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

Ph.D. thesis

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I. INTRODUCTION

I.1. Campylobacters

Campylobacter is the most common causative agent of bacterial zoonotic food-borne diarrheal diseases all around the world. Certain campylobacters are commensal, but the majority of them are pathogens, infecting the human and animal gastrointestinal tracts. Among them *Campylobacter jejuni* is the most important pathogen. According to Centers for Disease Control and Prevention (CDC), the reported incidence of infections caused by *Campylobacter* has increased in the USA by 14% compared within the period between 2006-2008. Similar increasing trends have been observed from 2008 till 2012 in numerous countries of the European Union (EU), just as well in Hungary (**Figure 1**).

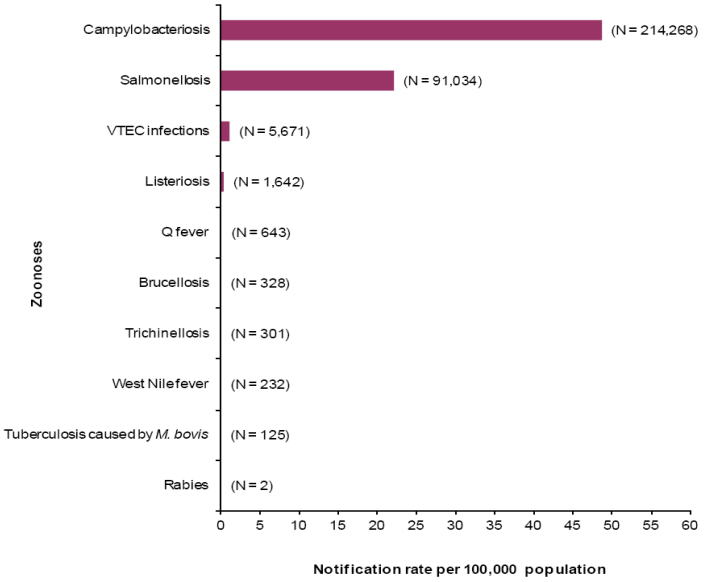

Figure 1. Reported notification rates of zoonosis of confirmed human cases in the EU, EU/EFTA 2012.
VTEC: verotoxin-producing *Escherichia coli*

Since *C. jejuni* is ubiquitous in the nature, and the cases are typically sporadic, detection of the sources of infection is troublesome. Epidemiologic studies based on numerous phylogenotyping methods are important to follow up emerged cases, and by this they are crucial in controlling this pathogen. The infection by this food-borne pathogen is mainly due to consumption of undercooked poultry meat, but raw milk and environmental water sources are all potential sources. The clinical spectrum of campylobacteriosis ranges from asymptomatic to mild and severe symptoms like bloody diarrhea. Rarely post infection sequels such as Miller-Fisher, or Gulillan-Barré syndromes can also develop. Although majority of the cases does not require antibiotic therapy, prolonged or severe

symptoms warrant medical intervention involving anti-microbial drugs. Currently macrolides are the drugs recommended in cases of proven etiology, however, over the last decade antibiotic resistance has widely been reported giving rise to serious public health concerns. For this reasons quest for alternative antimicrobials both in prevention and therapy is a high requisite.

The mechanism by which *C. jejuni* causes disease is enigmatic, the virulence factors leading to infection thought to be unique compared to other enteric pathogens (**Figure 2**). Although numerous potential virulence properties have been reported such as motility, chemotaxis, adhesion, invasion, intracellular survival, and production of toxin, but still, the exact molecular background of the process leading to campylobacteriosis is not fully understood. Furthermore, it is still an open question in what extent the particular host or bacterial factors contribute to the extensive variation of the individual clinical manifestations.

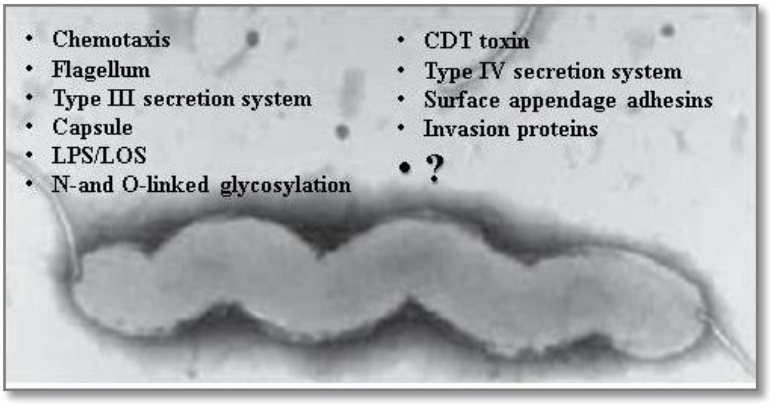

Figure 2. Virulence-associated factors of *Campylobacter jejuni*

To further complicate the question, hypervariable sequences are found in certain genes of *C. jejuni* coding for flagellum and cell surface carbohydrate structures, like capsule and lipooligosaccharide/lipopolysaccharide (LOS/LPS).

LOS/LPS structure of *C. jejuni*

One of the main morphologic 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.

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 (HMW) LPS-like structure at all has not yet been clarified. The major problem is that the traditional LPS isolation methods that are proper to reveal the pattern of analogous surface

associated components in *Salmonella*, *E. coli*, *Shigella*, etc. by silver staining are not applicable for *Campylobacter* spp.

I.2. Essential oils

Essential oils (EOs) are secondary metabolites playing a crucial role in the protection of plants as antimicrobials. Different mechanisms are known and hypothesized to play a role in their antimicrobial effect. Due to their hydrophobicity they can cause partition of the lipids in the bacterial cell membrane causing increased permeability, depolymerization, and decreased membrane potential disturbing the ion flow through the membrane. Certain EO components are also able to interfere with cell wall proteins usually involved in the transport of essential molecules into the cell. EOs can alter membrane fluidity by decreasing the proportion of the unsaturated fatty acids (UFAs) and also can act on quorum sensing (QS). Sub-lethal concentration of EO is the most suitable to elucidate those molecular changes that are behind the scene, and reveal their antibacterial mode of actions.

Clove essential oil is one of the most powerful antimicrobial agents. Eugenol, its main component is thought to be responsible for the strong biological and antimicrobial properties of this EO. 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 of the cell membrane. By the interaction with proteins eugenol is suggested to bind to and affect the properties of proteins at sub-lethal concentration.

II. AIMS OF THE THESIS

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. The clinical strains were isolated from patients having a wide variety of symptoms from mild diarrhea to dysentery with blood and mucus in the stool.

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

1. What is the clonal relationship among the characterized 190 isolates, and what differences can be evaluated among their virulence potential by phenotypic and genotypic tests.
2. Is there any correlation between the virulence factor pattern and the severity of the clinical symptoms of the investigated isolates?

3. Is whole transcriptomic analysis applicable to identify novel candidate genes that could play roles in the so far not completely understood invasion process of *C. jejuni*?
4. 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*?
5. What are those attendant molecular and phenotypic changes on and in the *C. jejuni* cell that are generated by clove EO eventually resulting in death of the bacterial cell?
6. We aimed to develop a simple detection method by which the existence of high molecular weight lipopolysaccharide structures of different *Campylobacter jejuni* isolates could be detected, and by this way to answer the question if it exists.

III. MATERIALS AND METHODS

The following methods were used for

- Analysis of the clonal relationship among the 190 clinical isolates (epidemiological studies):
- *FlaA*- Restriction fragment length polymorphism (*flaA*-RFLP)
- Pulsed-field gel electrophoresis (PFGE)
- Characterization of the virulence potential of the strain collection:
- Polymerase chain reaction (PCR)
- Solid-phase extracellular matrix protein binding assay
- INT407 cell adhesion and internalization assay
- Expression analysis of a highly virulent *C. jejuni* isolate during invasion:
- Whole transcriptome analysis (RNA-Seq)
- Detection of molecular and phenotypic changes of *C. jejuni* as a result of clove EO treatment:
- Determination of minimal inhibitory (MIC) and minimal bactericidal (MBC) concentration
- Electrophoretic protein microchip
- Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)
- Liquid chromatography–mass spectrometry (LC-MS) analysis
- Real-Time PCR (RT-PCR) analysis
- Motility assay
- Scanning electron microscopy (SEM)
- Gas chromatography–mass spectrometry (GC-MS) analysis
- Thin layer chromatography - direct bioautography (TLC-DB)
- Improvement of a protocol for isolation of *C. jejuni* high molecular weight polysaccharides

IV. RESULTS AND DISCUSSION

The clonal relationship among the 190 clinical isolates

By PFGE the minimal rate of homology was 31% for all the typeable isolates indicate that our studies were performed on a very diverse strain collection. Using the arbitrary value of ≥90% similarity of the banding patterns 69 RFLP and 122 PFGE groups could be established. However, 10 strains (6%) exhibited identical PFGE patterns but could be further divided based on their RFLP patterns. According to our results most of the isolates were related to sporadic cases.

Virulence factor pattern of the strain collection

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. We found that strains harboring non-sialylated LOS had the same ability to invade into INT407 cells as strains possessing the *csII/csIII* genes for sialylated LOS (**Table 1**).

Table 1. 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

All the *C. jejuni* strains showed high level of variability in their ability to bind extracellular matrix proteins (ECMPs). The binding to extracellular basement membrane proteins revealed to be strain specific. Strains were found to bind collagen type IV at the highest level, followed by fibronectin and laminin.

Adhesion and invasion ability varied considerably among the strains. We have grouped the isolates into (i) high adhesion but low invasion potential (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) categories (**Figure 3**).

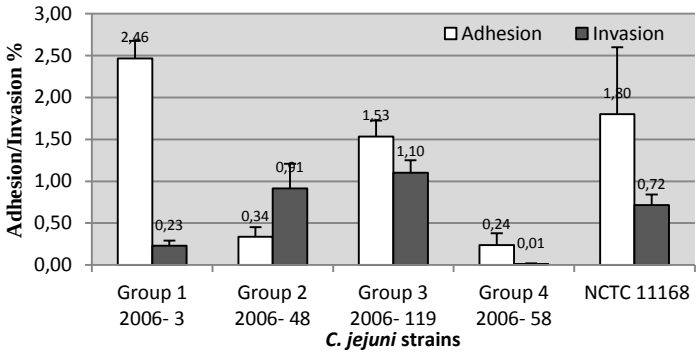

Figure 3. Comparison of the adhesion and invasion potentials of 1-1 representatives from the four groups.

No clear relation could be revealed among the results of phylogenic, phenotypic, genotypic tests and the symptoms of the patients. However, three strains (2006-119, 2006-134, and 2006-165) derived from patents with serious symptoms, possessed all the tested virulence genes. Furthermore, these strains showed higher affinity to bind ECMPs, adhere and invaded the INT407 cell line.

Expression analysis of a highly virulent *C. jejuni* isolate during invasion

High-throughput sequencing of cDNA libraries (RNAseq) was performed during the invasion process of the highly virulent *C. jejuni* strain 2006-119. 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 genes for 59 *membrane proteins*, 39 *periplasmic* and 134 *hypothetical proteins* were upregulated.

They belonged to the following groups:

- Transmembrane functions (e.g. *secY, secE, ompA,* and *omp50*)
- Adhesion (e.g. *jlpA, capA*, *cjaA*, and *flpA)*
- Bacterial shape determinants (e.g. *pbpC, rodA*, and *mreB)*
- Surface associated saccharides (*kpsM, kpsE, and galE)*
- Invasion (e.g. *ciaB, htrA* and *cetA*)
- Iron acquisition (e.g. *ceuBCDE*)
- Regulatory systems (e.g. *dccS* and *racS,)*
- Chemotaxis (e.g. *cheW* and *cheB*)
- Flagellar machinery (e.g. *flhA, flhB*, and *motA*)

Molecular and phenotypic changes of *C. jejuni* as a result of clove EO treatment

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 altogether 50 clinical isolates by reducing the living cell counts to one-third in 2 h.

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. 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. These spots correspond to proteins known to be involved in the synthesis of the following virulence associated factors: (i) Peb1, an important factor in host colonisation, (ii) Peb4, a temperature dependent colonisation factor, and (iii) HtrA a serine protease, which has a role in adherence and invasion. Additionally, two spots were revealed with elevated expression level compared to the control, they were identified as a chaperonin and the elongation factor Tu.

Altogether 45 genes were targeted by real time PCR in order to reveal their incidental alteration in expression due to clove EO treatment. Results of the RT-PCR have shown that at least two virulence associated genes (*galE* and *flhB*) were downregulated in the presence of clove EO. Furthermore, a threefold down regulation was observed in the case of *porA*, a major outer membrane protein possessing with strong antigenic feature. Although the 51.9 fold activation of *katA* could be a clear indication for clove EO resulted oxidative stress, the expression of three other oxidative stress genes (*dps*, *sodB*, and *ahpC*) was not elevated. Furthermore, overexpression of *groEL* and *dnaK*, two important molecular chaperons, characteristic for general stress response could be detected.

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 4-A, B**) presented with a shrunken and straightened outlook (**Figure 4-C, D**) if compared to the control.

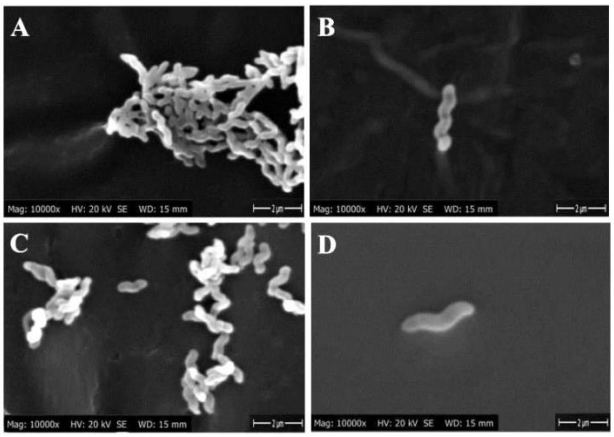

Figure 4. Scanning electron micrographs of non-treated (A, B) and clove EO treated (1: 3,000) (C, D) *C. jejuni* cells.

By using a functional assay we could also reveal the effect of clove EO oil on one of the most important virulence associated feature of *C. jejuni*. After 24-h microaerophilic incubation, untreated cells showed a 3 cm diameter swarming area. Survivor *C. jejuni* cells treated with the generally applied 1: 3,000 dilution of clove EO completely lost their ability to move. If another sub-inhibitory (1: 20,000) dilution of clove EO was applied bacterial cells showed a decreased capacity to swarm, while their living cell number did not change.

Compound composition of clove EO was determined by GC-MS analyses. Applying TLC with ethanolic vanillin–sulphuric acid reagent eight constituents could be visualized. 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. Besides eugenol, another five unidentified components of clove EO with antimicrobial effect against *C. jejuni* was detected by direct bioautography combined with TLC.

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 the expression of certain genes and proteins.

Improvement of a protocol for isolation of *C. jejuni* high molecular weight polysaccharides

We developed a method by which the presence of slow-migrating HMW polysaccharides (PSs) with a ladder-like structure characteristic to other Gram-negative bacteria can be visualized in *C. jejuni*. With our modified method we could detect the presence of LPS on strains showing only LOS structures by the other methods (**Figure 5)**.

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

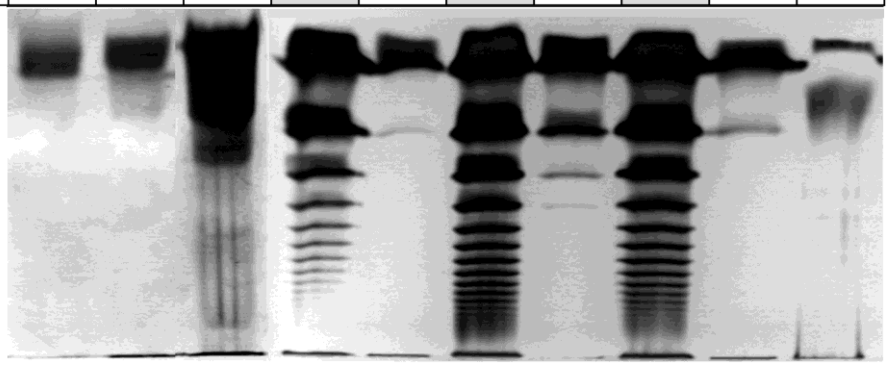

Figure 5. Comparison of the major steps and their effects on the isolation of the HMW fraction of *C. jejuni* NCTC 11168 by the published (lines 1-3) and the modified (lines 4-10) isolation methods. In accordance with the literature, no HMW structures were detected by silver staining when the methods of Szymanski, Salloway, and Preston and Penner (lines 1-3) were used.

In accordance with the literature, no HMW structures were detected by silver staining when the methods of Szymanski, Salloway, and Preston and Penner (**Figure 5, lines 1-3**) were used.

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 5**).

V. NOVEL FINDINGS

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.

- The severity of symptoms of *C. jejuni* infection did not correlate with the presence or absence of certain proposed virulence genes in the isolates.
- The adhesion and invasion ability varied considerably among the strains.
- In general, 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. However, three strains (2006-119, 2006-134, and 2006-165) were found among the clinical isolates possessing all the tested virulence genes and higher affinity to bind ECMPs, adhere and invaded the INT407 cell line.
- 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 function in the invasion and the intracellular survival of *C. jejuni*.
- Our results suggest that certain adhesion proteins may have an additional role in the invasion process and the intracellular life.

➢ 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.
- Eugenol and several other non-identified components of clove EO were revealed to possess bactericidal activity against *C. jejuni*

➢ Result of the improvement of a reliable HMW PSs isolation method:

- A modified isolation procedure was developed, by which HMW polysaccharide structure of *C. jejuni* can be visualized by silver staining.
- The presented modified receipt is simple, and hence proper to test large numbers of isolates.

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VII. 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; Sept 13. (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, 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, Keszthely, 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 Hungarian 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 Hungarian 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.

VIII. 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 hemolytic activity and matrix protein binding capacity of asymptomatic bacteriuria *Escherichia Coli* isolates. 16th International Congress of the Hungarian Society for Microbiology, Hungary, Budapest, 2011. July 20-22.