983), and *fliR* (7.173). Gene *fliF* (responsible for the MS-ring) and *flhF* also have an RpoN triggering function (Gilbreath et al., 2011). Beside its role of FlhF to activate sigma54, it has been reported to influence the motility through numerical control of flagellar biosynthesis *(*Kazmierczak and Hendrixson, 2013). Although, the class I gene regulator *rpoD* (sigma-70) (-1.794) was downregulated at this stage of the invasion, the ***early gene products (class I)*** were detected with an elevated expression (Figure 20). The sequent sigma factor, the rpoN (sigma54) was overexpressed, responsible for the ***class II gene products***, but the latters were not overexpressed at this stage of the invasion. In the group of the class II genes with the only exception of proximal rod genes (slightly upregulated), the other flagellar genes coding components of i) distal rod; ii) L-ring, iii) P-ring, iv) Hook, v) the minor flagellin FlaB and vi) FlgM protein (coding for repression of the sigma28 transcriptional regulator) were uniformly downregulated. The decreased level of *flgM* (repressor of

fliA) allows the activity of sigma28 (*fliA*) (Chadsey et al., 1998) was slightly increased in our experiment. This sigma factor is responsible for the activation of ***class III genes***, like flagellin genes (*flagG* (-2.247), major flagellin *flaA*) and the flagellar cap gene (*fliD*), which are not activated at this period of the invasion process.

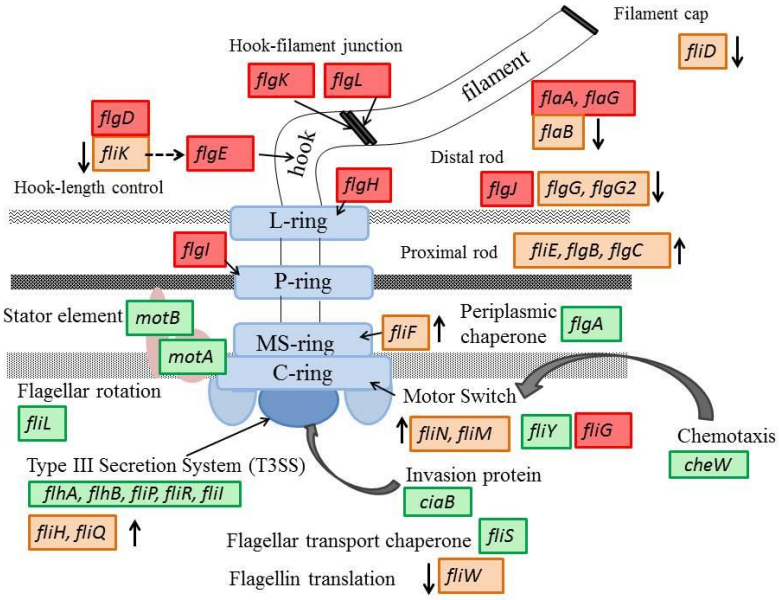

Figure 19. The expression changes of flagellar structure of the highly invasive *C. jejuni* strain 2006-119 during the invasion process. The changes in the expression level are presented with different colors: (i) upregulated genes (green color), (ii) downregulated (red color), and (iii) not significantly changed genes (orange color). In the latter case, direction of arrows is presenting the direction of changes in the expression.

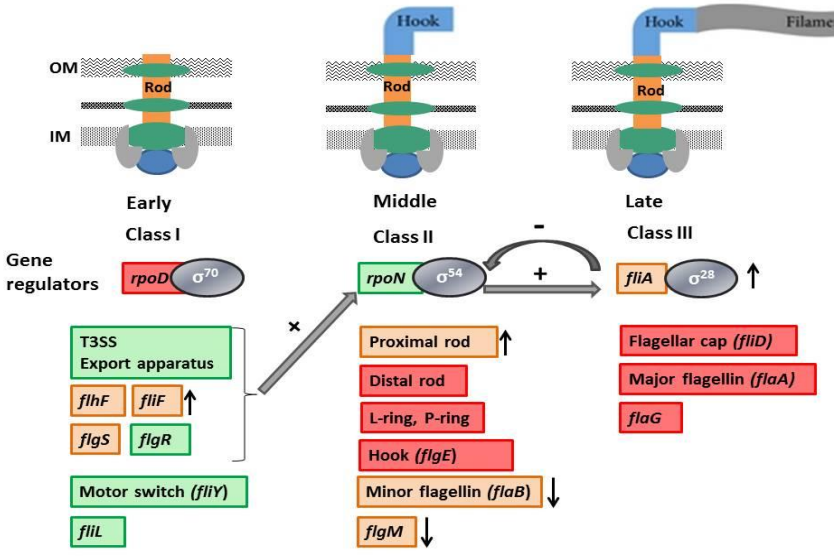

Figure 20. The expression changes of the flagellar regulatory cascades of the highly invasive *C. jejuni* strain 2006-119 during the invasion process

Furthermore, genes involved in (i) ***energy metabolism*** like Ni/Fe-hydrogenase small subunit (*hydA2*) (2.339); (ii) the ***O-linked glycolisation***; (iii) the ***N-linked glycolisation*** (Karlyshev et al., 2005), 200; (iv) ***respiration*** and (v) ***protein synthesis/modification/secretion*** were elevated.

4. Discussion

High-throughput sequencing of cDNA libraries (RNAseq) technologies provides unprecedented opportunities for mapping transcriptomes and profiling gene expression in diverse bacteria (Chaudhuri et al., 2011). RNAseq has several key advantages over microarray analysis, including 1) the ability to detect and quantify transcripts derived from all regions of the genome, 2) a large dynamic range that affords high sensitivity for low-abundance transcripts, and 3) single nucleotide resolution (Taveirne et al., 2013).

For successful internalisation adhesion is unavoidable for *C. jejuni*. Increased level of the RNAs complementer to the genetic determinants of known adhesion proteins (CapA, Peb3, FlpA, Peb2, CjaA, JlpA, CadF, Peb4, and Peb1A) in the third hour of the invasion experiment (A/5-G) is a strong hint, that these proteins are not only involved in successful adhesion, but also might promote internalisation. The role of these proteins during adhesion was formerly demonstrated (CadF: Monteville et al., 2003; FlpA: Flanagan et al., 2009), but data are contradictory mainly depending on the cell line on which the adhesion study was performed (JlpA: Jin et al., 2001; Flanagan et al., 2009; Novik et al., 2010). In addition, originally former studies have described these proteins as adhesion factors, but only subsequent studies started to reveal their possible involvement in the internalisation as well. CapA has been unequivocally dedicated as a factor, which not only influences adhesion, but also has a striking effect on invasion (Ashgar et al., 2007). In our experiments we could only partially justify this observation, since although the fold change of CapA, which we have measured was the highest among the upregulated adhesion factors, but because of its very low read number we could consider this value only with reservations.

A recent study proposed that co-acting of adhesion factors is a prerequisite for bacterial binding and subsequent invasion by *C. jejuni* (Eucker and Konkel, 2012). Elevated levels of a group of known adhesion factors (A/5-G) in the third hour in our experiment, also support the existence of such an additive effects of them.

Beside the above mentioned adhesion proteins we have also identified the upregulation of those genes that were referred in the literature as typical colonisation (A/5-I), and invasion (A/5-J) factors.

Beside proteins, saccharide based surface associated structures also play a role in invasion. Elevated levels of the three conservative capsule transport proteins (kpsM, kpsT, and kpsC) from our study (A/5-B) were revealed to be overexpressed during internalisation compared to control. This data is in harmony with a previous observation of Bacon et al. (2001) where the *kpsM* mutant of strain 81-176 showed a modest (10-fold) reduction in invasion of intestinal epithelial cells *in vitro*. Nevertheless, our data also confirm that capsule is not only important for adhesion, but its presence is also advantageous in the eukaryotic environment possibly solving as a protecting sheath around the bacterium cell in the intracellular environment.

Similarly to capsule, the role of another saccharide based surface antigen was demonstrated by Louwen et al. (2008) during invasion. They could show that *C. jejuni* with sialylated LOS structures showed significantly higher invasiveness than mutants impaired in sialysation, and only possessing LOS alone. This observation was also confirmed by our study (A/5-D) although the small read numbers are only indicative in these cases. Similar indicative results were gained in connection with the N- and O-linked glycosylation system, which is also an important surface antigen modifying system, and proved to affects not only adhesion but also invasion (Szymanski et al., 2002).

Once engulfed in the cell, the bacterium has to face to a new environment and reset its metabolic activity. The elevated expression level of several genes encoded for regulatory and signal transduction proteins (A/5-P) demonstrates this revved physiological situation.

During these circumstances a very critical point is the assurement of sustainable metabolism. The novel proteins require synthetic processes (A/5-U) and for these, energy is needed, which can be gained by revving up certain metabolic pathways. The moderate, but characteristic upregulation of the subunits of the NADH-quinone oxidoreductase (A/5-O), that pumps protons coming from metabolic processes across the plasma membrane of many bacteria (Brandt, 2006), and by this assures increased ATP synthesis, is an argument for this. For proper enzymatic functions availability of micronutrients can be limiting factors. One of the best micronutrient studied in bacteria is iron that's role and presence of different iron uptake mechanisms were demonstrated in *C. jejuni* as well (Miller et al., 2009). For this reason it is not surprising, that several genes potentially taking part in iron acquisition (A/5-L) were significantly upregulated during invasion. Iron metabolism and storage is an essential phenomenon in *C. jejuni* which involves several genes from multiple cellular processes particularly energy metabolism and oxidative stress response systems. It also have to be keep in mind that during certain conditions iron is also responsible for the generation of hydroxyl radical, which is particularly biotoxic (Pomposiello and Demple, 2002). This can be one reason why the upregulation of thioredoxin –capable of eliminating of -OH- coding gene was upregulated. Another reason for that can be the emergence of reactive oxygen species (ROS) during *C. jejuni*

infection as it was recently described (Corcionivoschi et al., 2012). The effects of emerging harsh environment around the internalised *C. jejuni* can also be deafened by increased capsule (Bacon et al., 2001; Keo et al., 2011) production and transport (A/5-B).

During stress situations *C. jejuni* was reported to transform from the spiral form to a coccoid form (Xie et al., 2011). Although we have not investigated if morphological changes of *C. jejuni* occur during invasion, our results demonstrate the elevated transcript levels for the Mre-based cytoskeleton proteins. Whether this means a change in the shape of the intruder or only means the tightening of the cell structure has to be elucidated. This second option is supported by the observation, that beside the elevated level of rod shape–determining proteins expression of the genes encoding for penicillin binding proteins (PbpC and PbpB) proved to be also elevated. Recently importance of a similar system during the pathogenic process was demonstrated in *Salmonella* Typhimurium (Doble et al., 2012).

Revealment of the intensive upregulation of genes coding for transmembrane and periplasmic proteins (A/5-A, A/5-S) strongly suggest, that invaded *C. jejuni* cells are in a state of requiring active transport in and out from the bacterial cell. Their activation however, is a clear indication for their roles in the intracellular survival of this zoonotic microorganism.

Upregulation the early (class I) and downregulation the middle (class II) and late (class III) phase genes encoding for the flagellar apparatus (A/5-V) is also a strong hint, that the internalised bacterium uses the secretory function (T3SS) of the flagellar apparatus (Figure 19). Being internalised the *C. jejuni* cell does not already need the hook and the filament, and therefore genes encoding for these structures become downregulated. In this situation the membrane integrated part of the T3SS export apparatus that forms a channel through the bacterial cell wall (Figure 20) is sufficient to turn over transport processes.

CDT is the only toxin that was reported from *C. jejuni* and its role during the pathogenic process is still contradictory. Some authors have demonstrated its importance (Jain et al., 2008), while others were not able to show correlation between invasion abilities and cytotoxicity in the investigated isolates (Gilbert et al., 2009). Nonetheless its 100% presence in our 190 human isolates is a strong indication of its involvement in the pathogenic process, and as it is hypothetised, also in the involvement of inflammatory diarrhea.

Genes located on the core genome may also have a function in the hyperinvasive phenotype in addition to the known *C. jejuni* pathogenesis related loci. Our results indicate that upregulation of metabolic and regulatory pathways during the invasion process might facilitate the hyperinvasive character of *C. jejuni* strains.

The data we have gained from the transcriptome analysis is only synoptic. We could get a little bit closer to see the major occurrences, but how these assure a successful internalisation for *C. jejuni* is still a mystery. For adequate answers certainly a proper model is needed. Although we cannot fully model the human epithelium, but similar results gained from almost similar experimental systems could already be more than hints. Keeping these criteria in mind the results we have gained show numerous similarities with the results of a recent whole transcriptomic analysis performed in mice (Bell et al., 2013).

CHAPTER V. MOLECULAR CHANGES AND VIRULENCE POTENTIAL MODIFICATION INDUCED BY CLOVE ESSENTIAL OIL ON *CAMPYLOBACTER JEJUNI*

1. Aims of the study

Although, the majority of the campylobacteriosis cases do not require antibiotic therapy, prolonged or severe symptoms warrant medical intervention involving antimicrobial drugs. Currently macrolides are the drugs recommended in cases of proven etiology (Hughes and Cornblath, 2005), however, over the last decade antibiotic resistance has widely been reported giving rise to serious public health concerns (Soonthornchaikul et al., 2006). For this reasons quest for alternative antimicrobials both in prevention and therapy is a high requisite.

Essential oils may represent the richest available reservoir of novel therapeutics. Although antibacterial effects of several essential oils (EOs) have already been reported (Burt, 2004) the effect of clove on *C. jejuni* has not yet been studied in detail (Smith-Palmer et al., 1998).

Based on the results of Smith-Palmer et al. (1998), clove EO proved to have a characteristic antibacterial effect on *C. jejuni*. We aimed to explore if clove EO could have a potential to control *C. jejuni.* Secondly, we wanted to reveal those attendant changes on and in the bacterial cell that eventually lead to cell death. For this reason cellular changes of treated and non-treated bacterial cells were analysed by phenotypic, proteomic and genomic approaches under a slightly antibacterial concentration of clove EO. The possible virulence modifying potential of clove EO was investigated considering the result of a former study (Derakhshan et al., 2010).

2. Materials and methods

2.1. Bacterial strains, essential oil and culture conditions

Antibacterial effect of clove EO was screened on four reference strains (NCTC 11168, 81168, 81-176, RM1221) and 50 human *C. jejuni* isolates representing different molecular clones (Sonnevend et al., 2011). For thorough analysis the reference strain NCTC 11168 was chosen. Bacteria were grown on CCDA, at 42 °C under microaerophilic condition for 24 h, if not otherwise stated. Before each experiment bacterial cell counts were synchronised by setting the optical density (OD) to 1.0 ($4x10^8$ cfu/ml), at 600 nm. For the motility assay *Luria-Bertani* (LB) plates were used with 0.3% agar concentrations. Clove EO was obtained from AROMAX Zrt. (Hungary). The quality of the essential oil was consistent with the standards described in the European Pharmacopoeia (4^{th} edition). For the proteomic, genomic and phenotypic experiments, *C. jejuni* was exposed to clove EO

treatment for 2 h, at 42 °C under microaerophilic condition.

2.2. Determination of minimal inhibitory (MIC) and minimal bactericidal (MBC) concentration

24-h cultures of the NCTC 11168 strain were harvested and suspended in LB medium. The suspensions (OD_{600}=1.0) were diluted 10-times, and 5 ml aliquots were placed into the wells of six-well tissue culture plates in three parallels. Clove EO was added in 0.25 (20,000x), 0.5 (10,000x), 1 (5,000x), 2 (2,500x), 4 (1,250x), 8 (625x), 16 (312x), 32 (156x), 64 (78x), 128 (39x) µl volumes, respectively. No EO was added to the control wells. After 24 h incubation under microaerophilic condition at 42°C samples were taken, and CFUs were determined. A minimal inhibitory concentration (MIC) was defined as the lowest concentration of clove EO completely inhibiting visible bacterial growth, while minimal bactericidal concentration (MBC) was defined as the lowest concentration that killed 99.9% of the initial inoculum. Based on preliminary studies, 1: 3,000 dilution was used for the proteomic and genomic analyses, assuring CFU reduction by one-third after 2 h. For the motility assay the 1: 20,000 dilution of EO was applied that caused no CFU reduction in 24 h.

2.3. Protein assays

2.3.1. Electrophoretic protein microchip

Microchip dithiothreitol (DTT) was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). All reagents were of analytical grade. The High Sensitivity Protein 250 LabChip kit was purchased from Agilent Technologies (USA). The kit included microchips and reagents, such as High Sensitivity Protein 250 Labeling Dye, Dimethyl sulfoxide (DMSO), ethanolamine, Protein 250 Standard Labeling Buffer (300 mM Tris/HCl, pH >8.5), Gel Matrix (4.5% polydimethylacrylamide-based linear polymer), Destaining Solution, and Sample Buffer. The fluorescent dye stock solution was prepared (reconstituted) by adding 54 µl DMSO to the vial containing the Labeling Dye pellet, and mixed carefully. A diluted dye solution (2 µl stock solution diluted ten times with 18 µl distilled water) was used daily in the labeling experiments. A denaturing solution containing sodium dodecyl sulfate (SDS) was prepared by adding 3.5 µl 1 M DTT (stock solution stored in a freezer) to 100 µl Sample Buffer.

For electrophoresis of the protein samples the Agilent 2100 Bioanalyzer system (Agilent Technologies, USA) was used. The protein solutions were diluted ten times with Standard Labeling Buffer. For the fluorescent labeling 0.5 µl fluorescent dye/DMSO solution was added to 4.5 µl diluted sample, and incubated for 10 min at room temperature. 2 µl of denaturing solutions were

added to 4 µl labeled samples and incubated at 100 ^{o}C for 5 min. Centrifuged supernatants were used for the electrophoretic analyses. For electrophoresis the original protein analysis protocol was used. 6 µl of labeled samples were loaded into the Gel Matrix.

Injection was carried out at 1,000 V for 80 sec, and separation continued at 1,000 V for 60 sec at 30 ^{o}C. Raw data were plotted by the 2100 Expert Software. Peak areas for the components were obtained by manual integration (Makszin et al., 2012).

2.3.2. Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Cell lysates of *C. jejuni* (control and treated with essential oil) were separated by 2D. 100 µg of the total protein content of the bacterium lysates were supplemented with 2D sample buffer (8 M urea (Bio-Rad), 2% CHAPS (Bio-Rad), 50 mM DTT (Bio-Rad, USA), 0.2% Bio-Lyte 3/10 ampholytes (Bio-Rad), and a trace of bromophenol blue (Bio-Rad, USA) to a total volume of 125 µl and then the IPG strips (7 cm, pH 3-10) were incubated for rehydration overnight (Bio-Rad, USA). Isoelectric focusing (IEF) was performed on an IEF cell (Bio-Rad, USA) using a following program: i) 250V, 2 h, linear, ii) 500V, 2 h, linear, iii) 4,000 V, 10,000 Vh, rapid. After IEF, the strips were equilibrated two times for 10 minutes in equilibration buffer (6 M urea, 2% SDS, 20% glycerol, a trace of bromophenol blue, and 2% DTT (Bio-Rad, USA)). In the second equilibration step 2.5% IAA was used instead of DTT. After equilibration, the strips were applied to the second dimension (12% SDS-PAGE, 8 x 6 cm). The separation of the proteins according to their masses was performed at 80 V for 20 min and 120 V until the end of the run (Bio-Rad). The SDS-PAGE gels were stained with Coomassie R-250 (Bio-Rad, USA). Protein marker (ladder, Bio-Rad Kaleidoscope Precision Plus, USA) was used as a molecular standard. The gels were scanned on Pharos FX laser scanner (Bio-Rad, USA). For protein identification and mass spectrometric analysis, the spots of interest were excised from the gels.

In-gel digestion

Protein bands were excised from the gel, and were cut to small pieces and digested using modified version of the protocol developed by Shevchenko et al. (2006). Coomassie and SDS were removed with 100 mM ammonium bicarbonate (Bio-Rad, USA), and then the gel slabs were dehydrated with acetonitrile. Disulfide bridges were reduced by 10 mM DTT (Bio-Rad, USA) then the free –SH groups were alkylated with 55 mM iodacetamide (Bio-Rad, USA) solutions. The modified proteins were in-gel digested with side-chain protected trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate overnight at 37 °C. The digested peptides were extracted from the gel with 5% formic acid (Sigma-Aldrich, USA) solution in acetonitrile (Sigma-Aldrich, USA): water 2:1

mixture. The extracted digests were evaporated to dryness, and before the mass spectrometric measurement they were dissolved in 10 µl 0.1% TFA in water.

2.3.3. Liquid chromatography–mass spectrometry (LC-MS) analysis

The samples were analysed with Waters nanoACQUITY ultra-performance liquid chromatography (UPLC) (Waters Corporation, Milford, MA, USA) coupled to nanoESI MS system. Aliquots (5 µl) of the samples were injected and separated on a 1.7 µm BEH130 C18 analytical column (75 µm x 100 mm) using gradient elution at a flow rate of 350 nl/min. The mobile phase was (A) aqueous formic acid solution (0.1%) with (B) acetonitrile formic acid solution (0.1%). Initial setting was 3% acetonitrile (v/v), which was increased to 10% over 1 min, then increased to 40% in 15 min. The total runtime was 30 min. The column temperature was set at 35 °C. The temperature of the samples was 4 °C. The nanoUPLC system was connected to Bruker Maxis 4G UHR-QTOF MS instrument (Bruker Daltonics, Bremen, Germany) coupled with a nano-ESI source. The instrument controls were performed via Compass 1.3 software package (Bruker Daltonics, Bremen, Germany). The mass spectrometer was operated in positive mode. The scanning mass to charge range was m/z 100–3,000 at 1 Hz acquisition rate. Nitrogen was used as nebulizer gas at a pressure of 0.6 bar, the drying gas flow was 4 L/min at 180 ºC, and the capillary voltage was set to 3.8 kV. Each intensive peptide was fragmented and the completed data were processed through the DataAnalysis 3.4 (Bruker Daltonics, Bremen, Germany) software. The identification of proteins was carried out by searching for bacteria taxonomically restricted in the databases of the National Center for Biotechnology Information (NCBI) (last accessed 20 February 2014) and Swiss-Prot (last accessed June 2014), using Mascot V2.4.1 (www.matrixscience.com, Matrix Science, London, UK). The search parameters allowed for one missed cleavage site and 80 ppm mass error for the MS and 0.3 Da for the MS/MS mode. In addition, variable modifications included methionine oxidation and fixed carbamidomethyl on cysteine (Shevchenko et al., 2006).

2.4. Nucleic acid assays

2.4.1. RNA isolation and cDNA synthesis

10 ml synchronised treated and non-treated cells of *C. jejuni* NCTC 11168 were centrifuged (8,000 x g for 15 min.) and suspended in RNazol (Molecular Research Center, USA). DNA remnants were removed by *DNase* (Roche, Switzerland) treatment for 20 min at 30 °C, and then stopped with 2 µl 0.2 M EDTA (10 min at 75 °C). RNA samples were purified by RNeasy Mini Kit (Qiagen, Germany), and served as a template for cDNA synthesis applying Superscript Reverse Transcriptase

III (Invitrogen, USA). For this, 10 pg-0.5 µg extracted total RNA, 50 µM random primers (Applied Biosystems, USA), and 10 mM dNTPs (Fermentas, Lithuania) were applied. The reaction was incubated for 5 min at 65 ^{o}C, followed by 1 min on ice. RNA amounts were quantified using the ND-1000 Nanodrop Spectrophotometer (Thermo Scientific, USA).

2.4.2. Real-Time PCR (RT-PCR) analysis

Primers for RT-PCR were designed with Vector NTI Software. SYBR green (Bio-Rad, USA) master mix was used for the PCR reactions performed in triplicates using the Rotor Gene, RG3000 (Qiagen, Germany) apparatus. Conditions were 15 sec at 96 °C, 15 sec at 50 °C followed by 25 sec at 72 °C with 45 repeats. Melting-curve analysis was performed immediately after each amplification. Each specific amplicon was verified both by the presence of a single melting temperature peak and by the presence of a single band of expected size on a 2% agarose gel after electrophoresis. In the negative control no RNA template was present. Samples were normalized using phosphoglucosamine mutase (*pgm*) as an internal standard. Relative *n*-fold changes in the transcription of the examined genes between the treated and non-treated samples were calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) Group wise comparison and statistical analysis of the relative expression results were performed with the Relative Expression Software Tool (REST) 2009 (Pfaffl et al., 2002).

2.5. Scanning electron microscopy (SEM)

The methods of Hazrin-Chong and Manefield (2012) and Xie et al. (2011) were combined and modified in order to pretreat *C. jejuni* NCTC 11168 cells for SEM analyses. 100 µl of 2.5% (v/w) glutaraldehyde (in PBS, pH 7.4) was added as a primary fixative solution to 100 µl-s of EO treated and non-treated bacteria (OD_{600}=1.0) followed by 2 h at room temperature. After centrifugation (12,000 x g for 15 min) the supernatant was discarded and a gradual dehydration of the bacteria was carried out by subsequent ethanol treatments (50%, 80%, and 96%) with three changes for 10 min in each concentration. Samples were dried with 50 and 100% hexamethyldisilazane (HMDS, Fluka, USA) for 30 min each. Bacterial cells were then mounted on aluminium stubs coated with a layer of gold using fine coat ion-sputter JFC 1100 (JEOL, UK), and viewed using an JSM 6300 Scanning Microscope (JEOL, UK) at 16 kV and magnification of x10,000, and x20,000.

2.6. Motility assay

Motility assay was performed in three parallels as previously described (Malik-Kale et al., 2007). 24-h culture of *C. jejuni* NCTC 11168 was set to OD_{600}=1.0 in LB broth, and 10 µl-s from this suspension were spotted in the middle of 0.3% agar plates lacking or containing clove EO (1: 3,000 and 1: 20,000). After 24 h incubation at 42 ^{o}C under microaerophilic condition the diameter of the growth zone was measured.

2.7. Determination of clove essential oil composition

2.7.1. Gas chromatography–mass spectrometry (GC-MS) analysis

Essential oil compounds were identified by an Agilent 6890N/5973N GC-MSD (USA) system equipped with an Agilent HP-5MS capillary column (30 m× 250 µm× 0.25 µm). The GC oven temperature was programmed to increase from 60 ºC (3 min isothermal) to 200 ºC in 8 ºC/ min (2 min isothermal), from 200–230 ºC in 10 ºC/ min (5 min isothermal), and finally from 230–250 ºC in 10 ºC/min (1 min isothermal). The following conditions were used for the measurements: high purity helium as carrier gas at 1.0 ml/min (37 cm/s) in constant flow mode; ionisation potential, 70 eV; and scan range 41–500 amp/s. Data were evaluated by the MSD ChemStation D.02.00.275 software (Agilent, USA). GC analysis was also performed using a Fisons GC 8000 gas chromatograph (Carlo Erba, Italy), equipped with a flame ionisation detector (FID). The oven temperature was increased at a rate of 8 °C/min from 60 °C to 230 °C, with a final isotherm at 230 °C for 5 min. Identification of peaks was made by retention time and standard addition; percentage evaluation was carried out by area normalisation. Three parallel measurements were carried out; RSD percentages were below 4.5% (Horváth et al., 2011).

2.7.2. Thin layer chromatography (TLC)

Active component visualisation and bioautography of clove EO was performed in parallel on two preconditioned (100 °C for 30 min) 5 x 10 cm 60 F_{254} thin layer chromatography (TLC) plates (Merck, Germany). 100 µl of essential oil was dissolved in 500 µl ethanol, and 3-5 µl aliquots were separated on the plates. Eugenol (Sigma-Aldrich, USA) was used as control (5 µl). After sample application, the TLC plates were developed with the mobile phase of toluene–ethyl acetate (95:5). Ascendant development was used in a saturated twin trough chamber (Camag, Switzerland). Ethanolic vanillin–sulphuric acid reagent was applied to visualise the separated compounds. The developed layers were dipped into this reagent, and heated for 5 min at 90 °C to remove the solvent completely.

Identification of the separated compounds was performed by R_f value and color of the standards.

2.7.3. Thin layer chromatography - direct bioautography (TLC-DB)

For bioautography the other plate was incubated for 1 h at 42°C under microaerophilic condition in 50 ml bacterium suspension (3 x 10^8 cells/ml). Than the plates were placed into an aqueous solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.05 g/90 ml) for 10 sec, and were further incubated for 2 h. Inhibition zones around the separated compounds were visualised by tetrazolium salt-based reagent. White spots against the bluish background point to the lack of dehydrogenase activity due to antibacterial activity of the separated compound. All measurements were performed in duplicates (Wagner and Bladt, 2001).

3. Results

3.1. MIC and MBC of clove essential oil against *C. jejuni*

MIC and MBC were determined as dilutions 1: 5,000 and 1: 2,500, respectively, in a 24-h experiment. Dilution 1: 3,000 proved to have a moderate antimicrobial effect not only on the four reference *C. jejuni* strains, but also on all the 50 clinical isolates by reducing the living cell counts to one-third in 2 h (Figure 21).

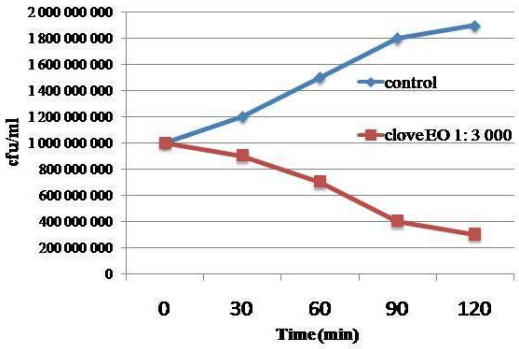

Figure 21. Effect of clove EO in 1: 3,000 dilution on the proliferation of *C. jejuni* strain NCTC 11168.

3.2. Clove essential oil induced changes in proteome

Marked differences were identified in the total protein profile of the clove EO treated *C. jejuni* strain NCTC 11168 by the protein chip assay, compared to that of the non-treated bacterium. 11 discrete peaks could be identified on the electropherogram of untreated *C. jejuni* (Figure 22.). In the profile of the treated cells basically the amplitude of the major peaks decreased, and in three cases (2, 6, and 10), they could not be detected at all as individual peaks. In addition, three new characteristic peaks (4, 9, and 13) appeared in the protein profile of the clove EO treated bacteria (Table 6).

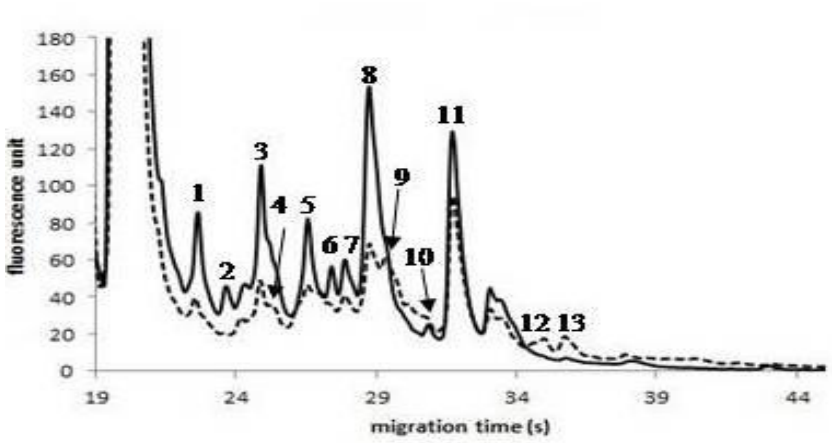

Figure 22. Protein chip-based electropherogram of clove EO-treated (dashed line) and untreated (continuous line) NCTC 11168 strain.

Table 6. Aligned migration times, molecular weights, and areas belonging to the dominant peaks presented in Figure 22.

	Control			Clove EO 1: 3,000		
	AMT[sec]	Size [kDa]	Area	AMT[sec]	Size [kDa]	Area
1	22.65	11.2	14.3	2254	10.9	2.4
2	23.64	13.4	3.5			
3	24.88	18.2	25.2	24.87	18.1	3.6
4				25.23	20.1	0.8
5	26.55	27.3	14.9	26.58	27.5	1.,2
6	27.38	32.7	3.3			
7	27.9	36.1	5.5	27.88	36	2.7
8	28.73	41.6	61.9	28.76	41.8	9.2
9				29.38	46	4.4
10	30.86	56.2	1.5			
11	31.69	61.9	52.8	31.71	62.1	37.4
12	33.04	75.8	19.5	33.06	76	9.9
13				34.97	96.4	2.9

In order to reveal the mostly affected proteins in the presence of clove EO, 2D polyacrylamide gel electrophoresis analysis and a subsequent LC/MS mass spectrometry were carried out. Six spots well definable on the control gel presented with drastically decreased expression in the profile of the clove EO treated counterpart (Figure 23). These spots correspond to proteins known to be involved in the synthesis of five virulence associated factors: (i) Peb1, an important factor in host colonisation (Pei et al., 1991), (ii) Peb4, a temperature dependent colonisation factor (Asakura et al., 2007), and (iii) HtrA a serine protease, which has a role in adherence and invasion (Rathbun et al., 2009). Additionally two spots were revealed with elevated expression level compared to the control, when cells were exposed to clove EO (Figure 23/B). They were identified as a chaperonin (Klancnik et al.,

2006), and the elongation factor Tu (Kjeldgaard et al., 1993) (Table 7). Peptide mass fingerprints of the digested proteins and detailed information of the identified proteins are presented in A/6.

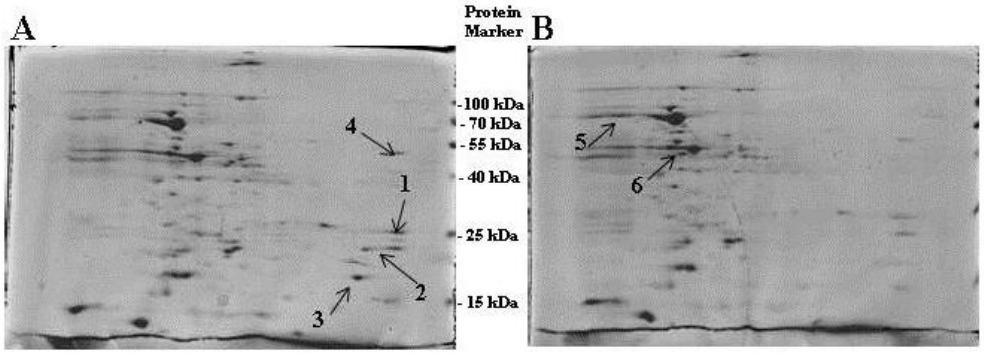

Figure 23. Differences in the protein expression patterns of the control (A.) and clove EO treated (B.) *C. jejuni* 11168 as shown by 2D PAGE analysis. Proteins designated by numbers display increased expression.

Table 7. Proteins identified by SDS-PAGE separation (pH 3.0–10.0 gradient gels) followed by in-gel digestion and LC/MS analysis.

Spot No.	Protein	Mascot Score	MW (kDa)	Peptides	Uniprot/ Acc. No
1	major antigen Peb4A	497,4	30,4	11,0	CBF2_CAMJJ
2	major cell-binding factor Peb1A	964,1	28,2	22,0	PEB1A_CAMJE
3	uncharacterised protein	201,6	21,0	5,0	Q5HW47_CAMJR
4	serine protease htrA	555,0	51,0	15,0	gi|57238208
5	60 kDa chaperonin	694,6	57,9	27,0	CH60_CAMJE
6	elongation factor Tu	269,5	44,9	9,0	gi|488955902

For Spot No. see Figure 23.

3.3. Gene expression profile of *C. jejuni* in response to clove essential oil

Altogether 45 genes (A/7) were targeted by reverse transcription PCR in order to reveal their incidental alteration in expression due to clove EO treatment. These genes are known to be involved in (i) stress responses, (ii) pathogenic processes, and (iii) basic metabolism (housekeeping genes).

Regarding the stress genes it was revealed that four of them were unambiguously upregulated (Table 8). Gene *katA* (VanVliet et al., 2002) encoding catalase, and *groEL (*Klancnik et al., 2006), encoding a molecular chaperone, proved to be upregulated 51- and 20-fold, respectively, when clove EO was present. Furthermore, *groES,* encoding a co-chaperonin and *dnaK* (Yoshimune et al., 2002), encoding another chaperone line up to them with a nearly 4-fold upregulation (3.937 and 3.704, respectively).

Table 8. Transcription intensity changes of the affected ORFs of *C. jejuni* 11168 in the presence of clove EO. Altogether 45 ORFs were investigated and from them 14 proved to be affected.

Gene ID/Name	Direction of change in expression level	Fold change	Specific function (Revez et al., 2012)
Cj1385/*katA*	Up	51.967	catalase
Cj1220/*groES*	Up	3.937	co-chaperonin GroES
Cj1131c/*galE*	Down	0.158	UDP-GlcNAc/Glc 4-epimerase
Cj0335/*flhB*	Down	0.131	flagellar biosynthesis protein FlhB
Cj0536/*oorA*	Down	0.104	2-oxoglutarate-acceptor oxidoreductase subunit OorA
Cj0415	Down	0.195	GMC oxidoreductase subunit
Cj1221/*groEL*	Up	20.350	molecular chaperone GroEL
Cj0759/*dnaK*	Up	3.704	molecular chaperone DnaK
C8J_0494/*sucC*	Down	0.050	succinyl-CoA synthetase subunit beta
Cj0268c	Down	0.206	transmembrane protein
Cj0891c/*serA*	Down	0.21	D-3-phosphoglycerate dehydrogenase
Cj1259/*porA*	Down	0.33	major outer membrane protein
Cj0670/*rpoN*	Down	0.32	RNA polymerase factor sigma54
Cj0061c/*fliA*	Up	1.73	flagellar biosynthesis sigma factor

$p < 0.001$ for all the listed genes

Results of the RT-PCRs have shown that at least two virulence associated genes were downregulated in the presence of clove EO. The UDP-glucose 4-epimerase (*galE*) (Fry et al., 2000) involved in the LOS biosynthesis and *flhB* (Matz et al., 2002), that codes for a flagellar biosynthesis protein were down regulated nearly by sevenfold. A threefold down regulation was observed in the case of *porA*, a major outer membrane protein possessing with strong antigenic feature (Goulhen et al., 2004). Expression of the three known global transcriptional regulators of *C. jejuni* (Carrillo et al., 2004) proved to be different. *RpoN* (σ^{54}) was downregulated by one third compared to *pgm* and in contrast to *fliA* (σ^{28}), that was 1.73 times upregulated while expression of *rpoD* (σ^{70}) remained unaltered (data not shown). The investigated housekeeping genes were transcribed in equal rates both in treated and non-treated cells.

3.4. Effect of clove essential oil on *C. jejuni* morphology

The influence of clove EO on *C. jejuni* morphology was examined by electron microscopy. After a 2-h clove EO treatment the originally curved *C. jejuni* cells (Figure 24-A, B) presented with a shrunken and straightened outlook (Figure 24-C, D) if compared to the control. We have not found any considerable amount of coccoid forms as they were described by others in relation with other environmental stresses (Xie et al., 2011).

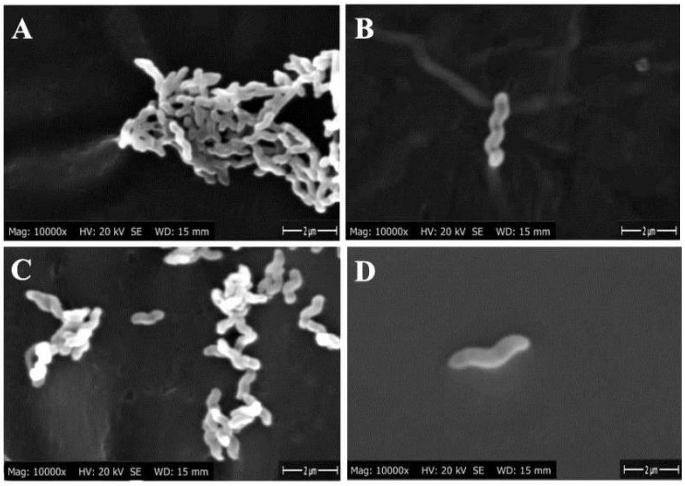

Figure 24. Scanning electron micrographs of non-treated (A, B) and clove EO treated (1: 3,000) (C, D) *C. jejuni* cells. Untreated *C. jejuni* showed the typical slightly curved spiral morphology, while clove EO treated cells became shortened and less intensely curved.

3.5. Effect of clove essential oil on the motility of *C. jejuni*

After 24-h microaerophilic incubation, untreated cells showed a 3 cm diameter swarming area (Figure 25-A). Survived *C. jejuni* cells treated with the generally applied 1: 3,000 dilution of clove EO completely lost their ability to move (Figure 25-C). If the sub-inhibitory (1: 20,000) dilution of clove EO was applied bacterial cells survived but they showed a decreased capacity to swarm (Figure 25-B).

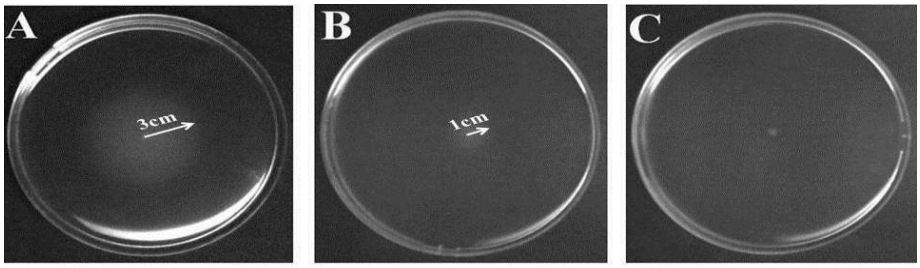

Figure 25. Motility of untreated (A) and treated (B, C) *C. jejuni* showed marked differences on 0.3% agar plates. Motile bacteria are represented by a diffuse cloud with a 3 cm diameter on the control agar plate (A). If clove EO was present in the media in a 20,000x dilution (B) bacterial growth was observable only around the inoculum stab while no swarming could be detected if the EO dilution 1: 3,000 (C) was applied.

3.6. Identification of the effective clove essential oil components

Compound composition of clove EO was determined by GC-MS analyses and presented in Table 9. Applying TLC with ethanolic vanillin–sulphuric acid reagent eight constituents could be visualised. The major component, eugenol was identified as an orange-brown zone (Rf=0.58), and β-caryophyllene appeared at the solvent front as a purple spot. Direct bioautography combined with TLC revealed another five unidentified components of clove EO with antimicrobial effect against *C.*

jejuni (Figure 26). By their Rf values they are supposed to be terpene alcohols (Wagner and Bladt, 2001).

Table 9. Volatile compound composition of clove EO determined by GC-MS.

	Compounds	t_R MS (min)	t_R FID (min)	%
1	Eugenol	14,3	15,8	88,6
2	β-caryophyllene	15,4	14,9	8,6
3	α-humulene	16	15,5	2,2
4	caryophylleneoxyde	18	18,9	0,5

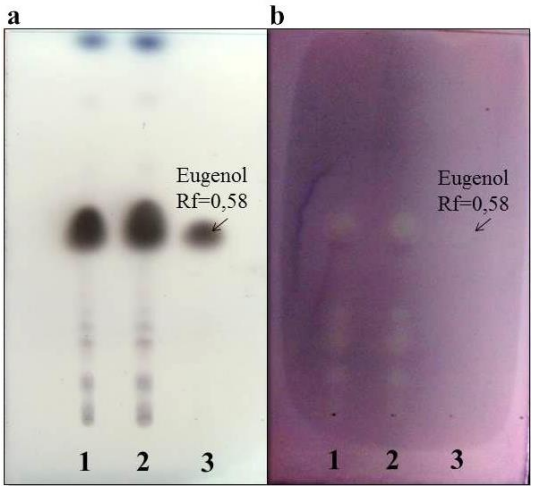

Figure 26. Thin layer chromatography separation and antibacterial effect of clove EO components. TLC separated components were visualised by ethanolic vanillin–sulphuric acid reagent (A.), while antibacterial effect of clove EO was revealed by bioautography developed with a tetrazolium salt-base reagent (MTT) (B.). Eugenol was used as standard.

4. Discussion

Appearance and spread of macrolide resistance among *Campylobacter* isolates (Soonthornchaikul et al., 2006) requires novel strategies and alternative agents both in prevention and treatment. Antibacterial effect of clove EO has been demonstrated on several bacterial species (Smith-Palmer et al., 1998).

It is a favorable view, that antibacterial effect of EOs is the consequence of pore-formation and a subsequent oxidative stress (Burt, 2004). Although the 51.9 fold activation of *katA* could be a clear indication for that (VanVliet et al., 2002), the expression of three other genes (*dps*, *sodB*, and *ahpC*) involved in oxidative stress responses (Xie et al., 2011) were not elevated (Table 8). We have tried to turn the scales of this question by using the EMA PCR (Xie et al., 2011), but ambiguities of the system and antibacterial effect of the ethidium monoazide itself queries the applicability of this

method. Furthermore, overexpression of *groEL* (Klancnik et al., 2006) and *dnaK* (Yoshimune et al., 2002), two important molecular chaperons, characteristic for general stress response could be detected. Our results are in harmony with the recent findings of Klancnik, (2006), arguing against the role of *groEL* in oxidative stress response. Additionally, the upregulation of *dnaK*, one of the three members of the prokaryotic Hsp70 family was demonstrated in chemical stresses (Yoshimune et al., 2002).

Campylobacter is prone to respond to stresses by forming rounded cells (Xie et al., 2011) however, this shrunkened and straightened outlook has not yet been described before (Figure 24). This observed definite change in the morphology may be the result of affected membrane protein or cytoskeletal changes. Clove EO has been reported to induce changes in the fatty acid profile of the cell membrane (decreasing the proportion of the UFAs), causing membrane fluidity resulting in morphological changes (Hyldgaard et al., 2012).

During this stress marked upregulation of the elongation factor Tu (EF-Tu) occurred supporting the hypothesis, that Tu being crucial in translational accuracy ensures proper function of housekeeping genes and by this keep cells alive till it is worth (Kjeldgaard et al., 1993).

Housekeeping genes are important to sustain the basic cellular functions in all circumstances. Transcriptomic level of the 11 tested housekeeping genes (Dingle et al., 2001) of *C. jejuni* proved to be similar compared among the clove EO treated and non-treated samples. Among them *pgm*, encoding for the phosphoglucosamine mutase proved to be the most stable, and for this reason, this gene was applied as an inner control in order to unequivocally define and evaluate the transcriptomic activity of the tested genes.

Chemotaxis, motility, adhesion, invasion and intracellular survival are the major stations during the pathogenic process of *C. jejuni* (Klancnik et al., 2006). Motility, mediated by the polar flagella has been shown to be crucial for colonisation in *C. jejuni* (Malik-Kale et al., 2007). Its function is based on a sophisticated chemosensory network and the effector flagella, which steer the bacterial cell toward the surface of the epithelial cells and help it to penetrate through the mucinous layer (Klancnik et al., 2006). Impaired function of one of its counterparts could lead to impaired motility. In this study we could reveal, that beyond its antibacterial feature, clove EO was able to intermeddle the expression at least one structural (*flhB*) component and two global regulators (*fliA, rpoN*) also affecting the motility apparatus. Although the revealment of detailed transcriptomic changes in the presence of clove EO affecting the flagellar apparatus was out of the scope of this study, we have shown by a phenotypic test that this EO impairs motility (Figure 25). Involvement of sub-sigma factorial targets is suggested by our results since *flhB*, a class I flagellar gene (Carrillo et al., 2004), proved to be down regulated although expression of its sigma factor *rpoD* (σ^{70}) was constant. As

flagellar motion requires energy, clove EO can hamper the motility inhibiting the function of ATPase, and PMF (Hyldgaard et al., 2012).

Here we have to emphasize that FlhB is not only necessary for the proper flagellar function, but also serves as part of an export apparatus through which secretion of the Cia proteins occurs (Rivera-Amill and Konkel, 1999). Loss of these proteins leads to invasion-deficiency and hereby impairs virulence (Malik-Kale et al., 2007).

Earlier and recent studies have demonstrated the role of Peb1 (Pei et al., 1991) and Peb4 (Asakura et al., 2007; Rathbun et al., 2009) in adhesion. Here we showed that, that these two proteins are potential targets of one or more components of clove EO, since their expressions are significantly decreased. Furthermore expression of the serine protease HtrA was also found to be impaired. During invasion a crucial role is attributed to this enzyme (Rathbun et al., 2009).

Seven volatile components possessing antibacterial activity were visualised (Figure 26.) by bioautography. Eugenol is thought to exert its inhibitory effect due to binding to and alter the activity of proteins (Burt, 2004).

We can conclude that as a result of clove EO treatment, the general stress response was dominant, but on the other hand, the oxidative stress was revealed to be notable as well. We have demonstrated that components of clove EO selectively influence the expression of certain genes involved in stress and virulence. In the case of flagellar function this observation was also confirmed by a functional assay. We presume that one or more components of clove EO can recognize specific proteins or genetic motifs, and by this it is able to inhibit gene expression and protein function. A more systematic study with separated clove EO components could reveal the affected groups of genes or proteins in order to precisely identify potential target sites on and in the *Campylobacter* cell.

CHAPTER VI. DETECTION OF HIGH- MOLECULAR- WEIGHT POLYSACCHARIDE STRUCTURES OF *CAMPYLOBACTER JEJUNI*

1. Aim of the study

There are several open questions regarding the composition and pathogenic role of the sugar constituents of *C. jejuni* associated with the cell surface. The simple question if *C. jejuni* has a high molecular weight LPS-like structure at all has not yet been clarified. The major problem is that the traditional LPS isolation receipts for silver staining are not ideal for *Campylobacter* spp. to reveal the typical structure of this component known to be suitable for other pathogenic bacteria (*Salmonella*, *E. coli*, etc.). However, since we have hypothetised that such polysaccharide structures have to be present in *C. jejuni* we aimed to systematically modify the already known receipts, and develop a reliable detection method by which the high molecular weight polysaccharide structures of *C. jejuni* can be visualized, and by which large-scale screening and comparative characterisation studies on different strain collections could be performed.

2. Materials and methods

2.1. Isolation of LPS-like structure

Strains were grown on CCDA, at 42 ^{o}C under microaerophilic condition for 24 h. After synchronisation to OD of 1.0 at 600 nm (5 ml) the cells were centrifuged at 4,000 x g, 4 ^{o}C for 10 minutes. The supernatant was discarded, and the pellet was washed three times in PBS, and then re-suspended in 300 µl distilled water. The cells were boiled at 100 ^{o}C for 30 minutes. After cooling to room temperature, lysozyme (Sigma-Aldrich, USA) was added to a final concentration of 3.0 mg/ml, and the bacterial suspension was incubated at 37 ^{o}C for 60 minutes to digest the peptidoglycan layer. After this step, 300 µl lysis buffer (2% SDS, 4%, 2-mercaptoethanol (Sigma-Aldrich, USA), 10% glycerol, 0.05% bromophenol blue (WVR, USA), 1M Tris-HCl, pH 6.8) was added, and the mixture was incubated at 100 ^{o}C for 10 minutes in order to liberate nucleic acids, proteins and cell wall components. Redundant proteins were eliminated by 0.5 mg/ml proteinase K (ROCHE, Switzerland) treatment in three steps at 65 ^{o}C for 60 minutes each time. The reaction was stopped by 600 µl 0.4 M $MgCl_2$ in cc. ethanol by overnight incubation at -20 ^{o}C. Next day the sample was pelleted (15,000 x g 4 ^{o}C for 15 min), suspended in 50 µl distilled water, and stored at -20 ^{o}C until use.

2.2. Silver staining

The isolated LPS-like structure was run by the discontinuous buffer system of Laemmli (1970) in a 15% tricine polyacrylamide gel (PAGE) at 40 mA for 30 min.

After SDS-PAGE the gels were fixed and stained by the silver staining method (Tsai and Frasch, 1982). Briefly: gels were fixed (1.3 v/w% sodium periodate dissolved in 50% isopropanol and 14% acetic acid) for 15 minutes and washed three times (45 minutes) with water. Staining solution (2 ml 1 M NaOH, 1.4% NH_3, 0.3 g $AgNO_3$) was added for 15 minutes, and the gel was washed three times for 15 minutes with water. Visualisation was carried out by the pre-warmed (40 ^oC) development solution (2.5% Na_2CO_3, 0.027% formaldehyde), and the reaction was terminated with 50 mM EDTA.

5. Results

In our comparative study two major groups of published methods were tested and compared. By the methods based on ***phenol extraction*** (Westphal and Jahn, 1965; Preston and Penner, 1987; Aspinall et al.1992; Rezania et al., 2011) only the low molecular weight (LMW) structures could be visualised, but the high molecular weight (HMW) structures seemed to be absent (data not shown). Furthermore, we tested three methods applying ***enzymatic digestions*** with no phenolic step as described by Preston and Penner, 1987 (i), Salloway et al, 1996 (ii) and Szymanski et al., 1999 (iii). None of these methods proved to be adequate in detecting a ladder-like HMW pattern in *C. jejuni*; however, by modifying certain conditions, we could demonstrate a typical ladder-like structure of HMW PS (Figure 27).

Sample treatment	1	2	3	4	5	6	7	8	9	10
Boiling the sample before adding the lysis buffer	no	no	no	yes	yes	no	no	yes	yes	yes
Digestion with lysozyme (mg/ml)	no	no	no	no	no	3,0	3,0	3,0	3,0	3,0
Concentra-tion of Tris in the lysis buffer (M)	0,0625	0,125	0,125	1,0	0,1	1,0	0,1	1,0	0,1	1,0
Digestion with proteinase K (mg/ml)	1,0	1,0	2,5	0,5	0,5	0,5	0,5	0,5	0,5	no

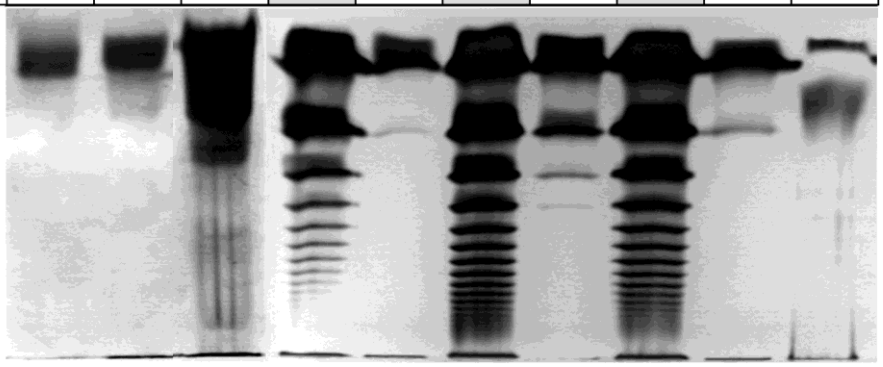

Figure 27. Comparison of the major steps and their effects on the isolation of the HMW fraction of *C. jejuni* NCTC 11168 by the published (lines 1-3) and the modified (lines 4-10) isolation methods.
In accordance with the literature, no HMW structures were detected by silver staining when the methods of Szymanski, Salloway, and Preston and Penner (lines 1-3) were used. The concentration values represent the final working concentrations of the solutions applied.

Critical steps of the modified method for the successful isolation (lines 4, 6, 8) of slow-migrating HMW structures are dependent on a higher Tris concentration (1 M), as well as the application of lysozyme and proteinase K (Figure 27). The modified method was tested on four reference strains (NCTC 11168, 81116, 81-176, 1221), on three strains isolated from patients suffering from diarrhea in Southwest Hungary, as well as on two standard *E. coli* and one *Salmonella* strain (Figure 28).

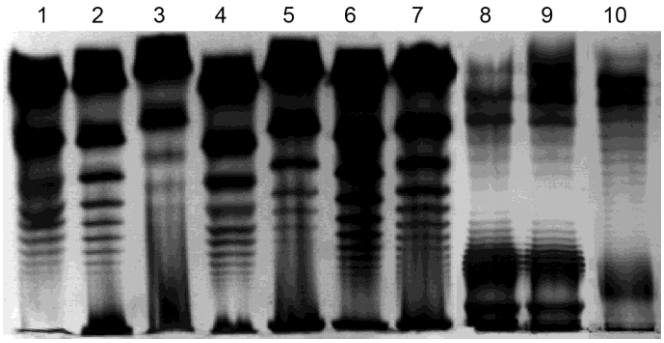

Figure 28. HMW PS fractions could be detected in different isolates of *C. jejuni* (1-7) and in other referenced Gram-negative bacteria (8-10) by using the modified method (Figure 27, lane 8). Lane 1, *C. jejuni* 81116; lane 2, *C. jejuni* NCTC 11168; lane 3, *C. jejuni* 81-176; lane 4, *C. jejuni* RM 1221; lane 5, *C. jejuni* 2006-2; lane 6, *C. jejuni* 2006-5; lane 7, *C. jejuni* 2006- 14; lane 8, Uropathogenic *E. coli* strain 536; lane 9, *E. coli* ATCC *25922* ; lane 10, *S. Typhimurium* ATCC 14028.

6. Discussion

One of the main characteristic features of *C. jejuni* is the production of a variety of glycoconjugates, located on the cell surface, including glycoproteins and glycolipids, taking part in its survival strategy, pathogenic process, and providing the basis for serotyping (Penner and Henessy, 1980). Preston and Penner were the first (Preston and Penner, 1987) to confirm that all *C. jejuni* serotypes possess LMW LPS detectable by silver staining method of Tsai and Frasch (1982), and a minority of the serotypes could also synthetise HMW LPSs. This HMW structure, presenting a characteristic ladder-like pattern however, could not be detected by SDS-PAGE but only by immune blotting with group specific antisera (Aspinall et al., 1994).

Despite significant efforts, the HMW LPS of *C. jejuni* could not be visualised for a long time, although the same or very similar extraction and silver staining methods have been applied by which the LPS of salmonellae (Perez Perez and Blaser, 1984) and *Escherichia coli* (Preston and Penner, 1987) could unequivocally be detected.

Some authors have thought that *C. jejuni* expresses either LPS or LOS in a strain–dependent manner (Preston and Penner, 1987; Muldoon et al., 2002) while others (Karlyshev et al.; 2000) were on the opinion that *C. jejuni* produces LOS, and the previously published HMW LPS O-chains were simply capsular polysaccharides (PSs). Supporting this hypothesis the genome sequencing of *C. jejuni* NCTC 11168 (Parkhill et al., 2000) revealed a homologous gene cluster coding for CPS transport, and *cps (kpsM)* mutants lost their ability to be typed by the Penner scheme (Bacon et al, 2001). However, these authors also reported a *kpsM* independent ladder-like HMW PS structure revealed by the laborious immunoblot technique. Although there are unresolved discrepancies concerning the nature of HMW structures in *C. jejuni*, it seems evident from the literature that several isolates possess these slow-migrating ladder-like PS structures. However, a reliable and standardized method has not yet been developed up to now by which the HMW fraction could be detected among isolates presenting with different antigenicity.

In this study we describe a method by which the presence of a slow-migrating HMW PS with a ladder-like structure characteristic to other Gram-negative bacteria can be visualised in *C. jejuni*. With our modified method we could detect the presence of LPS on strains showing LOS structures by the other methods. We think that the results of former publications on this subject are worth revisiting. Furthermore, LOS structures of taxa other than *Campylobacter* might also be reinvestigated. It is also an advantage of the method that the hazardous phenol is avoided from protocol. Since our method does not require a time consuming immunoblotting step by specific and

expensive antiserum, it offers a widely applicable and robust alternative that can initiate or revitalize studies focusing on the incidence, variability, and the pathogenic function of LPS/LOS molecules.

CHAPTER VII. FINAL CONCLUSIONS

Our main findings can be summarized as follows:

- We have revealed the genetic relatedness among the *C. jejuni* clinical isolates from the South Transdanubian region: 69 RFLP groups and 122 PFGE groups were established.
- We have confirmed by *flaA*-RFLP and PFGE, that all strains are independent isolates, mainly representing sporadic cases in the region.
- No correlation could be revealed between the phylogenetic groups and the severity of symptoms caused by clinical isolates.
- We have found, that the severity of symptoms of *C. jejuni* isolates did not correlate with the presence or absence of certain proposed virulence genes.
- We have found, that the adhesion and invasion ability varied considerably among the strains
- No correlation could be revealed between the results of phenotypic virulence assays (*in vitro* adhesion/invasion assay, ECMP-binding assay), and the clinical status of the patients.

➢ Main findings of the investigation of virulence- associated factors contributing to a highly pathogenic phenotype of *C. jejuni*:

- Beside the upregulation of several known and putative virulence associated genes results of the whole transcriptomic analysis revealed the possible importance of cell-shape determining genes during or after the invasion.
- Our results strongly suggest the active involvement of transmembrane processes and early flagellar proteins (T3SS) export funcion in the invasion and the intracellular survival of *C. jejuni*.
- According to our results, an additional role of certain adhesional proteins during the invasion and the intracellular life is suggested.
- Genes with known and unknown functions have been identified during cell internalisation representing candidates for future studies.

- Main findings of the investigation of the antimicrobial mode of action of clove EO against *C. jejuni:*
  - Clove EO markedly influenced the morphology and motility of *C. jejuni*
  - Dominance of general stress response and suppression of certain virulence associated factors (flagella, adhesins…) was also revealed as a result of clove EO treatment.
  - Two volatile clove EO components (eugenol and several other non-identified) components possess bactericidal activity on *C. jejuni* were revealed.

- Result of the improvement of a reliable HMW PS isolation method:
  - A modified procedure was developed, by which of HMW polysaccharide structure of *C. jejuni* can be visualised.
  - This modified receipt is more simple, than the previously used immunoblot method.
  - This method can contribute to solve some old questions related to PS structures of *C. jejuni.*

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APPENDICES

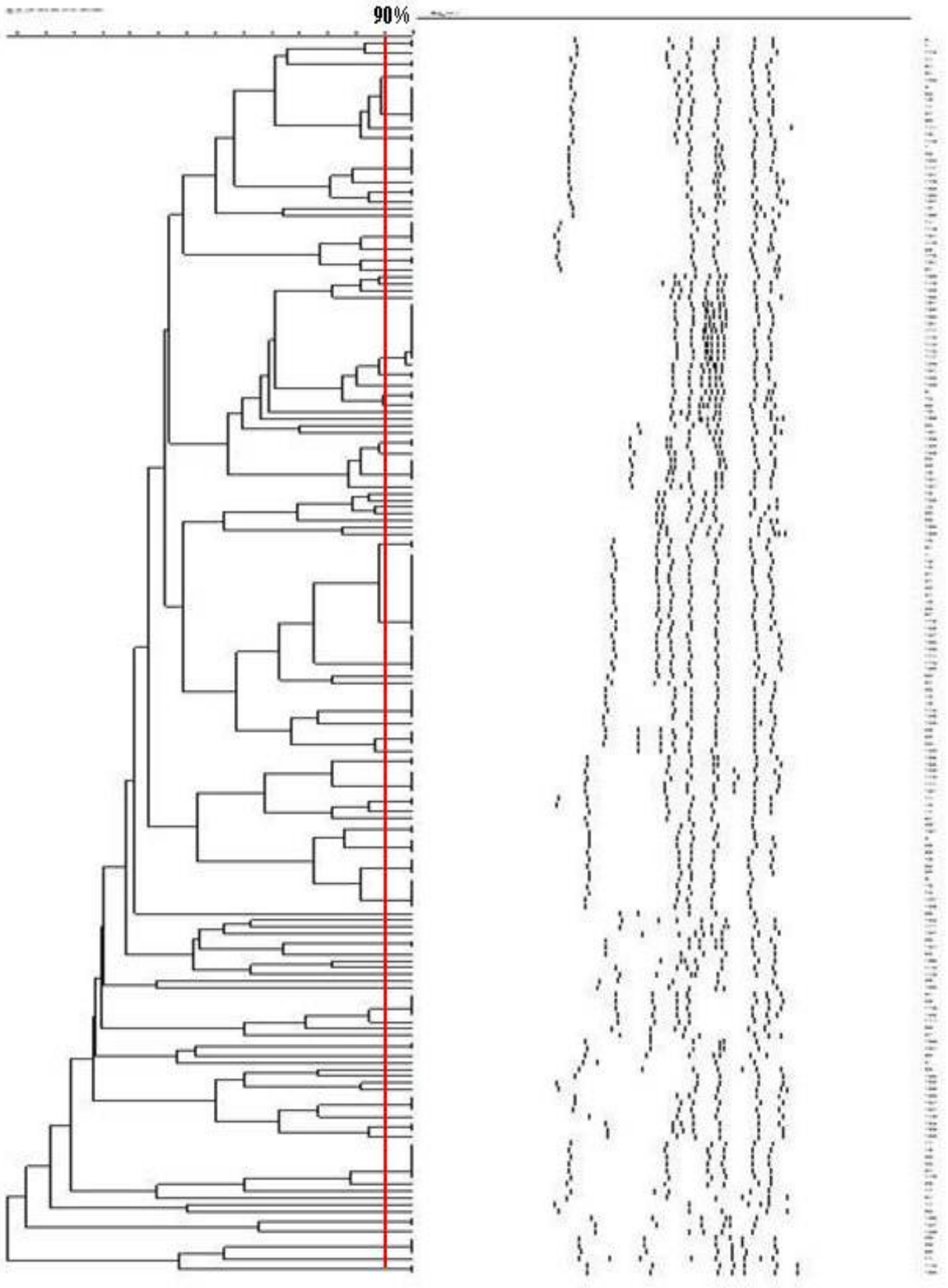
A/1 - List of abbrevations

A/…	Appendice
AFLP	Amplified fragment length polymorphism
AIDP	Acute inflammatory demyelinating polyneuropathy
AIDS	Acquired Immune Deficiency Syndrome
AMAN	Acute motor axonal neuropathy
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CCDA	Charcoal Cefoperazone Deoxycholate
CCV	Campylobacter-containing vacuole
CDC	Centers for disease Control and Prevention
CDT	Cytolethal distending toxin
CFUs	Colony forming units
CPSs	Capsular pliysaccharides
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECDC	European Centre for Disease Prevention and Control
ECMP	Extracellular matrix protein
EDTA	Ethylene diamine tetra acetic acid
EFSA	European Food Safety Authority
EFTA	European Free Trade Association
EMA	Ethidium monoazide bromide
EO	Essential oil
ERK	Extracellular Signal-regulated Kinase
ETs	Electrophoretic types
EU	European Union
FAK	Focal adhesion kinase
GBS	Guillain- Barré syndrome
GC	Gas Chromatography
GPA	Gentamicin protection assay
HL	Heat labile
HMW	High molecular weight
HS	Heat stabile

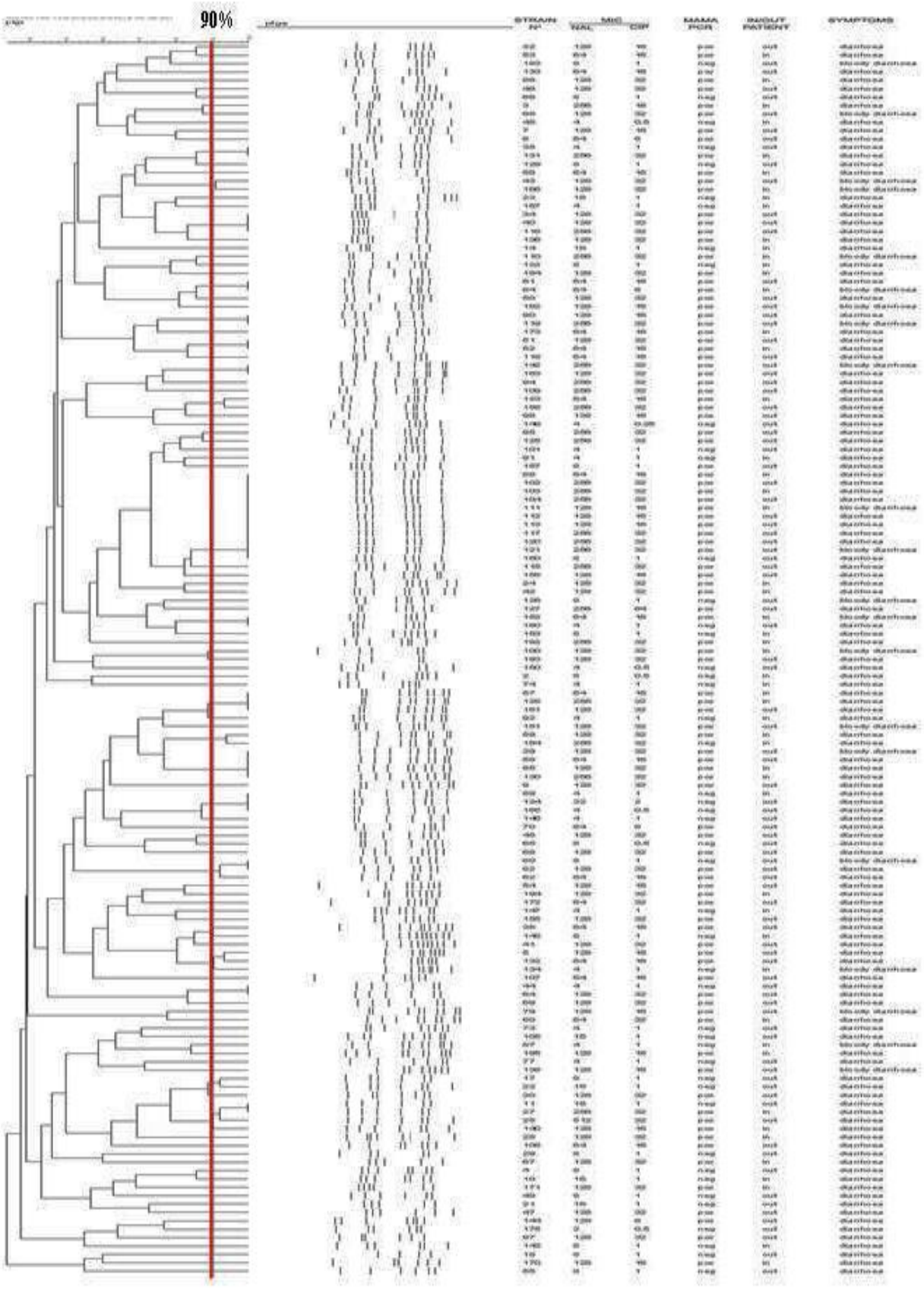
Hsp	Heat shock protein 90
IC	Intracellular
IEF	Isoelectric focusing
IL	Interleukin
LMW	Low molecular weight
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MACPs	Methyl-accepting chemotaxis proteins
MAPK	Mitogen-activated protein kinase
MBC	Minimal bactericidal concentrations
MEE	Multilocus Enzyme Electrophoresis
MFS	Miller Fischer Syndrome
MIC	Minimal inhibitory concentrations
MLST	Multi-locus sequence typing
MOI	Multiplicity of infection
MOMP	Major outer membrane protein
MS	Mass Spectroscopy
MTs	Molecule types
NCBI	National Center for Biotechnology Information
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PKC	Protein kinase C
PMF	Proton motive force
PS	Polysaccharide
PV	Phase-variation
QS	Quorum sensing
RFLP	Restriction fragment length polymorphism
ROS	Reactive Oxygen Species
RT-PCR	Real-time Polymerase chain reaction
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SNPs	Single nucleotide polymorphisms
SSR	Simple sequence repeats

STs	Sequence types
TLC	Thin layer chromatography
UPLC	Ultra-performance liquid chromatography
USA	United States of America
VNC	Viable but not culturable
VNTR	Variable number tandem repeat
WHO	World Health Organization

A/2 - Dendrogram of *flaA*-RFLP analysis



A/3 - Dendrogram of PFGE analysis



A/4 - List of primers and annealing temperatures used for PCR analysis

Target gene	Primers	Sequence	Annealing temp. (°C)	PCR- product (bp)	Reference
cgtB	*DL39*	5′-tta aga gca aga tat gaa ggt g-3′	54	*420*	*Linton et al. (2000a)*
	cgtBrev	5′-gca cat aga gaa cgc tac aa-3′			
flaB	FlaB3	5′-ata aac acc aac atc ggt gca-3′	53	1670	Smith et al. (1999)
	FlaB4	5′-gtt acg ttg act cat agc ata-3′			
flhB	flhB-q-F	5′-cag gtg cgg atg tgg tga tc-3′	59	101	Müller et al (2006)
	flhB-q-R	5′-cac tcc ttt ggc aac aac cct-3′			
flgB	flgB-q-F	5′-gca cga ttt acc aaa gct gtt tca a-3′	59	123	Müller et al (2006)
	flgB-q-R	5′-cac tgg tgc ttt agc ggg tag a-3′			
flgE2	flE2-q-F	5′-cat ctc acc acg acc tcc tgt tc-3′	55	132	Müller et al (2006)
	flgE2-q-R	5′-gca aaa atc gca atg gct tca-3′			
wlaN	Cj1139cF	5′-tgc tgg gta tac aaa ggt tgt g-3′	55	330	Wassenaar et al. (2002)
	Cj1139cR	5′-aat ttt gga tat ggg tgg gg-3′			
ciaB	CiaB-F	5′-ttt cca aat tta gat gat gc-3′	48	1165	Rivera-Amill et al.(1999)
	CiaB-R	5′-gtt ctt taa att ttt cat aat gc-3′			
iamA	Cia3f	5′-gca caa aat ata tca tta caa-3′	47	518	Müller et al (2006)
	Cia5r	5′-ttc acg act act atg agg-3′			
virB11	VirB11F	5′-gaa cag gaa gtg gaa aaa cta gc-3′	54	709	Bacon et al. (2002)
	VirB11R	5′-ttc cgc att ggg cta tat g-3′			
cadF	cadF-F2B	5′-ttg aag gta att tag ata tg-3′	46	400	Konkel et al. (1999)
	cafF-R1B	5′-cta ata cct aaa gtt gaa ac-3′			
docA	docA1	5′-ata agg tgc ggt ttt ggc-3′	48	725	Müller et al (2006)
	docA2	5′-gtc ttt gca gta gat atg-3′			
docB	docB1	5′-cgg aga gtt tag agg cac c-3′	53	1418	Müller et al (2006)
	docB2	5′-ccg caa att cca tag cag-3′			
docC	docC1	5′-tga gct acg cta tca ttg-3′	51	1835	Müller et al (2006)
	docC2	5′-gct tac gct atg ggt tgg-3′			
cdtB	WMI-R	5′-gtt ggc act tgg aat ttg caa ggc-3′	58	495	Bang et al. (2003)
	Vat2	5′-gtt aaa atc ccc tgc tat caa cca-3′			
cstII	cstII-IIIF	5’-gta ttt aga tgy art car ttt tat ttt gaa g-3’	55	624	This study
	csIIR	5’-tat grt aaa att tga att taa att tgg ygc-3’			
csIII	cstII-IIIF	5’-gta ttt aga tgy art car ttt tat ttt gaa g-3’	51	793	This study
	cstIIIR	5’-tat ttt tat ttg cat att ttt cct tta agt ag-3’			

A/5 - Transcriptional analysis of *C. jejuni* 2006-119 ORFs during invasion

Only genes with at least 1.5 fold up- and downregulation detected after normalization were considered for further analysis with the exception of *certain genes. In this table, generally the upregulated genes are considered, but in some cases the unchanged or downregulated genes are also demonstrated to get a whole picture of a special mechanisms. The color code represents the degree of the up-or downregulation. 2006-119 hour 3, normal culture bacterial - trimmed RNA-Seq - Normalized expression values (**a**); 2006-119 hour 3, normal culture bacterial - trimmed RNA-Seq - Unique gene reads (**b**); 2006-119 hour 3, during invasion bacterial - trimmed RNA-Seq - Normalized expression values (**c**); 2006-119 hour 3 during invasion bacterial - trimmed RNA-Seq - Unique gene reads (**d**). Only genes with at least 1.5 fold up- and downregulation detected after normalisation were considered for further analysis with the exception of genes marked with (*) were choosen to obtain a whole picture of the changes regarding the virulence associated and flagellar genes. Main functional categories and specific functions are as indicated in the 2006 re-annotation of *C. jejuni* NCTC 11168 genome [1]; GenBank accession AL11168 [1]. ORFs are organized in sections containing the following functional categories: **A.** Membranes/surface molecules/lipoproteins/porins/transport/binding, **B.** Capsule, **C.** Toxin, **D.** LOS/Invasion, **E.** Chemotaxis, **F.** Efflux, induced by deoxycholate, colonisation, **G.** Adherence/Colonisation, **H.** Bacterial shape determinant genes, **I.** Colonisation, **J.** Invasion, **K.** Motility/Invasion, **L.** Iron acquisition, **M.** N-linked glycolisation, **N.** O-linked glycosylation, **O.** Colonisation/ Respiration, **P.** Regulator/Signal transduction, **Q.** Restriction/Modification, **R.** Mucin degradation, **S.** Periplasmic proteins, **T.** Energy metabolism, **U.** Protein synthesis/modification/secretion, **V.** Flagellar proteins, **W.** Hypothetical proteins, **X.** Other protein coding ORFs.

Feature ID	Experiment - Fold Change (normalized values)	a	b	c	d	Specific Funcion [1]
Genes coding						
A. Membranes/surface molecules/lipoproteins/porins/transport/binding						
Cj0201c	14,346	0,889	5	12,747	14	integral membrane protein
Cj1187c	12,809	1,506	18	19,292	45	arsenical pump membrane protein
Cj0181	12,168	1,153	8	14,034	19	TonB transport protein
Cj0472	10,713	52,648	88	564,014	184	protein translocase subunit SecE
Cj1373	10,247	0,958	22	9,821	44	integral membrane protein
Cj0186c	8,384	4,936	33	41,381	54	TerC family integral membrane protein
Cj0959c	7,970	11,336	36	90,346	56	membrane protein insertion efficiency factor
Cj1316c	7,685	0,189	2	1,456	3	pseudaminic acid biosynthesis protein PseA
Cj0692c	6,785	4,243	37	28,792	49	membrane protein
Cj1200	6,382	7,780	57	49,651	71	NLPA family lipoprotein
Cj1688c	5,655	27,050	318	152,974	351	protein translocase subunit SecY
Cj1245c	5,331	6,658	74	35,493	77	membrane protein
Cj0801	5,124	3,560	48	18,240	48	integral membrane protein
Cj0025c	4,955	18,881	243	93,551	235	sodium:dicarboxylate family transmembrane symporter
Cj0789	4,658	5,293	55	24,654	50	poly(A) polymerase family protein
Cj1168c	4,538	12,501	70	56,731	62	integral membrane protein
Cj0619	4,099	2,453	30	10,055	24	MATE family transport protein
Cj0182	4,029	9,197	103	37,058	81	transmembrane transport protein
Cj1665	3,790	15,598	73	59,116	54	lipoprotein thiredoxin
Cj0950c	3,751	27,727	112	104,009	82	lipoprotein
Cj0599	3,622	6,547	58	23,713	41	OmpA family membrane protein
Cj1170c	3,564	13,935	184	49,666	128	outer membrane protein Omp50
Cj0294	3,564	3,753	23	13,376	16	MoeB/ThiF family protein

Cj0616	3,522	2,325	16	8,191	11	phosphate ABC transporter ATP-binding protein PstB
Cj0552	3,328	21,289	137	70,860	89	membrane protein
Cj1093c	3,209	24,794	364	79,570	228	protein translocase subunit SecD
Cj0649	3,137	11,422	49	35,828	30	OstA family protein
Cj1092c	3,067	15,178	137	46,547	82	protein translocase subunit SecF
Cj0110	2,882	8,836	32	25,466	18	ExbD/TolR family transport protein ExbD3
Cj0268c	2,881	23,041	233	66,372	131	transmembrane protein
Cj0430	2,626	7,021	80	18,437	41	integral membrane protein
Cj0430	2,626	7,021	80	18,437	41	integral membrane protein
Cj0461c	2,386	11,875	131	28,331	61	MFS transport protein
Cj0355c	2,312	181,085	1130	418,741	510	two-component regulator
Cj0917c	2,183	17,591	345	38,403	147	integral membrane protein- carbon starvation
Cj0958c	2,139	7,804	115	16,688	48	membrane protein insertase YidC
Cj0982c	2,094	37,948	296	79,479	121	amino acid transporter substrate-binding protein CjaA
Cj1074c	2,062	40,882	246	84,296	99	lipoprotein
Cj0235c	1,950	65,424	226	127,556	86	protein translocase subunit SecG
Cj0942c	1,780	24,541	590	43,688	205	protein translocase subunit SecA
Cj0112	1,749	36,787	413	64,348	141	translocation protein TolB
B. Capsule						
Cj1431c	33,303	0,246	4	8,202	26	capsular polysaccharide heptosyltransferase HddD
Cj1448c	4,240	7,977	58	33,824	48	capsule polysaccharide ABC transporter permease KpsM
Cj1447c	3,897	11,532	71	44,939	54	capsule polysaccharide ABC transporter ATP-binding protein KpsT
Cj1414c	3,447	2,861	55	9,862	37	capsule polysaccharide modification protein KpsC
Cj1445c	1,882	31,181	324	58,676	119	capsule polysaccharide ABC transporter permease KpsE
C. Toxin						
*Cj0079c	1,230	26,689	200	32,818	48	cytolethal distending toxin CdtA
*Cj0078c	-1,025	28,339	210	27,657	40	cytolethal distending toxin CdtB
Cj0077c	-1,561	10,580	56	6,776	7	cytolethal distending toxin CdtC
D. LOS/Invasion						
Cj1140	20,494	0,122	1	2,494	4	alpha-2,3 sialyltransferase CstIII
Cj1141	15,371	0,104	1	1,604	3	sialic acid synthase NeuB1
Cj1136	11,528	0,367	4	4,233	9	glycosyltransferase
Cj1137c	8,966	0,432	4	3,878	7	glycosyltransferase
Cj1139c	6,148	0,590	5	3,630	6	beta-1,3 galactosyltransferase WlaN/cgtB
Cj1143	4,099	0,334	5	1,370	4	bifunctional beta-1,4-N-acetylgalactosaminyltransferase/CMP-Neu5Ac synthase NeuA
Cj1131c	4,054	27,168	249	110,127	197	UDP-GlcNAc/Glc 4-epimerase GalE
Cj0288c	3,718	22,226	226	82,637	164	ipid-A-disaccharide synthase LpxB
Cj1148	3,357	3,253	29	10,920	19	heptosyltransferase II WaaF
Cj1135	2,417	3,687	53	8,911	25	glucosyltransferase
Cj1150c	1,549	6,682	86	10,350	26	bifunctional D-beta-D-heptose 7-phosphate kinase/D-beta-D-heptose 1-phosphate adenylyltransferase hldE
E. Chemotaxis						
Cj0924c	1,868	55,882	288	104,386	105	MCP protein-glutamate methylesterase CheB

Cj0283c	1,616	69,317	336	112,042	106	chemotaxis protein, CheW
*Cj0284c	1,416	100,231	2150	141,879	594	chemotaxis histidine kinase CheA
*Cj0285c	1,402	120,068	1067	168,351	292	chemotaxis protein CheV
*Cj0923c	1,325	23,749	174	31,469	45	MCP protein methyltransferase CheR
*Cj1564	1,164	109,855	888	127,882	231	methyl-accepting chemotaxis signal transduction protein Tlp3
*Cj1118c	1,062	129,611	473	137,587	98	chemotaxis protein CheY
*Cj0262c	-1,011	3,072	25	3,038	4	methyl-accepting chemotaxis signal transduction protein DocC
*Cj0019c	-1,012	34,202	565	33,806	109	MCP-domain signal transduction protein DocB
F. Efflux, induced by deoxcholate, colonisation						
Cj0366c	1,567	8,345	242	13,074	74	multidrug efflux pump protein CmeB
*Cj0367c	1,314	18,631	191	24,489	49	multidrug efflux pump protein CmeA
*Cj0365c	-1,086	9,320	128	8,580	23	multidrug efflux pump protein CmeC
G. Adherence/colonisation						
Cj0628	12,809	0,063	0	0,803	2	lipoprotein CapA
Cj1677	5,124	0,128	1	0,656	1	lipoprotein CapB
Cj0289c	4,861	33,465	234	162,668	222	major antigenic peptide Peb3
Cj1279c	2,909	55,849	640	162,490	362	fibronectin domain-containing lipoprotein FlpA
Cj0778	2,443	59,974	411	146,536	196	major antigenic peptide Peb2
Cj1349c	2,160	8,398	102	18,139	43	fibronectin/fibrinogen-binding protein
Cj0091	2,132	23,643	137	50,401	57	lipoprotein
Cj0982c	2,094	37,948	296	79,479	121	amino acid transporter substrate-binding protein CjaA
Cj0090	1,879	8,755	30	16,448	11	lipoprotein
Cj0983	1,803	12,030	125	21,695	44	lipoprotein JlpA
Cj1478c	1,535	156,487	1395	240,242	418	outer membrane fibronectin-binding protein CadF
*Cj0596	1,333	329,357	2514	438,986	654	peptidyl-prolyl cis-trans isomerase Peb4
*Cj0921c	1,034	618,663	4481	639,468	904	bifunctional adhesin/ABC transporter aspartate/glutamate-binding protein Peb1A
H. Bacterial shape determinant genes						
Cj0652	4,455	4,114	69	18,331	60	penicillin-binding protein PbpC
Cj0277	2,831	10,913	76	30,898	42	rod shape-determining protein MreC
Cj0525c	2,661	20,062	337	53,376	175	penicillin-binding protein PbpB
Cj1282	2,242	3,130	32	7,016	14	rod shape-determining protein RodA
Cj0276	1,533	28,345	274	43,462	82	rod shape-determining protein MreB
I. Colonisation						
Cj1222c	2,638	12,116	134	31,966	69	two-component sensor histidine kinase DccS signaling colonisation
Cj1018c	4,639	23,575	243	109,356	219	branched-chain amino acid ABC transporter substrate-binding protein LivK
Cj1019c	4,207	26,440	274	111,241	225	branched-chain amino acid ABC transporter substrate-binding protein LivJ
Cj1260c	3,705	6,239	65	23,113	47	chaperone protein DnaJ
Cj0561c	3,052	5,442	47	16,612	28	periplasmic protein- fitness in chickens
Cj0379c	2,885	55,411	460	159,848	259	sulfoxide reductase catalytic subunit
Cj0020c	1,770	55,198	469	97,687	162	cytochrome C551 peroxidase DocA
*Cj1351	1,397	3,590	33	5,016	9	phospholipase A PldA

J. Invasion						
Cj1073c	3,057	10,560	233	32,278	139	ATP-dependent protease Lon
Cj0685c	2,616	3,741	47	9,787	24	invasion protein CipA
Cj1171c	2,515	12,263	55	30,843	27	peptidyl-prolyl cis-trans isomerase Ppi
Cj1189c	2,350	47,141	218	110,794	100	bipartate energy taxis response protein CetB
Cj1190c	2,329	63,521	811	147,934	370	bipartate energy taxis response protein CetA
Cj0497	2,242	9,438	112	21,155	49	lipoprotein
Cj0914c	2,036	18,624	317	37,927	126	invasion antigen CiaB
Cj0588	1,992	2,544	18	5,069	7	hemolysin tlyA
Cj0192c	1,931	46,389	252	89,601	95	ATP-dependent protease proteolytic subunit ClpP
Cj0762c	1,524	39,302	427	59,891	127	aspartate aminotransferase AspB
*Cj1228c	1,437	88,186	1162	126,759	326	serine protease HtrA
*Cj1647	1,409	5,958	40	8,395	11	ABC transporter ATP-binding protein IamA
*Cj1646	1,067	2,328	24	2,485	5	ABC transporter permease IamB
K. Motility/Invasion						
Cj1011	2,562	2,524	18	6,466	9	CorA-like Mg2+ transporter protein
*Cj1565c	-1,012	14,149	311	13,986	60	paralysed flagellum protein PflA
L. Iron acquisition						
Cj1353	6,587	0,803	7	5,288	9	enterochelin uptake permease CeuC
Cj0173c	5,726	2,014	17	11,533	19	iron-uptake ABC transporter ATP-binding protein CfbpC
Cj1616	5,124	1,525	11	7,811	11	hemin uptake ABC transporter ATP-binding protein ChuC
Cj0174c	4,347	2,198	33	9,554	28	iron-uptake ABC transporter permease CfbpB
Cj1617	3,119	3,069	23	9,572	14	hemin uptake system substrate-binding protein ChuD
Cj1615	2,989	1,309	12	3,913	7	hemin uptake ABC transporter permease ChuB 132
Cj1354	2,846	1,282	9	3,649	5	enterochelin uptake ATP-binding protein CeuD
Cj0177	2,759	1,643	13	4,533	7	iron transport protein
Cj1397	2,684	10,051	21	26,975	11	ferrous iron transport protein FeoA
Cj1398	2,337	6,665	114	15,576	52	ferrous iron transport protein FeoB
Cj1355	2,292	8,242	76	18,892	34	enterochelin uptake substrate-binding protein CeuE
Cj0175c	2,116	59,149	552	125,174	228	iron-uptake ABC transporter substrate-binding protein CfbpA
M. N-linked glycolisation						
Cj1121c	2,868	34,969	377	100,276	211	UDP-N-acetylbacillosamine transaminase PglE
Cj1122c	2,415	11,526	70	27,841	33	integral membrane protein WlaJ
Cj1130c	2,379	5,337	84	12,695	39	protein glycosylation PglK
Cj1670c	2,242	10,395	64	23,302	28	glycoprotein CpgA
Cj1128c	2,005	5,327	46	10,679	18	GalNAc(5)-diNAcBac-PP-undecaprenol beta-1,3-glucosyltransferase pglI WlaD
Cj1120c	1,612	12,937	213	20,850	67	UDP-N-acetyl-alpha-D-glucosamine C6 dehydratase PglF
Cj1125c	1,601	6,094	64	9,757	20	N, N'-diacetylbacillosaminyl-diphospho-undecaprenol alpha-1,3-N-acetylgalactosaminyltransferase PglA
N. O-linked glycosylation						
Cj1315c	17,932	0,355	2	6,373	7	imidazole glycerol phosphate synthase subunit HisH
Cj1303	8,539	0,304	3	2,598	5	3-oxoacyl-ACP synthase FabH2

Cj1298	7,319	0,952	7	6,967	10	N-acetyltransferase
O. Colonisation/ Respiration						
Cj1571c	2,049	11,742	70	24,064	28	NADH-quinone oxidoreductase subunit I
Cj1567c	1,971	4,704	65	9,270	25	NADH-quinone oxidoreductase I subunit M NuoM
Cj1569c	1,708	4,351	12	7,431	4	NADH-quinone oxidoreductase I subunit K
Cj1570c	1,708	6,847	33	11,694	11	NADH-quinone oxidoreductase subunit J
Cj1568c	1,667	4,991	83	8,318	27	NADH-quinone oxidoreductase subunit L NuoL
Cj1573c	1,574	13,379	306	21,058	94	NADH-quinone oxidoreductase subunit G
Cj1510c	1,559	34,722	207	54,144	63	formate dehydrogenase iron-sulfur subunit FdhA
*Cj1577c	1,464	13,275	98	19,433	28	NADH-quinone oxidoreductase subunit C
P. Regulator/Signal transduction						
Cj0571	14,678	4,564	37	66,994	106	transcriptional regulator
Cj0293	6,244	4,435	32	27,694	39	stationary phase survival protein SurE
Cj1198	2,734	119,438	549	326,593	293	S-ribosylhomocysteine lyase LuxS
Cj1222c	2,638	12,116	134	31,966	69	two-component sensor histidine kinase DccS signaling colonisation
Cj1272c	2,204	16,526	337	36,432	145	guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase SpoT
Cj1262	1,733	18,035	207	31,248	70	two-component sensor histidine kinase RacS
*Cj0400	1,370	55,207	243	75,662	65	ferric uptake regulator Fur
*Cj0643	1,291	56,310	651	72,681	164	two-component response regulator CbrR
*Cj1223c	1,228	27,650	171	33,967	41	two-component regulator DccR
*Cj1261	1,139	131,247	819	149,433	182	two-component regulator RacR
Cj1230	-1,641	159,380	555	97,108	66	heat shock transcriptional regulator HspR
Cj0368c	-1,644	27,220	160	16,561	19	transcriptional regulator CmeR
Q. Restriction/Modification						
Cj0031	3,652	2,712	94	9,903	67	type IIS restriction/modification enzyme
R. Mucin degradation						
Cj0256	1,988	9,376	134	18,643	52	sulfatase family protein
Cj1344c	1,708	7,051	66	12,042	22	tRNA N6-adenosine threonylcarbamoyltransferase
S. Periplasmic proteins						
Cj1668c	12,484	18,336	71	228,905	173	periplasmic protein
Cj1637c	5,623	8,947	82	50,312	90	periplasmic protein
Cj0784	4,010	8,121	69	32,562	54	periplasmic protein
Cj0168c	2,835	252,558	394	715,965	218	periplasmic protein
Cj1380	2,800	76,488	505	214,183	276	periplasmic protein
Cj0781	2,277	35,315	243	80,417	108	quinol dehydrogenase periplasmic subunit
Cj1666c	2,255	88,266	359	199,034	158	periplasmic protein
T. Energy metabolism						
Cj1664	9,678	3,964	18	38,363	34	thiredoxin
Cj1020c	5,903	22,776	92	134,450	106	cytochrome C
Cj0147c	2,622	319,992	936	839,015	479	thioredoxin
Cj0239c	2,738	64,481	582	176,538	311	nitrogen fixation protein NifU
Cj1399c	2,339	17,372	241	40,624	110	Ni/Fe-hydrogenase small subunit HydA2

U. Protein synthesis/modification/secretion						
Cj1453c	9,820	1,338	12	13,137	23	tRNA(Ile)-lysidine synthase TilS
Cj0470	9,564	232,969	2596	2228,163	4846	elongation factor Tu
Cj0493	6,726	52,755	1017	354,812	1335	elongation factor G
Cj1105	4,845	21,871	92	105,966	87	SsrA-binding protein
Cj0856	3,900	8,498	67	33,144	51	signal peptidase I LepP
Cj0551	3,608	26,828	142	96,799	100	elongation factor P
Cj0460	3,481	30,853	312	107,412	212	transcription elongation factor NusA
Cj1181c	3,411	74,099	739	252,758	492	elongation factor Ts
Cj0518	2,748	20,218	343	55,568	184	chaperone protein HtpG
Cj1379	2,657	3,220	54	8,554	28	selenocysteine-specific elongation factor SelB
Cj1030c	2,281	9,828	164	22,414	73	elongation factor EF-4
Cj0578c	2,264	12,549	86	28,410	38	Sec-independent protein translocase TatC
Cj0757	1,841	145,483	1074	267,895	386	heat-inducible transcription repressor HrcA
V. Flagellar proteins						
Cj1179c	7,173	2,804	20	20,116	28	flagellar biosynthesis protein FliR
Cj0335	6,904	37,281	377	257,383	508	flagellar biosynthesis protein FlhB
Cj1340c	5,124	0,296	1	1,517	3	motility protein
Cj0336c	4,534	23,883	165	108,274	146	flagellar motor protein MotB
Cj1024c	4,262	19,189	232	81,788	193	sigma54 associated transcriptional activator FlgR
Cj0882c	3,697	8,714	176	32,217	127	flagellar biosynthesis protein FlhA
Cj1332	3,416	0,838	6	2,863	4	flagellin modification protein ptmA
Cj0109	3,240	13,194	68	42,748	43	MotA/TolQ/ExbB proton channel family protein ExbB3
Cj1408	2,590	54,346	271	140,764	137	flagellar basal body-associated protein FliL
Cj0337c	2,586	43,381	313	112,197	158	flagellar motor protein MotA
Cj0195	2,508	7,459	96	18,710	47	flagellum-specific ATP synthase FliI
Cj0059c	2,189	27,210	213	59,561	91	flagellar motor switch protein FliY
Cj0670	2,021	6,112	71	12,349	28	RNA polymerase factor sigma-54
Cj0820c	1,983	13,626	93	27,025	36	flagellar biosynthesis protein FliP
Cj0769c	1,590	9,421	58	14,980	18	flagellar basal body P-ring biosynthesis protein FlgA
Cj0549	1,556	68,732	247	106,929	75	flagellar protein FliS
*Cj0318	1,438	21,883	342	31,473	96	flagellar MS-ring protein fliF
*Cj0064c	1,330	15,395	208	20,477	54	flagellar biosynthesis regulator FlhF
*Cj1675	1,250	32,706	82	40,871	20	flagellar biosynthesis protein FliQ
*Cj0061c	1,233	121,057	806	149,289	194	flagellar biosynthesis RNA polymerase sigma factor FliA
*Cj0526c	1,203	466,293	1286	561,042	302	flagellar hook-basal body protein FliE
*Cj0351	1,156	46,352	133	53,568	30	flagellar motor switch protein fliN
*Cj0060c	1,141	43,874	440	50,066	98	flagellar motor switch protein FliM
*Cj0528c	1,040	407,825	1636	424,032	332	flagellar basal body rod protein FlgB
*Cj0527c	1,022	740,557	3404	756,849	679	flagellar basal body rod protein FlgC
*Cj0793	1,009	33,785	320	34,079	63	signal transduction histidine kinase flgS
*Cj1338c	-1,009	364,792	4019	361,416	757	flagellin FlaB
*Cj0320	-1,038	13,089	101	12,615	19	flagellar assembly protein FliH
*Cj1075	-1,041	216,484	784	207,968	147	flagellar assembly protein FliW

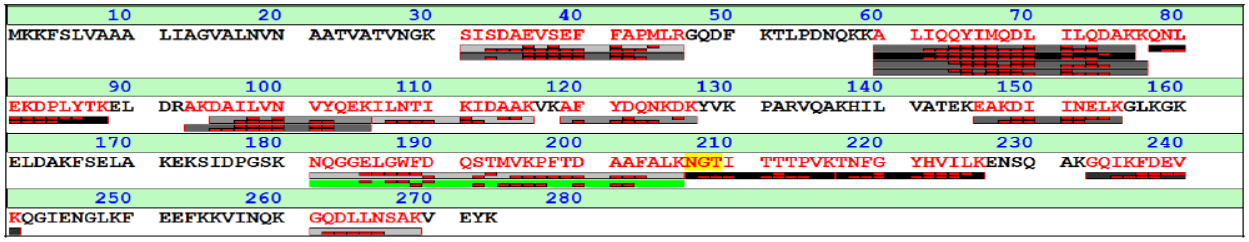
*Cj0063c	-1,093	13,911	112	12,728	20	ATP-binding protein FlhG
*Cj0720c	-1,101	540,458	3764	490,692	667	flagellin C FlaC
*Cj1464	-1,133	1379,842	2537	1217,758	437	flagellar biosynthesis protein FlgM
*Cj0698	-1,188	541,168	3980	455,614	654	flagellar basal body rod protein FlgG
*Cj0697	-1,266	983,777	7427	777,069	1145	flagellar basal-body rod protein FlgG2
*Cj0041	-1,350	106,132	1771	78,603	256	flagellar hook-length control protein FliK
*Cj0548	-1,354	546,766	9793	403,875	1412	flagellar hook-associated protein FliD
*Cj1729c	-1,423	197,928	4775	139,106	655	flagellar hook protein FlgE
Cj1466	-1,507	290,768	4933	192,978	639	flagellar hook-associated protein FlgK
Cj1462	-1,520	551,099	5358	362,565	688	flagellar basal body P-ring protein FlgI
Cj0887c	-1,657	377,463	7897	227,754	930	flagellin FlgL
Cj0043	-1,750	221,033	3362	126,317	375	flagellar hook protein FlgE
Cj1001	-1,794	113,855	1976	63,471	215	RNA polymerase sigma factor RpoD
Cj1339c	-1,875	1076,082	11953	573,900	1209	flagellin A FlaA
Cj0042	-1,932	887,679	7295	459,482	737	flagellar hook assembly protein
Cj0042	-1,932	887,679	7295	459,482	737	flagellar hook assembly protein
Cj0687c	-1,951	938,086	6089	480,712	609	flagellar basal body L-ring protein FlgI
Cj0319	-1,981	28,675	274	14,477	27	flagellar motor switch protein fliG
Cj1463	-2,177	161,534	513	74,212	46	flagellar biosynthesis protein FlgJ
Cj0547	-2,247	1940,768	6596	863,810	573	flagellar protein FlaG
W. Hypothetical proteins						
Cj1418c	81,977	0,046	1	3,773	16	hypothetical protein - capsule
Cj0008	66,606	0,061	1	4,094	13	conserved hypothetical protein
Cj1145c	46,112	0,127	1	5,863	9	hypothetical protein - LOS/ Invasion
Cj0752	35,865	0,261	3	9,359	21	pseudogene (IS element)
Cj0305c	27,326	0,528	3	14,425	16	hypothetical protein - unknown function
Cj1322	15,371	0,310	2	4,763	6	hypothetical protein - O-linked glycosylation
Cj0030	13,508	2,542	33	34,337	87	hypothetical protein
Cj0170	10,247	0,434	3	4,444	6	hypothetical protein - colonisation
Cj0988c	10,247	0,909	2	9,312	4	hypothetical protein
Cj1555c	10,247	0,169	1	1,735	2	hypothetical protein - Invasion
Cj0046	7,416	2,692	38	19,965	55	hypothetical protein - transport/binding protein
Cj0522	6,742	1,243	19	8,380	25	hypothetical protein - unknown fucntion
Cj0286c	6,569	6,796	39	44,640	50	hypothetical protein - chemotaxis?
Cj0568	6,262	0,838	9	5,246	11	hypothetical protein
Cj0742	5,518	0,827	13	4,563	14	hypothetical protein - membrane protein
Cj1340c	5,124	0,296	1	1,517	3	hypothetical protein - O-linked glycosylation
Cj0939c	4,919	14,132	50	69,512	48	hypothetical protein
Cj0706	4,747	20,426	136	96,961	126	conserved hypothetical protein
Cj1069	4,729	1,615	13	7,637	12	hypothetical protein - VirK
Cj0705	4,315	2,818	19	12,160	16	conserved hypothetical protein
Cj1392	3,605	2,500	27	9,014	19	hypothetical protein - Putative cystathionine beta-lyase
Cj0125c	3,586	5,933	20	21,280	14	hypothetical protein - DksA
Cj1633	3,367	3,830	35	12,897	23	putative ATP-binding protein

Cj0621	3,210	5,877	83	18,863	52	hypothetical protein
Cj0427	3,151	64,101	200	201,981	123	hypothetical protein
Cj1497c	3,054	13,053	52	39,870	31	hypothetical protein - motility FliJ
Cj0444	2,996	6,133	118	18,374	69	hypothetical protein - colonisation CfrB
Cj0380c	2,966	2,603	19	7,722	11	hypothetical protein
Cj0760	2,836	17,685	168	50,159	93	hypothetical protein
Cj1533c	2,829	20,853	201	59,002	111	hypothetical protein - helix-turn-helix domain
Cj1449c	2,650	7,654	29	20,285	15	hypothetical protein
Cj1627c	2,641	14,040	97	37,080	50	hypothetical protein
Cj0248	2,591	33,010	263	85,528	133	hypothetical protein
Cj0984	2,562	3,779	26	9,680	13	hypothetical protein
Cj1132c	2,365	5,283	39	12,493	18	hypothetical protein - LOS/ Invasion
Cj0881c	2,145	4,423	43	9,486	18	hypothetical protein
Cj0428	2,064	365,416	1303	754,350	525	hypothetical protein - motility a
Cj1384c	2,049	8,547	25	17,516	10	hypothetical protein
Cj0617	1,971	1,139	13	2,245	5	hypothetical protein - poly G tract-colonisation
Cj0676	1,966	5,589	86	10,988	33	putative KdpA
Cj0993c	1,951	43,120	197	84,109	75	hypothetical protein
X. Other protein coding ORFs						
Cj0636	6,404	1,538	12	9,853	15	NOL1/NOP2/sun family protein
Cj0275	3,621	11,422	133	41,359	94	ATP-dependent protease ATP-binding subunit ClpX
Cj1461	2,365	8,187	52	19,360	24	DNA methylase
Cj1521c	2,074	10,470	42	21,713	17	CRISPR-associated endoribonuclease Cas2
Cj0891c	1,508	18,016	265	27,170	78	D-3-phosphoglycerate dehydrogenase SerA

A/6 - Proteins identified by LC/MS analysis

Protein 1: **Putative peptidyl-prolyl cis-trans isomerase Cbf2 OS=Campylobacter jejuni subsp. jejuni serotype O:23/36 (strain 81-176) GN=cbf2 PE=4 SV=1**

Accession:	CBF2_CAMJJ	**Score:**	568.0
Database:	SwissProt(SwissProt_2014_02.fasta)	**MW [kDa]:**	30.4
Database Date:	2014-03-02	**pI:**	9.7
Modification(s):	Oxidation	**Sequence Coverage [%]:**	55.7
		No. of unique Peptides:	18



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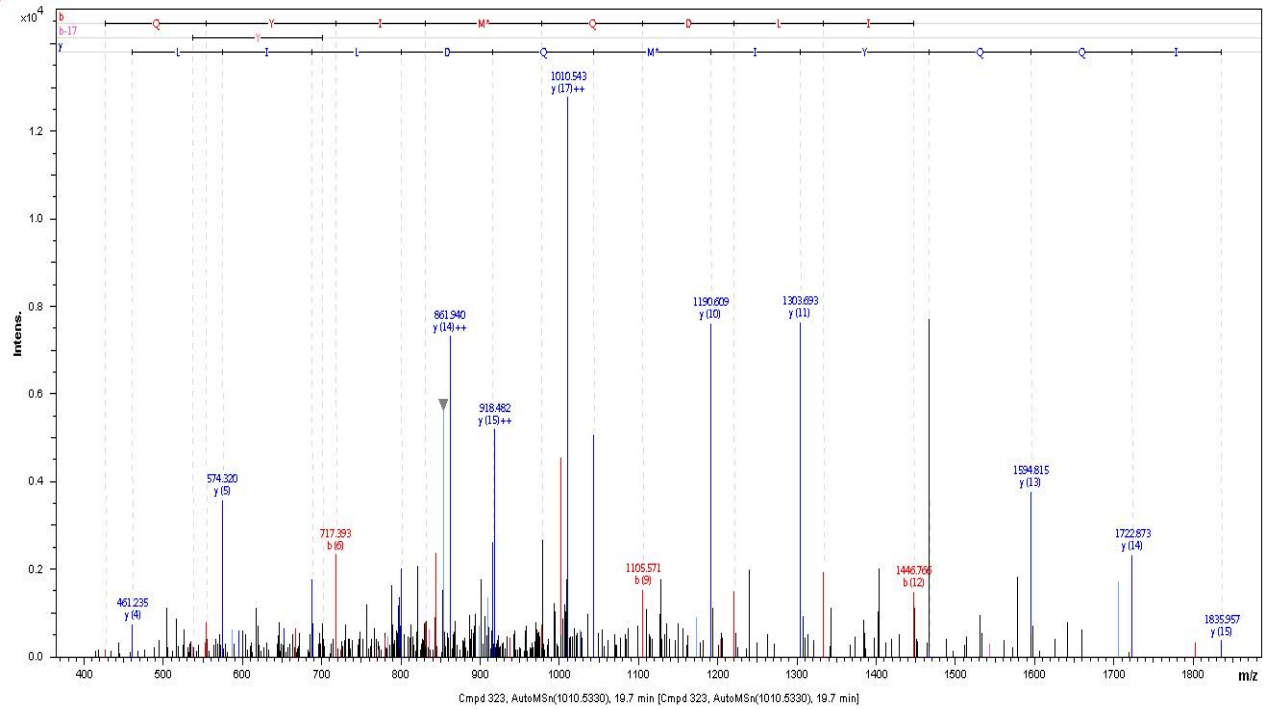
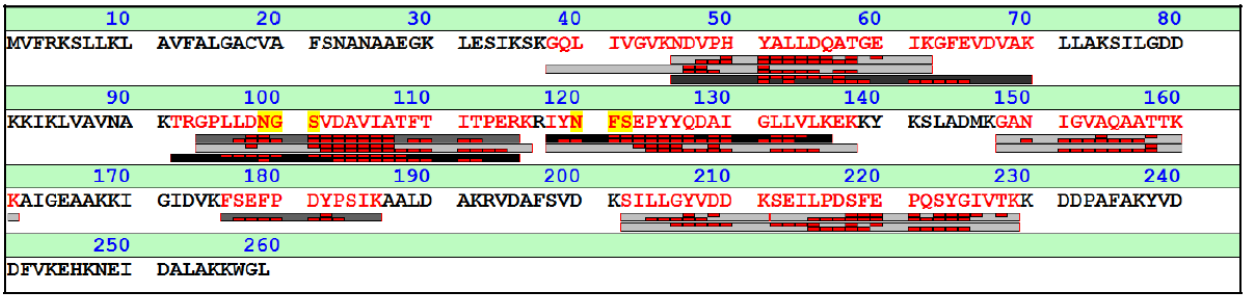
356	1	899.9285	-10.14	2	21.5	76.2	0	31-46	K.SISDAEVSEFFAPMLR.G	
320	1	907.9264	-9.56	2	19.6	68.1	0	31-46	K.SISDAEVSEFFAPMLR.G	Oxidation: 14
349	1	1002.5357	-9.63	2	21.2	78.2	0	60-76	K.ALIQQYIMQDLILQDAK.K	
323	1	1010.5330	-9.70	2	19.7	88.0	0	60-76	K.ALIQQYIMQDLILQDAK.K	Oxidation: 8
333	1	711.3906	-9.88	3	20.2	43.4	1	60-77	K.ALIQQYIMQDLILQDAKK.Q	
308	1	716.7228	-8.99	3	18.9	50.4	1	60-77	K.ALIQQYIMQDLILQDAKK.Q	Oxidation: 8
233	1	674.8529	-9.01	2	14.8	22.1	1	78-88	K.QNLEKDPLYTK.E	
247	1	745.9068	-10.01	2	15.5	64.5	1	93-105	R.AKDAILVNVYQEK.I	
267	1	646.3420	-9.72	2	16.6	34.0	0	95-105	K.DAILVNVYQEK.I	
239	1	600.3655	-9.99	2	15.0	27.1	1	106-116	K.ILNTIKIDAAK.V	
221	1	564.7640	-10.00	2	13.9	30.0	1	119-127	K.AFYDQNKDK.Y	
248	1	586.8237	-10.28	2	15.6	23.5	1	146-155	K.EAKDIINELK.G	
348	1	953.7901	340.53	3	21.1	40.3	0	181-206	K.NQGGELGWFDQSTMVKPFTDAAFALK.N	
332	1	958.7874	-10.10	3	20.2	39.3	0	181-206	K.NQGGELGWFDQSTMVKPFTDAAFALK.N	Oxidation: 14
223	1	516.7791	946.98	2	14.1	51.5	0	207-216	K.NGTITTTPVK.T	
250	1	596.3238	-9.87	2	15.7	16.4	0	217-226	K.TNFGYHVILK.E	
229	1	532.2875	-9.82	2	14.5	20.2	1	233-241	K.GQIKFDEVK.Q	
228	1	473.2490	-9.76	2	14.4	26.2	0	261-269	K.GQDLLNSAK.V	

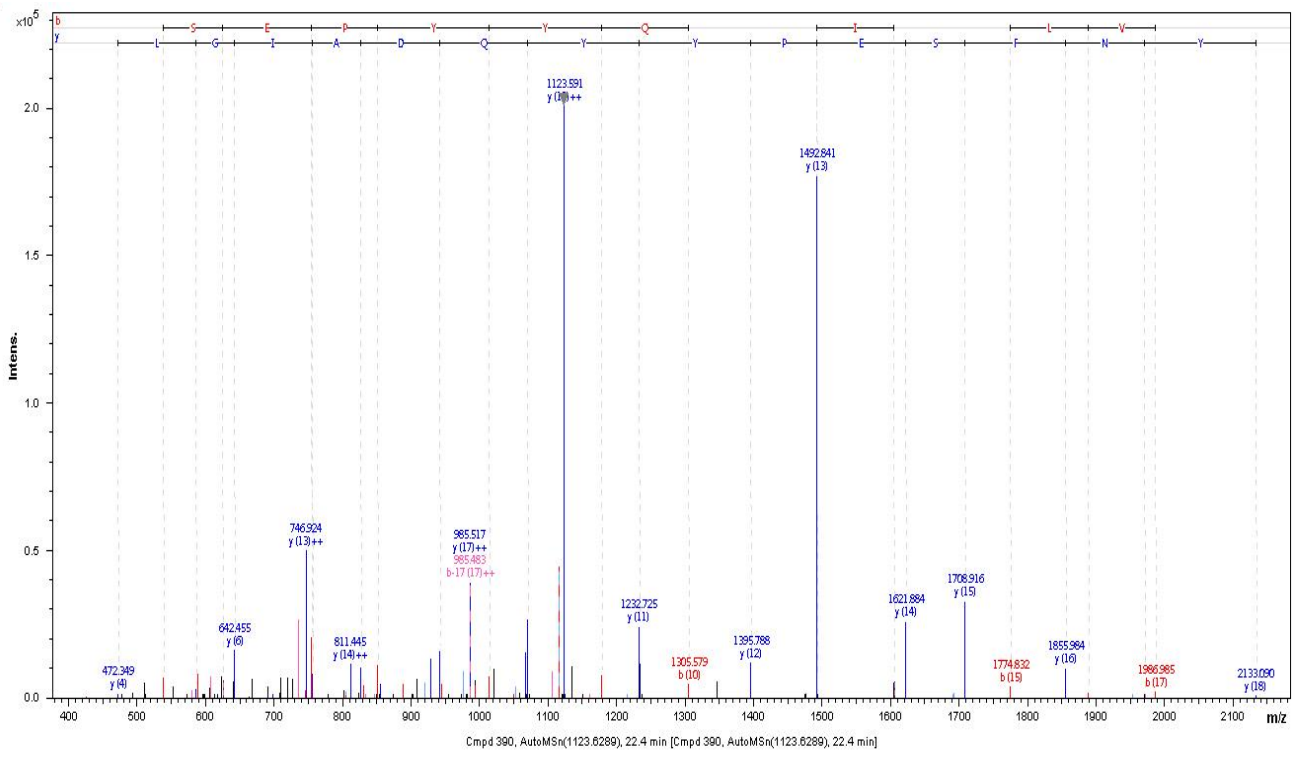


Protein 1: **Major cell-binding factor OS=Campylobacter jejuni subsp. jejuni serotype O:2 (strain NCTC 11168) GN=peb1A PE=1 SV=1**

Accession:	PEB1A_CAMJE	**Score:**	964.1
Database:	SwissProt(SwissProt_2014_02.fasta)	**MW [kDa]:**	28.2
Database Date:	2014-03-02	**pI:**	9.4
		Sequence Coverage [%]:	51.0
		No. of unique Peptides:	14



Cmpd.	No. of Cmpds.	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	P	Range	Sequence	Modification
318	1	893.5178	32.71	3	18.3	39.8	1	38-62	K.GQLIVGVKNDVPHYALLDQATGEIK.G	
305	1	942.5096	32.93	2	17.6	67.6	0	46-62	K.NDVPHYALLDQATGEIK.G	
350	1	910.4953	34.12	3	20.0	70.3	1	46-70	K.NDVPHYALLDQATGEIKGFEVDVAK.L	
372	1	848.4833	34.91	3	21.3	100.8	1	92-115	K.TRGPLLDNGSVDAVIATFTITPER.K	
389	1	1143.6405	33.31	2	22.3	121.6	0	94-115	R.GPLLDNGSVDAVIATFTITPER.K	
379	1	805.4622	32.84	3	21.5	61.0	1	94-116	R.GPLLDNGSVDAVIATFTITPERK.R	
390	1	1123.6289	33.92	2	22.4	105.8	0	118-136	R.IYNFSEPYYQDAIGLLVLK.E	
371	1	835.1371	33.91	3	21.2	64.5	1	118-138	R.IYNFSEPYYQDAIGLLVLKEK.K	
252	1	601.3492	31.18	2	14.3	60.8	0	148-160	K.GANIGVAQAATTK.K	
248	1	665.3971	28.93	2	14.1	40.3	1	148-161	K.GANIGVAQAATTKK.A	
307	1	665.3447	34.58	2	17.7	69.2	0	176-186	K.FSEFPDYPSIK.A	
311	1	561.8235	31.65	2	17.9	16.3	0	202-211	K.SILLGYVDDK.S	
355	1	1038.5720	34.79	3	20.4	87.3	1	202-229	K.SILLGYVDDKSEILPDSFEPQSYGIVTK.K	
330	1	1005.5403	33.21	2	19.0	60.0	0	212-229	K.SEILPDSFEPQSYGIVTK.K	

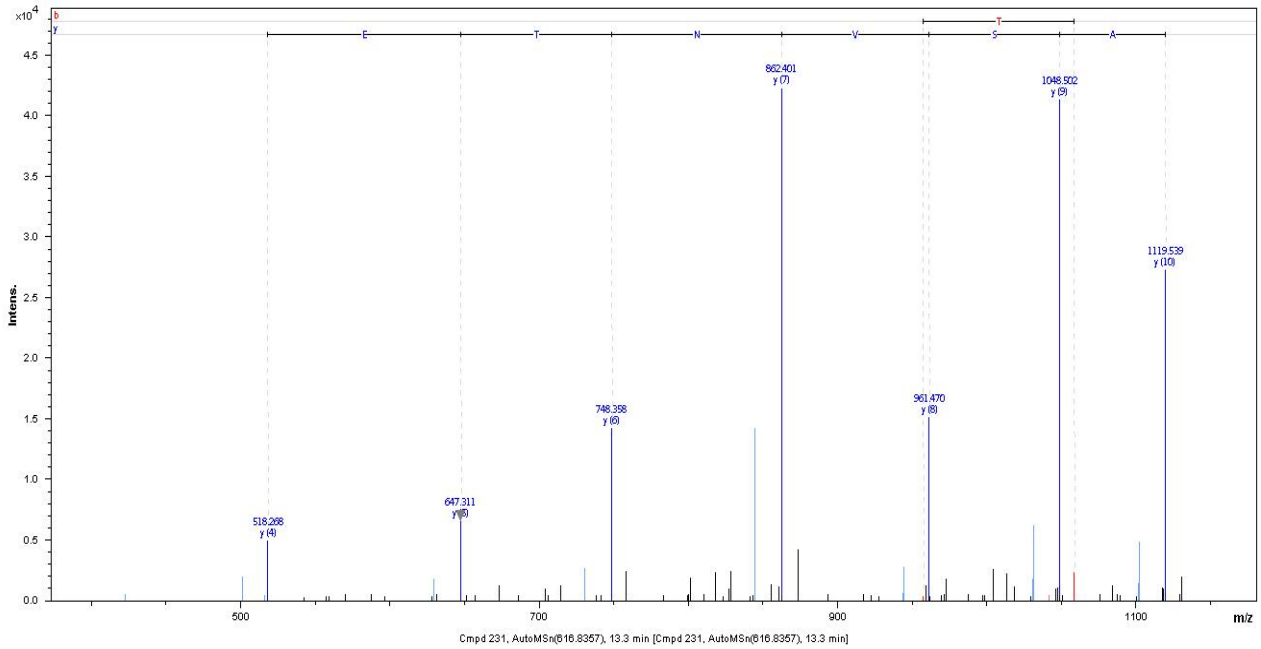


Protein 1: **hypothetical protein CJE0469 [Campylobacter jejuni RM1221] >gi|218562078|ref|YP_002343857.1| hypothetical protein Cj0420 [Campylobacter jejuni subsp. jejuni NCTC 11168 = ATCC 700819] >gi|315123975|ref|YP_004065979.1| periplasmic protein [Campylobacter jejuni subsp. jejuni ICDCCJ07001] >gi|384442755|ref|YP_005659007.1| Putative periplasmic protein [Campylobacter jejuni subsp. jejuni S3] >gi|384447707|ref|YP_005655758.1| hypothetical protein CJSA_0392 [Campylobacter jejuni subsp. jejuni IA3902] >gi|403055201|ref|YP_006632606.1| hypothetical protein BN148_0420 [Campylobacter jejuni subsp. jejuni NCTC 11168-BN148] >gi|407941867|ref|YP_006857507.1| hypothetical protein A911_02045 [Campylobacter jejuni subsp. jejuni PT14] >gi|525912725|ref|YP_008294194.1| hypothetical protein M635_06415 [Campylobacter jejuni 32488] >gi|543945861|ref|YP_008538365.1| periplasmic protein [Campylobacter jejuni subsp. jejuni 00-2538] >gi|543947628|ref|YP_008540029.1| periplasmic protein [Campylobacter jejuni subs**

Accession:	gi|57237475	**Score:**	201.6
Database:	NCBInr(NCBInr_20140220.fasta)	**MW [kDa]:**	21.0
Database Date:	2014-03-03	**pI:**	9.4
Modification(s):	Oxidation	**Sequence Coverage [%]:**	33.2
		No. of unique Peptides:	5

```
        10         20         30         40         50         60         70         80
MKKVLLSSLV AVSLLSTGLF AKEYTLDKAH TDVGFKIKHL QISNVKGNFK DYSAVIDFDP ASAEFKKLDV TIKIASVNTE
        90        100        110        120        130        140        150        160
NQTRDNHLQQ DDFFKAKKYP DMTFTMKKYE KIDNEKGKMT GTLTIAGVSK DIVLDAEIGG VAKGKDGKEK IGFSLNGKIK
       170        180        190        200
RSDFKFATST STITLSDDIN LNIEVEANEK
```

Cmpd.	No. of Cmpds.	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	P	Range	Sequence	Modification
368	1	887.9407	32.15	2	19.9	49.4	0	51-66	K.DYSAVIDFDPASAEFK.K	
231	1	616.8357	33.40	2	13.3	59.3	0	74-84	K.IASVNTENQTR.D	
290	1	703.8436	32.91	2	16.3	41.4	0	85-95	R.DNHLQQDDFFK.A	
274	1	597.8424	31.51	2	15.4	34.1	0	119-130	K.MTGTLTIAGVSK.D	Oxidation: 1
379	1	825.8105	32.11	3	20.5	17.3	1	119-143	K.MTGTLTIAGVSKDIVLDAEIGGVAK.G	Oxidation: 1

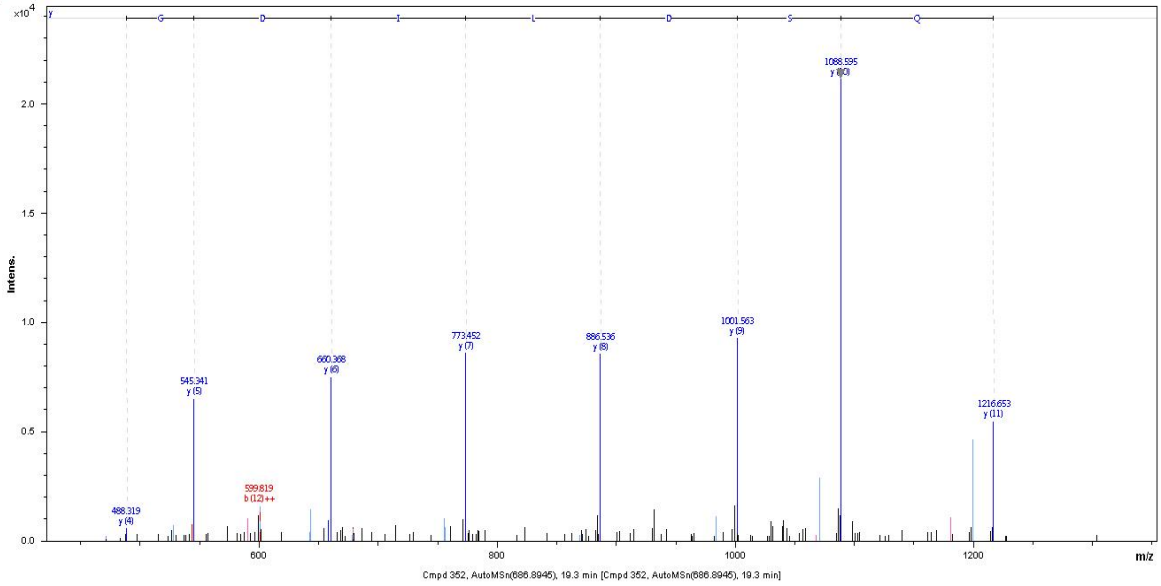


Protein 1: **serine protease [Campylobacter jejuni subsp. jejuni NCTC 11168 = ATCC 700819] >gi|384448471|ref|YP_005656522.1| serine protease (protease DO) [Campylobacter jejuni subsp. jejuni IA3902] >gi|403055963|ref|YP_006633368.1| serine protease [Campylobacter jejuni subsp. jejuni NCTC 11168-BN148] >gi|543946619|ref|YP_008539123.1| serine protease [Campylobacter jejuni subsp. jejuni 00-2538] >gi|543948400|ref|YP_008540801.1| serine protease [Campylobacter jejuni subsp. jejuni 00-2426] >gi|543950179|ref|YP_008542523.1| serine protease [Campylobacter jejuni subsp. jejuni 00-2544] >gi|544062837|ref|YP_008562542.1| serine protease [Campylobacter jejuni subsp. jejuni 00-2425] >gi|488942160|ref|WP_002853235.1| serine protease [Campylobacter jejuni] >gi|85838958|gb|EAQ56224.1| protease DO [Campylobacter jejuni subsp. jejuni CF93-6] >gi|88191191|gb|EAQ95163.1| protease DO [Campylobacter jejuni subsp. jejuni 84-25] >gi|112360546|emb|CAL35343.1| serine protease (protease DO) [Campylobacter jejuni subsp. j**

Accession:	gi|218562840	**Score:**	555.0
Database:	NCBInr(NCBInr_20140220.fasta)	**MW [kDa]:**	51.0
Database Date:	2014-03-03	**pI:**	9.3
		Sequence Coverage [%]:	30.1
		No. of unique Peptides:	13

10	20	30	40	50	60	70	80
MKKIFLSLSL	ASALFAASIN	FNESTATANR	VNPAAGNAVL	SYHDSIKDAK	KSVVNISTSK	TITRANRPSP	LDDFFNDPYF
90	**100**	**110**	**120**	**130**	**140**	**150**	**160**
KQFFDFDFSQ	RKGKNDKEVV	SSLGSGVIIS	KDGYIVTNNH	VVDDADTITV	NLPGSDIEYK	AKLIGKDPKT	DLAVIKIEAN
170	**180**	**190**	**200**	**210**	**220**	**230**	**240**
NLSAITFTNS	DDLMEGDVVF	ALGNPFGVGF	SVTSGIISAL	NKDNIGLNQY	ENFIQTDASI	NPGNSGGALV	DSRGYLVGIN
250	**260**	**270**	**280**	**290**	**300**	**310**	**320**
SAILSRGGGN	NGIGFAIPSN	MVKDIAKKLI	EKGKIDRGFL	GVTILALQGD	TKKAYKNQEG	ALITDVQKGS	SADEAGLKRG
330	**340**	**350**	**360**	**370**	**380**	**390**	**400**
DLVTKVNDKV	IKSPIDLKNY	IGTLEIGQKI	SLSYERDGEN	KQASFILKGE	KENPKGVQSD	LIDGLSLRNL	DPRLKDRLQI
410	**420**	**430**	**440**	**450**	**460**	**470**	**480**
PKDVNGVLVD	SVKEKSKGKN	SGFQEGDIII	GVGQSEIKNL	KDLEQALKQV	NKKEFTKVWV	YRNGFATLLV	LK

Cmpd.	No. of Cmpds.	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	P	Range	Sequence	Modification
374	1	681.6776	28.19	3	20.4	60.8	0	65-81	R.ANRPSPLDDFFNDPYFK.Q	
424	1	841.4160	625.48	4	23.4	49.0	1	65-91	R.ANRPSPLDDFFNDPYFKQFFDFDFSQR.K	
375	1	668.8201	27.79	2	20.4	21.7	0	82-91	K.QFFDFDFSQR.K	
347	1	681.9105	29.16	2	19.0	61.3	0	234-246	R.GYLVGINSAILSR.G	
391	1	766.9601	29.09	2	21.4	57.4	0	278-292	R.GFLGVTILALQGDTK.K	
376	1	554.3414	27.82	3	20.5	61.2	1	278-293	R.GFLGVTILALQGDTKK.A	
290	1	658.3634	26.02	2	15.6	42.2	0	297-308	K.NQEGALITDVQK.G	
264	1	545.7939	29.06	2	14.3	20.8	1	309-319	K.GSSADEAGLKR.G	
313	1	618.3519	27.02	2	17.0	19.8	0	339-349	K.NYIGTLEIGQK.I	
352	1	686.8945	28.12	2	19.3	66.8	0	376-388	K.GVQSDLIDGLSLR.N	
345	1	664.3596	28.17	3	18.8	18.3	0	420-438	K.NSGFQEGDIIIGVGQSEIK.N	
315	1	586.3522	24.65	2	17.2	48.2	1	439-448	K.NLKDLEQALK.Q	
392	2	538.8364	942.21	2	21.4	28.8	0	463-472	R.NGFATLLVLK.-	

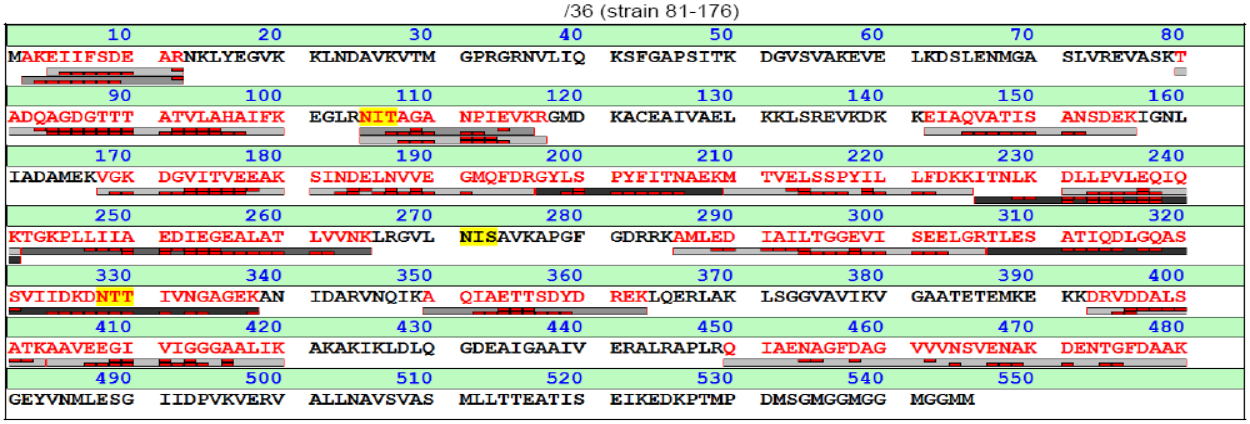


Protein 1: **60 kDa chaperonin - Campylobacter jejuni**

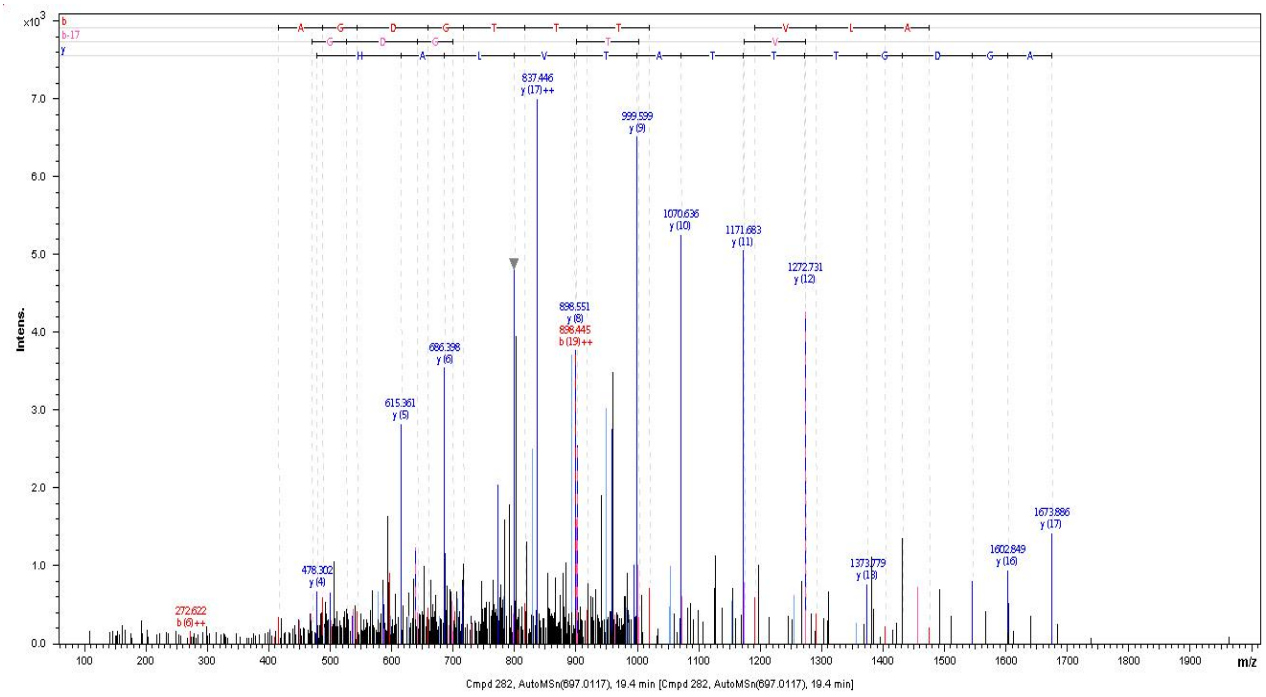
Accession:	CH60_CAMJE	**Score:**	694.6
Database:	SwissProt(SwissProt_55.3.fasta)	**MW [kDa]:**	57.9
Database Date:	2008-09-07	**pI:**	4.9
Modification(s):	Oxidation	**Sequence Coverage [%]:**	52.1
		No. of unique Peptides:	19

Alias proteins:

Accession	Name	Description
CH60_CAMJJ	SwissProt(SwissProt_55.3.fasta)	60 kDa chaperonin - Campylobacter jejuni subsp. jejuni serotype O:23 /36 (strain 81-176)



Cmpd.	No. of Cmpds.	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	P	Range	Sequence	Modification
208	1	639.8277	-16.14	2	14.9	52.3	1	2-12	M.AKEIIFSDEAR.N	
226	1	540.2634	-15.90	2	16.1	22.5	0	4-12	K.EIIFSDEAR.N	
282	1	697.0117	-16.92	3	19.4	84.8	0	80-100	K.TADQAGDGTTTATVLAHAIFK.E	
211	1	613.8300	-17.35	2	15.2	27.8	0	105-116	R.NITAGANPIEVK.R	
199	1	461.5878	-18.96	3	14.4	41.3	1	105-117	R.NITAGANPIEVKR.G	
206	1	788.3833	-16.94	2	14.8	28.7	0	142-156	K.EIAQVATISANSDEK.I	
192	1	672.8614	-15.92	2	14.1	41.9	1	168-180	K.VGKDGVITVEEAK.S	
255	1	941.4199	-17.05	2	17.9	38.4	0	181-196	K.SINDELNVVEGMQFDR.G	Oxidation: 12
269	1	751.8674	-16.66	2	18.7	52.5	0	197-209	R.GYLSPYFITNAEK.M	
293	1	633.9980	-18.03	3	20.2	18.1	1	210-225	K.MTVELSSPYILLFDKK.I	Oxidation: 1
309	1	622.3646	-16.79	3	21.0	40.0	1	226-241	K.ITNLKDLLPVLEQIQK.T	
296	1	648.3707	-17.59	2	20.4	15.0	0	231-241	K.DLLPVLEQIQK.T	
362	1	836.4686	-16.71	3	23.8	55.1	0	242-265	K.TGKPLLIIAEDIEGEALATLVVNK.L	
330	1	744.7062	-17.31	3	22.1	34.7	0	286-306	K.AMLEDIAILTGGEVISEELGR.T	Oxidation: 2
291	1	822.9112	-17.49	4	20.0	20.5	1	307-338	R.TLESATIQDLGQASSVIIDKDNTTIVNGAGEK.A	
183	1	813.8699	-17.19	2	13.5	58.6	1	350-363	K.AQIAETTSDYDREK.L	
191	1	595.7938	-17.57	2	14.0	24.5	1	393-403	K.DRVDDALSATK.A	
266	1	784.4439	-15.81	2	18.5	16.0	0	404-420	K.AAVEEGIVIGGGAALIK.A	
265	1	1060.8234	-16.46	3	18.5	25.5	1	450-480	R.QIAENAGFDAGVVVNSVENAKDENTGFDAAK.G	

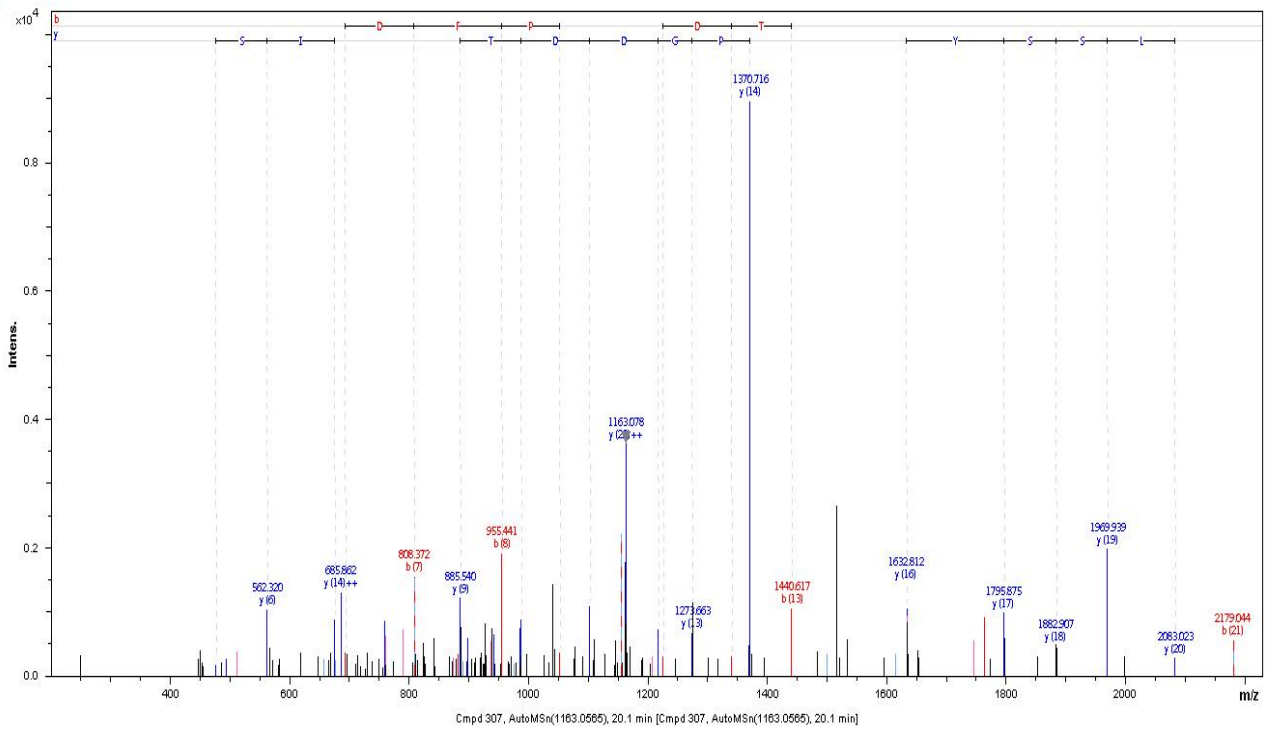

x10^3
Intens.
m/z
b
b-17
y
A G D G T T T V L A
G D G T V
H A L V T A T T T G D G A
837.446
y (17)++
999.599
y (9)
1070.636
y (10)
1171.683
y (11)
1272.731
y (12)
898.551
y (8)
896.445
b (19)++
686.398
y (6)
615.361
y (5)
478.302
y (4)
272.622
b (6)++
1373.779
y (13)
1602.849
y (16)
1673.886
y (17)
Cmpd 282, AutoMSn(897.0117), 19.4 min [Cmpd 282, AutoMSn(897.0117), 19.4 min]

Protein 1: **elongation factor Tu [Campylobacter jejuni]**

Accession:	gi|488955902	**Score:**	269.5
Database:	NCBInr(NCBInr_2013_06.fasta)	**MW [kDa]:**	44.9
Database Date:	2013-06-13	**pI:**	5.3
Modification(s):	Oxidation	**Sequence Coverage [%]:**	22.7
		No. of unique Peptides:	8

10	20	30	40	50	60	70	80
MLNLFFIRRK	KWLKKISRNK	PHVNIGTIGH	VDHGKTTLTA	AISAVLSRRG	LAELKDYDNI	DNAPEEKERG	ITIATSHIEY
90	**100**	**110**	**120**	**130**	**140**	**150**	**160**
ETDNRHYAHV	DCPGHADYVK	NMITGAAQMD	GAILVVSAAD	GPMPQTREHI	LLSRQVGVPY	IVVFMNKADM	VDDAELLELV
170	**180**	**190**	**200**	**210**	**220**	**230**	**240**
EMEIRELLSS	YDFPGDDTPI	ISGSALKALE	EAKAGQDGEW	SAKIMDLMAA	VDSYIPTPTR	DTEKDFLMPI	EDVFSISGRG
250	**260**	**270**	**280**	**290**	**300**	**310**	**320**
TVVTGRIEKG	VVKVGDTIEI	VGIKDTQTTT	VTGVEMFRKE	MDQGEAGDNV	GVLLRGTKKE	EVIRGMVLAK	PKSITPHTDF
330	**340**	**350**	**360**	**370**	**380**	**390**	**400**
EAEVYILNKD	EGGRHTPFFN	NYRPQFYVRT	TDVTGSIKLA	DGVEMVMPGE	NVKITVSLIA	PVALEEGTRF	AIREGGKTVG
410							
SGVVSKIIK							

Cmpd.	No. of Cmpds.	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	P	Range	Sequence	Modification
330	1	652.3697	-19.84	2	21.3	54.9	0	36-48	K.TTLTAAISAVLSR.R	
318	1	487.6170	668.60	3	20.7	23.9	1	36-49	K.TTLTAAISAVLSRR.G	
194	1	569.9095	-18.58	3	13.9	21.4	1	56-69	K.DYDNIDNAPEEKER.G	
307	1	1163.0565	-18.89	2	20.1	77.7	0	166-187	R.ELLSSYDFPGDDTPIISGSALK.A	
348	1	733.6781	-19.98	3	22.4	15.8	1	221-239	R.DTEKDFLMPIEDVFSISGR.G	
340	1	739.0093	-20.51	3	22.0	33.5	1	221-239	R.DTEKDFLMPIEDVFSISGR.G	Oxidation: 8
191	1	461.2389	-19.65	2	13.8	40.4	0	350-358	R.TTDVTGSIK.L	
204	1	824.8711	-19.35	2	14.6	17.8	0	359-373	K.LADGVEMVMPGENVR.I	Oxidation: 7, 9



A/7 - Genes and primers selected for RT-PCR

Primer sequences [1] were designed at this study

Gene function	*Gene*	Primer	Sequence(5'-3') [1]
Oxidative stress response genes Peroxide sensing regulator			
DNA protection during starvation protein	*dps*	Forward	CTCTATTGTCTTGATTAGGA
		Reverse	AATCTTGCGACAAATCCAAA
superoxide dismutase	*sodB*	Forward	TGGCGGTTCATGTCAAAGTA
		Reverse	ACCAAAACCATCCTGAACCA
alkyl hydroperoxide reductase	*ahpC*	Forward	CTTGCTTGATGCTGATGGAA
		Reverse	GGGTTAGCTTTCATACCTTC
catalase	*katA*	Forward	ATCCTGATGCACAAAGATAT
		Reverse	GACTAAATCAGGTTCAAGAT
General stress response genes Pobable thiol peroxidase			
co-chaperonin	*groES*	Forward	AACCTTTAGGAAAGCGTGTT
		Reverse	CACCATTTGCAATATCAGTG
molecular chaperone	*groEL*	Forward	CTATGCTTGAAGATATAGCG
		Reverse	CTAGAAGCTTGTCCAAGATC
chaperone	*dnaK*	Forward	CTCTACTCGTGTTCCTTTAGT
		Reverse	CTAGCATCTACAGCTTCTTTG
chemotaxis regulatory protein	*cheY*	Forward	AACTTGTGGAGTAAAAGGTT
		Reverse	AGATTGGAATATGCCAGAAA
Virulence factors and toxins			
flagellin	*flaB*	Forward	CATATCAGCATTGATAAAGG
		Reverse	TATGAAGATGGTGATGGAAA
flagellar biosynthesis protein	*flhB*	Forward	AATTTATGCAAGAGCTTCCA
		Reverse	TACGAATTCTACCTTTAACC
flagellar basal body rod protein	*flgB*	Forward	AGAGCTGTTATCATAACTGT
		Reverse	TGGTAAATCGTGCAAATGAA
outer membrane fibronectin-binding protein	*cadF*	Forward	TCAAGTTCATTAGCAACACT
		Reverse	TTGGAAGGTCATTTTGGTTT
periplasmic cytochrome C peroxidase	*docA*	Forward	TAAATCTTCTTGGTTTGGGT
		Reverse	TGCAGTTGCTGAATTTGAAA
methyl-accepting chemotaxis proreins (MCPs)	*docB*	Forward	GTTGTCATTGATTTGTTGGA
		Reverse	CCAAAGTGCGGATAATATTA
methyl-accepting chemotaxis proreins (MCPs)	*docC*	Forward	AAGGCAAGAAATTCTTTAGC
		Reverse	CCATTTGGTTGAGCAATATA
cytolethal distending toxin	*cdtB*	Forward	TCATTTCCATTGCGAATTCC
		Reverse	GGAATTTAGGAACTCTTTC
β-1,3 galactosyltransferases to synthesize ganglioside mimics	*wlaN*	Forward	TGCTGGGTATACAAAGGTTGTG
		Reverse	AATTTTGGATATGGGTGGGG
flagellar hook protein	*flgE2*	Forward	CATCTCACCACGACCTCCTGTTC
		Reverse	GCAAAAATCGCAATGGCTTCA
LPS sytnhesis genes			
UDP-glucose 4-epimerase	*galE*	Forward	GCTTCAATCACTTCTTTTAC
		Reverse	TTTGGCGATGATTATGATAC
ABC transporter, permease/ATP-binding protein	*wlaB*	Forward	ATCCATGATTTTTGCTTCAC
		Reverse	AATATCACTTTTGGAGATGC

Putative virulence genes			
oxidoreductase	*Cj0415*	Forward	CCTTGTCAATACTGTGCGTAT
		Reverse	GTTCCAAGCTGAACGCTATAA
D-3-phosphoglycerate dehydrogenase	*serA*	Forward	CCTAAAATTCCACCCAAAGCA
		Reverse	GATTGTACACAGAAGAGGCTT
quinone-reactive Ni/Fe-hydrogenase, large	*hydB*	Forward	ATCGATCACATAAGGAGCACA
		Reverse	AATCGGAAGAGATGAATGGCT
RND efflux system, inner membrane transporter	*cmeB*	Forward	CCATCTTTCATATTGGGCAG
		Reverse	GATGAATGCAACTATAGGCAC
major outer membrane protein	*porA*	Forward	CAGTGCTGCTATAGCTGATAA
		Reverse	CCTAAGTAAGCACCTTCAAGT
2-oxoglutarate-acceptor oxidoreductase subunit	*oorA*	Forward	CCTATTACTCCTAGTAGTGAG
		Reverse	CCATGATGAAGTCCTGTAACA
methyl-accepting chemotaxis signal transduction protein	*Cj1564*	Forward	CGATCTCCATCTTGAGTAAGA
		Reverse	CTGCTAATCGTTCTATGGCTA
sucC succinyl-CoA synthase, beta subunit	*sucC*	Forward	GCTATCCTATCACAACGAACA
		Reverse	GGACTTGAAGTAGCGAGAGTT
thiamine biosynthesis protein	*thiC*	Forward	GCTATGATAGGAACAGTTCCT
		Reverse	CCTGGACCTTCTATCATTACT
putative transmembrane transport protein	*C8J_1184*	Forward	AGCTTGTGCATCTGCTTCTAT
		Reverse	ATCGATCCAGTTGTTCGTGAT
Housekeeping genes			
hippurate hydrolase	*hipO*	Forward	ATCTAATGCTCTAACACTCA
		Reverse	GCAAAAGATCCTATTTATGC
glutamine synthetase	*gln*	Forward	TTCATTTTCTGGTCCAAAGT
		Reverse	CTGATCCTACTATCATAGTA
citrate synthase	*glt*	Forward	GCATACCTTCATGGATAAAA
		Reverse	ATGTTTTCTTATGATGAGGG
serine hydroxymethyltransferase	*gly*	Forward	ACTCTAGCTATTGAAAGATG
		Reverse	AGAACTTACTTTTGCACCAT
transketolase	*tkt*	Forward	TCAACTCTTGGAGTAGAAAT
		Reverse	GCTAAAGAACAAGCTTCATA
phosphoglucosamine mutase	*pgm*	Forward	GGAAAAGATACAAGAAGAAG
		Reverse	CAACGCATATCTTCAGTTAA
F0F1 ATP synthase subunit alpha	*uncA*	Forward	GTAGGTATTGTTATACTTGG
		Reverse	TTCATTAGCATTGATCACAC
DNA gyrase subunit A	*gyrA*	Forward	CATCATAAACTGCTGTATCT
		Reverse	GTTATTATAGGTCGTGCTTT
ketol-acid reductoisomerase	*ilvC*	Forward	GGTAGTGTAAGTGCTGTTAA
		Reverse	TGAATTTCATCAGGAGCTAA
DNA-directed RNA polymerase subunit alpha	*rpoA*	Forward	GCCAACAGAATTTACAATAG
		Reverse	AAGTGCTACATCTTCAAGCA
FKBP-type peptidyl-prolyl cis-trans isomerase SlyD	*slyD*	Forward	AAAGCTTAGAAGAAGAAGTG
		Reverse	AATACCTGCAAATTGCTCTT
rRNA-16S ribosomal RNA	*rrs*	Forward	AAATCCGTAGATATCACCAA
		Reverse	TAGCTGCATTACTGAGATGA
Transcription regulator			
RNA polymerase factor sigma70	*rpoD*	Forward	TTTAGAATACCGCTTACCTA
		Reverse	GCGTTTAATTTGCTCCAAAA
RNA polymerase factor sigma54	*rpoN*	Forward	GGAGTTAGATGAAGATATTG
		Reverse	CCATCAGTTTGGATTGAAAT

flagellar biosynthesis factor sigma28	*fliA*	Forward	GCCTTCAAGTATAGATGTTA
		Reverse	TCCATGATTGCATCAATATC

A/8 - List of publications and presentations related to the present work

Original articles

- Sonnevend Á, **Kovács J**, Pál T, Akawi N, Nagelkerke N, Schneider G. Lack of correlation between the 257C-to-T mutation in the gyrA gene and clinical severity of *Campylobacter jejuni* infection in a region of high incidence of ciprofloxacin resistance Scand J Infect Dis, 2011; Dec; 43(11-12):905-11. **(IF: 1,722)**

- **Kovács JK**, Felső P, Emődy L, Schneider Gy, Kocsis B. Improved Isolation Protocol to Detect High Molecular Weight Polysaccharide Structures of *Campylobacter jejuni.* J Microbiol Meth 2014; 107:55-57. **(IF: 2,096)**

Oral presentations

- **Kovács JK**, Horváth Gy, Kocsis B, Schneider Gy. Antibakteiális Illóolajok Hatásmódjainak Kísérletes Vizsgálata. Congress of Hungarian Society for Microbiology, Hungary, Keszthely, 2014. October 15-17. **(Best young author award)**

- **Kovács JK**. Mode of Antimicrobial Action of Clove Essential Oil Against the Foodborne Pathogen *Campylobacter jejuni*. 3rd Interdisciplinary Doctoral Conference, Hungary Pécs, 2014. April 15-17. **(Best presenter award)**

- **Kovács JK**, Emődy L, Schneider Gy. *Campylobacter jejuni* patomechanizmusában szerepet játszó virulencia faktorok és gazda-parazita intearakció vizsgálata. 2nd Interdisciplinary Doctoral Conference, Hungary Hungary, Pécs, 2013. May 15-17.

Poster presentations

- **Kovács JK**, Felső P, Emődy L, Schneider Gy, Kocsis B. Lipopolysaccharide structure based comparative analysis of different *Campylobacter jejuni* strains. 4th Central European Forum for Microbiology, Hungary, Keszhely, 2013. October 16-18. **(Best young author award)**

- **Kovács JK**, Felső P, Horváth Gy, Emődy L, Schneider Gy. *Campylobacter jejuni* törzsek lipopoliszacharid mintázatának vizsgálata növényi illóolajok hatására. Congress of Hunagrian Society for Microbiology, Hungary, Keszthely, 2012. October 24-26.

- Dorn Á, Horváth Gy, **Kovács JK**, Schneider Gy. Effect of herbal extracts on the growth of pathogenic bacteria. Congress of Hungarian Society for Microbiology, Hungary, Keszthely, 2010. October

- **Kovács JK**, Emődy L, Schneider Gy. Adhesion potential of human isolate *Campylobacter jejuni* to the extracellular matrix proteins type IV collagen, fibronectin and laminin. Congress of Hunagrian Society for Microbiology, Hungary, Keszthely, 2010. October

- **Kovács JK**, Beer B, Emődy L, Schneider Gy. Virulence potential of inpatient *Campylobacter jejuni* isolates in South- West Hungary. FEMS, NoE EuroPathoGenomics and ERA-NET PathoGenoMics Conference, Hungary, Pécs, 2010. April 22-24.

- **Kovács JK**, Sonnevend Á, Buruncz A, Schneider Gy. Comparative genetic analysis of human *Campylobacter jejuni* strains isolated from the Southern Region of Hungary by *flaA*-RFLP and PFGE. NoE EPG 4th Student Meeting, Spain, Palma de Mallorca, 2009. April 27-29.

A/9 - List of additional publications and presentations

Poster presentations

- **Kovács JK,** Dorn Á, Schneider Gy, Emődy L, Molnár J, and Makovitzky J. Analysis of the cell surface of *Campylobacter spp.* and *Helicobacter pylori* by charge transfer reactions. 54th Symposium of the Society for Histochemistry, Austria, Vienna, 2012. September 5-8.

- Kovács B, Dorn Á, **Kovács J**, Kerényi M, Emődy L. Investigation on the haemolític activity and matrix protein binding capacity of asymptomatic bacteriuria *Escherichia Coli* solates. 16th International Congress of the Hungarian Society for Microbiology, Hungary, Budapest, 2011. July 20-22.