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# EVALUATING THE EFFECT OF DIFFERENT GROWTH CONDITIONS ON GLYCOCONJUGATE EXPRESSION IN *CAMPYLOBACTER COLI*: A METHODOLOGICAL PERSPECTIVE

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Tiivistelmä — Referat — Abstract

*Campylobacter* species, particularly *C. jejuni* and *C. coli*, are the most common cause of human gastroenteritis worldwide. Lipooligosaccharides (LOS) are heat-stable amphiphilic glycolipids essential for viability and outer membrane stability of Gram-negative bacteria. LOS trigger the activation of both innate and adaptive immune systems. This project aimed to investigate variability of *C. coli* LOS expression under different growth conditions and its impact on immunogenicity. Moreover, it aims to highlight possible method-based biases in investigating LOS-host interaction.

In this study the LOS of eight *C. coli* strains were extracted by three different methods. Despite differences in growth conditions, the electrophoretic mobility of analyzed LOS was consistent within strains. SDS-PAGE analysis and TLR4 activation assay revealed a significant overestimation of the LOS concentration in the samples by the commonly used LOS quantitation method – purpald assay. Furthermore, it was shown that standardization of lyophilized LOS samples by weight lead to an incorrect estimation of LOS concentration.

In conclusion, growth conditions did not cause a visible change in the electrophoretic mobility, suggesting no significant changes in LOS expression. Crude LPS extraction was suitable for LOS profiling and immune response evaluation in HEK-TLR4 cells. LOS quantitation by weight and/or by the purpald assay may result in incorrect estimation of LOS concentration and it might significantly affect downstream immunological analyses. The results of this thesis suggest that the validity of data produced using LOS quantified with either purpald assay or weight of lyophilized material should be critically reassessed.

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*Campylobacter coli*; growth conditions; lipooligosaccharides; TLR4; immune response

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## PREFACE

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Finally, I want to express my very profound gratitude to my family for the continuous support and encouragement throughout the years of study, this accomplishment would not have been possible without them.

## ABBREVIATIONS

| | |
|---|---|
| AMPs | antimicrobial peptides |
| APC | antigen-presenting cell |
| CD14 | cluster differentiation antigen 14 |
| CPS | capsular polysaccharide |
| *cst* | *Campylobacter* sialyltransferase |
| DCs | dendritic cells |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid |
| EFSA | European Food Safety Agency |
| EU | European Union |
| GalNAc | N-acetylgalactosamine |
| GBS | Guillain-Barré syndrome |
| GlcN | D-glucosamine |
| GlcN3N | 2,3-diamino-2,3-dideoxy-D-glucopyranose |
| IRF3 | interferon regulatory factor 3 |
| IRAK | IL-1 receptor-associated kinase |
| Kdo | 3-deoxy-D-*manno*-oct-2-ulosonic acid |
| L-Ara4N | 4-amino-4-deoxy-L-arabinose |
| L-α-D-Hep | L-*glycero*-D-*manno*-heptose |
| LAL | Limulus amoebocyte lysate |
| LOS | lipooligosaccharide |
| LPS | lipopolysaccharide |
| LBP | LPS-binding protein |
| MD2 | myeloid differentiation factor 2 |
| MLST | multilocus sequence typing |

| | |
|---|---|
| MyD88 | myeloid differentiation factor 88 |
| $M_r$ | relative molecular mass |
| NA | nutrient agar |
| NBA | nutrient agar supplemented with defibrinated horse blood |
| Neu5Ac | N-Acetylneuraminic acid, sialic acid |
| NCTC | National Collection of Type Cultures (UK) |
| NF-κB | nuclear factor kappa B |
| NOD1 | nucleotide-binding oligomerization domain protein 1 |
| OS | core oligosaccharide |
| PAGE | polyacrylamide gel electrophoresis |
| PAMPs | pathogen-associated molecular patterns |
| PEtN | O-phosphorylethanolamine, 2-aminoethyl dihydrogen phosphate |
| PRR | pattern recognition receptor |
| SEAP | secreted embryonic alkaline phosphatase |
| SDS | sodium dodecyl sulfate |
| spp. | species |
| ST | sequence type |
| TIR | Toll/interleukin-1 receptor |
| TLR | Toll-like receptor |
| TNF | tumor-necrosis factor |
| UTVG | unsubstituted terminal vicinal glycol groups |
| WHO | World Health Organization |

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## 1 INTRODUCTION

*Campylobacter* species are the most common cause of human bacterial gastroenteritis. *Campylobacter coli* is prevalent worldwide, with most infections related to consumption of undercooked food and environmental exposure (WHO 2013). In most cases symptoms of campylobacteriosis are generally mild and the disease is self-limiting, however, it has been associated with a range of complications, such as Guillain-Barré syndrome (Skirrow 1977; Blaser *et al.* 1979; Allos 1997). EFSA and ECDC (2015a) reported about 56 deaths attributed to campylobacteriosis in 2013, resulting in a EU case-fatality rate of 0.05%, which was the highest observed in five years. Although *C. coli* contributes to a minority of *Campylobacter* infections (approximately 10%), its health burden is considerable and greater than previously thought (Tam *et al.* 2003).

The lipooligosaccharide (LOS) is a glycolipid essential for bacterial survival, and is found in the outer membrane of some Gram-negative bacteria, including *Campylobacter*. LOS molecules, composed of an oligosaccharides core and a lipid A, contribute to structural integrity of the cell membrane, immune evasion, host cell adhesion, and molecular mimicry (Moran *et al.* 1996; Guerry and Szymanski 2008; Naito *et al.* 2010). It has been suggested that sialylation of the LOS increases invasive potential and reduces immunogenicity of *C. jejuni* (Guerry *et al.* 2000; Louwen *et al.* 2008). Furthermore, environmental and host physiological temperature affects *C. jejuni* LOS expression which may be an adaptive mechanism for colonization of different hosts (Semchenko *et al.* 2010).

While most of glycoconjugate research is related to *C. jejuni*, little is known about LOS expression in *C. coli*. In *C. coli* 76339, a human strain, the presence of sialic acid was detected by chromatography analysis of the purified LOS, which corroborates genomic findings (Skarp-de Haan *et al.* 2014). The main objective of this study was to investigate whether different growth conditions affect LOS expression in *C. coli* and to explore their role in the host-pathogen interaction.

In the first part of the thesis, the general endotoxin structure and the *C. jejuni* and *C. coli* LOSs structures are described. The variations in *Campylobacter* LOS expression, methods of LPS extraction, and TLR4 signaling pathway are explored in the second part of this thesis. In the final chapters, the purity of obtained LOS fractions, the accuracy of the purpald assay in estimating LOS concentration, and the effect of growth conditions on *C. coli* LOS expression are discussed.

# 2 LITERATURE REVIEW

## 2.1 Background

*Campylobacter coli* is a Gram-negative, thermo- and microaerophilic, non-spore forming bacterium from the *Campylobacteraceae* family. Morphologically *C. coli* is a curved, spiral or S-shaped rods 0.2-0.8 μm wide and 0.5-5 μm long, which move by a corkscrew-like motion, propelled by a flagellum at the end (or both ends) of the cell (Allos 2001). Due to their similar morphology campylobacters were initially classified as vibrios (Smith and Taylor 1919). A vibrio-like bacterium was isolated by Doyle (1944) from pigs suffering from dysentery, he proposed it to be a causative agent of this disease and suggested the name, *Vibrio coli* (Doyle 1948). Decades later, Véron and Chatelain (1973) revised the taxonomy of a recently established genus *Campylobacter* and included *V. coli* as *C. coli* (Doyle) comb. nov.

Campylobacters captured the attention of clinical microbiologists since 1950s, after pioneering work of Vinzent and colleagues (1947) who isolated *V. fetus* (now *C. fetus*) from the blood of three pregnant women hospitalized with fever of unknown origin; two of them were thereafter aborted. King (1957, 1962) detected a presence of "related vibrios" in blood samples of patients suffering of diarrhea and established the methods for laboratory diagnostics, biochemical testing, and culturing. The development and application of selective media resulted in the discovery of *Campylobacter* in the stools of patient hospitalized with diarrhea and fever (Dekeyser *et al.* 1972). The relationship between *Campylobacter* and the acute diarrheal disease was reaffirmed by Skirrow (1977).

*Campylobacter* spp., particularly *C. jejuni* and *C. coli*, are zoonotic pathogens that exist as a commensal in most wild and domestic animals (Waldenström *et al.* 2002; Stanley and Jones 2003; Horrocks *et al.* 2009). Typical symptoms of campylobacteriosis include watery diarrhea, fever, abdominal cramps, inflammatory enterocolitis, nausea, and vomiting (Altekruse *et al.* 1999). However, asymptomatic *Campylobacter* infections have been also reported (Calva *et al.* 1988). Rarely, serious sequelae, such as Miller-Fisher syndrome, reactive arthritis, and irritable bowel may develop after campylobacteriosis (Allos 2001; WHO 2013).

According to the European Food Safety Authority (EFSA), campylobacteriosis is the most frequently reported food-borne disease in EU: the number of confirmed cases in 2014 was 236,851 which was an increase of 22,067 cases compared to 2013 (EFSA and ECDC 2015b). Among the 52.6% of human isolates identified to species level, *C. jejuni* accounted

for 81.8% of the cases whereas *C. coli* was associated with 7.13% of the cases. In comparison to *Salmonella* Typhimurium, *C. coli* was responsible for 3.5 times more cases of indigenous foodborne disease and 4 times more hospitalizations reported in England and Wales in 2000 (Tam *et al.* 2003). Although *C. coli* is recognized as an important human pathogen, its impact on public health is most likely underestimated. For example, Gürtler *et al.* (2005) revealed 18.6% prevalence of diverse *C. coli* strains among patients with campylobacteriosis, which was higher than the respective data of the previous clinical studies conducted in Germany and other countries (Steinhauserova and Fojtikova 1999; Sopwith *et al.* 2003; Alpers *et al.* 2004). Gillespie *et al.* (2002) noted the absence of routine species characterization of human isolates may hamper the systematic study of the *C. coli* epidemiology.

### 2.2 Source attribution of *C. coli* infection

Most cases of campylobacteriosis are sporadic and occur in late summer-early fall in developed countries (Horrocks *et al.* 2009), although, a winter peak of human *C. coli* cases has been also reported (Gillespie *et al.* 2002; Gürtler *et al.* 2005). Consumption of undercooked food and unpasteurized milk, environmental exposure, direct contact with animals, and travelling abroad are considered to be the most significant risk factors of acquiring *Campylobacter* infections (Studahl and Andersson 2000; Potter *et al.* 2003; Friedman *et al.* 2004; Stafford *et al.* 2007).

Gillespie *et al.* (2002) proposed that *C. coli* might have different sources within the food chain and a different age distribution of infection compared to *C. jejuni*. Accordingly, the study of Doorduyn and colleagues (2010) revealed differences in campylobacteriosis risk factors in the Netherlands: a higher incidence of *C. coli* infection were found in the 45–59 years age group and in urban regions. Additionally, consumption of game and internal organs of animals, swimming, and ownership of cats were identified as specific risk factors for *C. coli* (Doorduyn *et al.* 2010). Indeed, 42.4% occurrence of *C. coli* within pigs' liver samples obtained in UK retail has been reported previously (Kramer *et al.* 2000). *C. coli* has been also linked to waterborne outbreaks in Finland which implies a fecal contamination of groundwater wells (Hänninen *et al.* 2003).

Nevertheless, poultry and ruminants were recognized as the principal source of human *C. coli* infection (Harris *et al.* 1986; EFSA 2010; Sheppard *et al.* 2010; Kittl *et al.* 2013; Roux *et al.* 2013). Siemer *et al.* (2005) confirmed contribution of poultry (chicken, duck, turkey, and ostrich) to *C. coli* infection; while Miller *et al.* (2006) described a prevalence of host-associated MLST alleles among 488 *C. coli* isolates of the different food animal origin (cattle, chickens, swine, and turkeys). In an EU-wide survey *C. coli* was detected in 13.1%,

9.5% and 87.1% of the isolates from broilers, cattle, and pigs, respectively (EFSA and ECDC 2009). It should be noted, that despite the high (up to 99%) recovery of *C. coli* from swine fecal samples (Thakur and Gebreyes 2005; Horrocks *et al.* 2009), pig meat at retail is rarely contaminated with *Campylobacter* (Stern *et al.* 1985; EFSA and ECDC 2009).

### 2.3 Complications of campylobacteriosis

Although uncommon, *Campylobacter* infection may have extraintestinal manifestations such as bacteremia, neonatal sepsis, meningitis, endocarditis, osteomyelitis, and septic shock (Allos 2001). In an 11-year surveillance study, Skirrow and colleagues (1993) estimated an incidence of 1.5 bacteremia cases per 1000 campylobacteriosis patients, and 89% of bacteremia cases were associated to *C. jejuni* and *C. coli*. According to Louwen *et al.* (2012) neonates, elderly and immunocompromised individuals are the population at risk. Fernández-Cruz *et al.* (2010) reported about 16.4% mortality due to *Campylobacter* bacteremia, while complications appeared in 23.9% of patients. In a Finnish nationwide study, severe complications (Guillain-Barré syndrome and cervical spondylodiscitis) were found in two patients hospitalized with *C. jejuni* bacteremia (Feodoroff *et al.* 2011).

Guillain-Barré syndrome (GBS) is the most important post-infectious sequel of campylobacteriosis. GBS is an autoimmune acute demyelinating polyneuropathy resulting in muscle weakness and neurological damage. Rees *et al.* (1995) reported that one third of the patients with severe GBS characterized by extensive axonal injury and respiratory failure had preceding *C. jejuni* infection. Molecular mimicry between GM-1 gangliosides of peripheral nerves and terminal structures on the lipooligosaccharide (LOS) of *C. jejuni* has been observed (Willison and Yuki 2002; Yuki *et al.* 2004).

GBS induced by campylobacteriosis is widely recognized as a true case of molecular mimicry-mediated disease (Moran *et al.* 1996; Ang *et al.* 2004; WHO 2013). The crucial role of *C. jejuni* genes related to LOS biosynthesis and sialylation in the induction of anti-ganglioside antibodies was elucidated (Gilbert *et al.* 2000; Godschalk *et al.* 2004). Bersudsky *et al.* (2000) claimed that LOS of *C. coli* isolated from the patient with GBS had a ganglioside mimicry. In contrast, Funakoshi *et al.* (2005) and van Belkum *et al.* (2009) postulated that there is no relationship between *C. coli* enteritis and GBS.

### 2.4 History of endotoxin research

The heat-stable toxic component of Gram-negative bacteria was discovered and termed “endotoxin” by Richard Pfeiffer at the end of the 19th century (Morrison 1983). Pfeiffer experimentally showed that lysates of heat-inactivated *Vibrio cholerae* are able to

induce shock and death in laboratory animals; he proposed that the unknown toxic substance is present inside the bacterium and being released only after destruction of the bacterial cell wall. In this way, he coined the term “endotoxin” to distinguish it from an exotoxins released during bacterial growth in culture (Bone 1993). Hort and Penfeld (1912) developed the first rabbit pyrogen test and discovered that lysates of various Gram-negative bacteria (but not Gram-positive) were pyrogenic despite prolonged heat treatment.

The first endotoxin extraction method employing trichloroacetic acid was introduced by Boivin and Mesrobeanu (1933). Endotoxins of several Gram-negative bacteria were isolated and identified as carbohydrate-lipid complexes (Landsteiner and Chase 1937). However, extraction of relatively pure endotoxin was only feasible after the introduction of a novel hot phenol-water method by Westphal *et al.* (1952). Westphal and colleagues (1952) revealed the endotoxin nature of lipopolysaccharides (LPS) and their immunogenic properties. Soon after, the lipid A, a subunit of LPS, was identified as the actual toxic moiety (Westphal and Lüderitz 1954).

After the investigation of *C. fetus* LPS toxicity and chemical structure by Vigdahl (1967), the association between lipid A and a toxic manifestations was corroborated. Furthermore, it was suggested that the LPS conformation was important for the immune response (Vigdahl 1967). Later on, artificial *E. coli* lipid A exhibiting identical endotoxic activity to its natural counterpart was synthesized (Galanos *et al.* 1985). In this way, the previous results were verified and an important toxin of Gram-negative bacteria was completely identified.

### 2.5 General aspects of endotoxin structure

LPS is the major component of the outer membrane of Gram-negative bacteria, contributing to its structural integrity. Hence, LPS is absolutely essential for bacterial survival. Moreover, LPS is involved in various physiological and ecological bacterial events, such as nutrient diffusion, membrane permeability, surface adhesion, bacteriophage sensitivity, and interactions with other cells, including mammalian immune cells (Nikaido and Vaara 1985).

Endotoxins of Gram-negative bacteria share a common general structure. Chemically, LPS is an amphiphilic macromolecule, comprised of three distinct domains: a lipophilic moiety (lipid A), and the two hydrophilic parts – core oligosaccharide (OS) and the O-specific polysaccharide (O-antigen) (Figure 1). Bacteria expressing the O-antigen have a smooth shape, thus this form of LPS is called smooth. In contrast, the rough LPS form (R-,

or LOS) is lacking the O-antigen (Holst and Molinaro 2009). Both forms contain lipid A and a core oligosaccharide that is comprised of up to 15 sugar residues (Holst 1999).

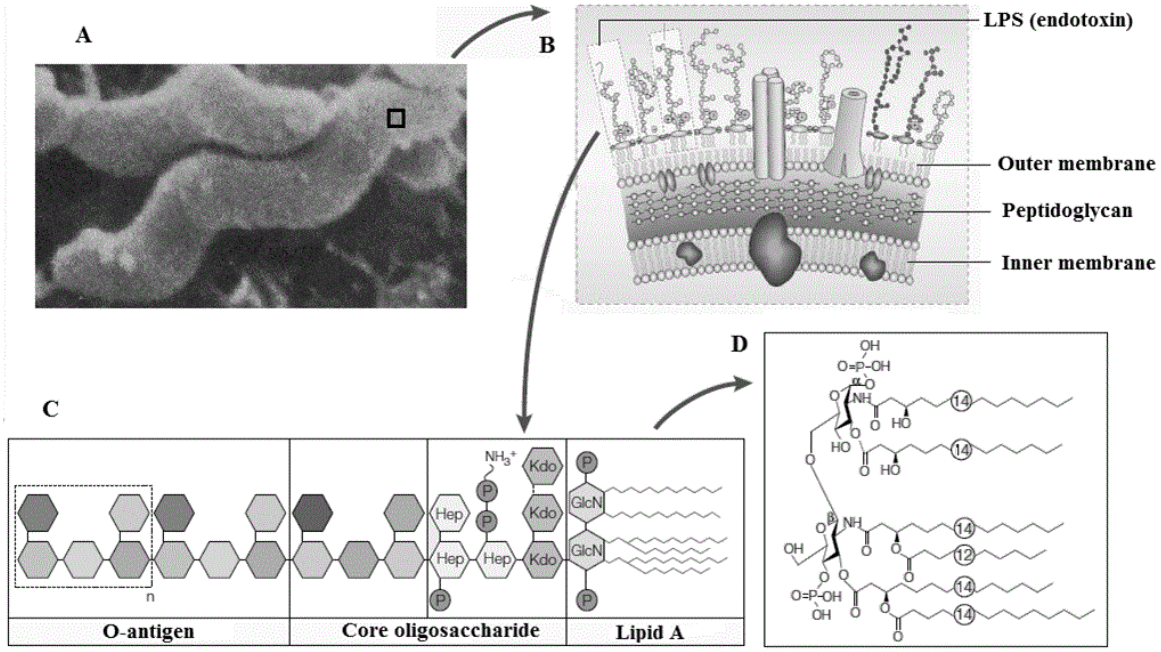



**Figure 1**. Electron micrograph of *Campylobacter coli* (A) and schematic representation of enterobacterial LPS in the bacterial cell wall (B), LPS structure (C) and the lipid A structure (D). GlcN, D-glucosamine; Hep, L-*glycero*-D-*manno*-heptose; Kdo, 2-keto-3-deoxy-octulosonic acid; P, phosphate. Adapted from Ng *et al.* (1985); Beutler and Rietschel (2003) with permission from the publishers.

Lipid A, in most cases, has a β-(1→6)-linked D-glucosamine (GlcN) disaccharide backbone, phosphorylated at positions 1 of the proximal a-GlcN and 4′ of the distal b-GlcN (Figure 1, D). Both GlcN residues are connected at positions 2 and 3 with 3-hydroxy fatty acids via amide and ester linkages; these fatty acids anchor the LPS into the outer leaflet of the outer membrane by hydrophobic interactions (Takayama *et al.* 1983; Silipo and Molinaro 2011). Lipid A is the most conserved and essential for bacterial viability part of LPS which determines endotoxic effects of bacteria (Galloway and Raetz 1990).

Lipid A can stimulate the innate immune system via recognition by the toll-like receptor TLR4 (see Chap. 2.10), sometimes causing sepsis and septic shock due to a systemic uncontrolled pro-inflammatory state (Takeuchi and Akira, 2010). The primary structure of lipid A determines its immunogenic properties. The lipid A containing two GlcN with an asymmetric (4 + 2) distribution of the saturated fatty acids and two phosphoryl groups represents the most active TLR4 agonistic structure (Rietschel *et al.* 1994). Any variations in this "canonical" lipid A structure, e.g. in number, position, length of acyl groups, phosphorylation pattern or tilt angle of the GlcN backbone, affect a physicochemical and biological behavior of the lipid A and, consequently, its endotoxin bioactivity (Seydel *et al.* 2000).

Due to their amphiphilic nature, LPS form three-dimensional aggregates (LPS micelles) in aqueous environments above a critical aggregation concentration, displaying a thermotropic polymorphism (Seydel *et al.* 1989; Aurell and Wistrom 1998). Brandenburg *et al.* (1993) reported that LPS and free lipid A with a nonlamellar inverted (cubic or hexagonal) structure exhibited full endotoxicity, while laminar aggregates were endotoxically inactive.

The core OS can be divided into two parts: one covalently linked to lipid A (inner core OS), and one attached to the O-antigen (outer core OS) (Figure 1, C). The structure of the inner core is rather conserved within a genus or family (Raetz and Whitfield 2002). At least one residue of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and several heptoses (usually, the L-*glycero*-D-*manno* isomers) are present in the inner core of the OS of most Gram-negative bacteria (Wilkinson 1996). Kdo links the inner core to the lipid A by the ketosidic bond (α2→6) which is prone to acid cleavage, hence, it is possible to separate the lipid A from LPS by mild acid treatment (Osborn 1963; Rosner *et al.* 1979). Kdo is an unusual sugar rarely found in other glycoconjugates, thus Kdo has been effectively utilized as a diagnostic marker for the LPS (Osborn *et al.* 1964; Lüderitz *et al.* 1966; Osborn *et al.* 1972; Lee and Tsai 1999). The inner core OS often contains a non-stoichiometric amounts of other substituents, such as (di)phosphate, 2-aminoethyl phosphate (PEtN), uronic acids and 4-amino-4-deoxy-L-arabinose (L-Ara4N) (Boll *et al.* 1994; Silipo and Molinaro 2011). These substitutes along with Kdo provide a negative charge to LPS and contribute to membrane stability (Holst 2011).

The outer core OS is a docking site for the O-antigen made of common hexoses, such as D-glucose, D-mannose, D-galactose, and N-acetylhexosamines (Wilkinson 1996). The outer core regions of *Enterobacteriaceae* LPS display structural microheterogeneity: one core type was identified in *Salmonella*, while five core types were observed in *E. coli* (R1–R4 and K-12) (Jansson *et al.* 1981). LOS of mucosal pathogens, such as *Neisseria*, *Haemophilus*, *Bordetella*, and *Campylobacter*, contain a different type of outer core OS: substituted glycans are attached to heptose residues, while linear neutral hexoses determine the length of the oligosaccharide (Griffiss *et al.* 1988; Phillips *et al.* 1990; Aspinall *et al.* 1993a,b,c). Furthermore, in absence of the protecting O-antigen, these LOS allow bacteria to evade host's defense (e.g. phagocytosis and complement-mediated killing) by structural mimicry of epitopes of mammalian glycosphingolipids and incorporation of N-acetyl neuraminic acid (Neu5Ac) to the outer core region (Mandrell and Apicella 1993).

The O-antigen is the most variable component of LPS, and it is made of oligosaccharide repeating units (O-units) composed of several identical or different monosaccharides, which may be linear or branched. Variations in the O-antigen gene clusters cause a high diversity of O-antigens structures and compositions. Moreover, heterogeneity may be expanded by lateral glycosylation or O-acetylation introduced by prophage genes (Keenleyside and Whitfield 1999). The broad variability of O-antigens facilitates immune evasion and enables serological typing of different bacterial strains (Reeves 1995).

The newly synthesized LPS molecules are translocated across the periplasm and through the outer membrane occupying 75% of its surface. The polysaccharide region is oriented outwards from the membrane and extends up to 10 nm from the surface (Caroff and Karibian 2003). The quantity of LPS is lower in rough than in smooth strains, it was estimated that *E. coli* possess ~ 2-3.5x$10^6$ LPS molecules per cell (Raetz 1986; Rietschel *et al.* 1994). As part of the bacterial outer membrane the LPS molecules are not toxic *per se*. However, free LPS released during division or lysis of bacteria evokes an immune response (van Amersfoort *et al.* 2003).

### 2.6 *Campylobacter* LOS structure

The detailed structure of *C. jejuni* lipid A was elucidated by Moran *et al.* (1991a). *C. jejuni* lipid A contains a biphosphorylated hybrid 2,3-diamino-2,3-dideoxy-D-glucopyranoses (GlcN3N) and GlcN backbone (Moran *et al.* 1991a). Furthermore, heterogeneity of the lipid A polar head groups (e.g., substitution by ethanolamine phosphate) was observed (Figure 2). In spite of the presence of a long-chain fatty acids (16:0) in the lipid A, *C. jejuni* LPS had slightly lower biological activity compared to other enterobacterial endotoxins (Naess and Hofstad 1984a; Moran 1995). Moran (1997) suggested that a lower fluidity of *Campylobacter* lipid A and variations in its supramolecular structure (due to the length of acyl groups and/or GlcN3N substitution) may influence the lipid A activity. Later on, a relationship between low agonistic activity of *C. jejuni* and conical/concave shape of its lipid A has been established (Schromm *et al.* 2000).

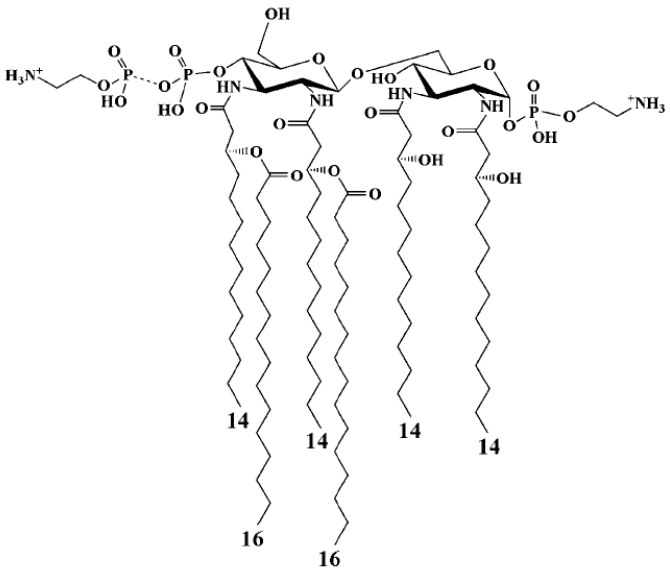


**Figure 2**. Chemical structure of the *C. jejuni* CCUG 10936 lipid A. Numbers indicate the lengths of acyl chains; dashed lines represent a partial substitution due to microheterogeneity. Adapted from Moran *et al.* (1991a).

It is well known that lipid A fine structure varies among different bacterial genera, and also within species of the same genus (Rietschel *et al.* 1994; Silipo and Molinaro 2011). GlcN instead of GlcN3N and a different fatty acid composition was detected in *C. fetus* lipid A (Moran *et al.* 1994). These findings were in accordance with previous study of Vigdahl (1967) who discovered the occurrence of Kdo in *C. fetus* LPS and estimated $LD_{50}$ of its lipid A (5900 μg/mouse). Naess and Hofstad (1984b) found that *C. coli* lipid A resembled an enterobacterial "classical" variant: it contained the common fatty acids (tetradecanoic, 3-hydroxytetradecanoic and hexadecanoic acids) and both glucosamine and phosphate. Accordingly, GlcN-GlcN disaccharide backbones were detected in the lipid A of nine *C. coli* isolates (Culebro *et al.* 2016).

Naess and Hofstad (1984b) proposed that *C. coli* contain R-type oligosaccharide (LOS) composed of Kdo, L-*glycero*-D-*manno*-heptose, glucose, galactose, and glucosamine. Such monosaccharide composition of *C. coli* LOS was confirmed by Beer *et al.* (1986), additionally, 3-amino-3.6-dideoxyglucose was detected. The core OS structure of *C. coli* serotype 0:30 was solved by Aspinall *et al.* (1993a): notably, a phosphate substitutes (either as monoester or PEtN) were absent in the inner core, while the general disaccharide unit of L-*glycero*-α-D-*manno*-heptose (L-α-D-Hep) was expanded by the indirectly attached third L-α-D-Hep. Furthermore, Neu5Ac residues were absent in *C. coli* LOS, and both (R)-3-hydroxybutyryl and 3-hydroxy-2,3-dimethyl-5-oxoprolyl groups acted as the N-acyl substituents (Figure 3, A). The core OS structures of a different *C. jejuni*

strains were elucidated and a molecular basis for the serotypic differences and ganglioside mimics was established (Aspinall *et al.* 1993b,c).

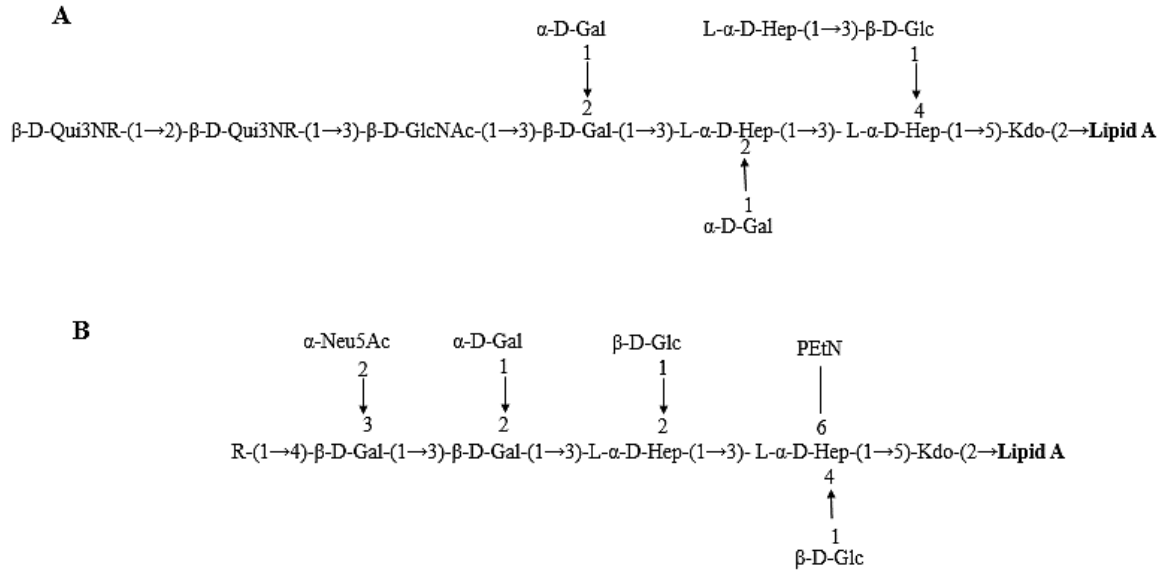



**Figure 3**. Chemical structures of the core regions of *C. coli* serotype O:30 (A) and *C. jejuni* serotypes O:1 (R = β-D-GalNAc) and O:2 (R = H) (B). Adapted from Aspinal *et al.* (1993a,b,c).

Penner *et al.* (1983) described the 42 thermostable serotypes of *C. jejuni* and 17 of *C. coli*, which were identified by a passive hemagglutination assay similarly to the LPS antigens of other Gram-negative bacteria. Nevertheless, Logan and Trust (1984) confirmed a low molecular mass ($M_r$) of *C. jejuni* and *C. coli* LOS and the absence of O-antigen. On the contrary, Mandatori and Penner (1989) reported about isolates of *C. coli* possessing both low and high $M_r$-LPS: O-antigens were detected by immunoblotting with homologous antisera. Chart *et al.* (1996) suggested that these heat-stable antigens were not long-chain S-LPS, but a capsular polysaccharide (CPS). Later, Karlyshev *et al.* (2000) confirmed that assumption and demonstrated that the high $M_r$ LPS of *C. jejuni* is biochemically and genetically unrelated to LOS and actually belong to CPS. Following studies revealed a complex nature and strain-dependent variations of polysaccharides produced by *Campylobacter*: e.g., *C. jejuni* 81116 produces a LPS-associated neutral polysaccharide B and a CPS-related acidic polysaccharide A (Kilcoyne *et al.* 2006).

### 2.7 Variation of LOS

#### 2.7.1 Genetic basis of *Campylobacter* LOS diversity

Modulation of the surface glycoconjugate expression is an important strategy for *Campylobacter* adaptation to microenvironments and survival in the host (Karlyshev *et al.* 2005a). A high genetic diversity in the LOS and CPS loci was found in *C. jejuni* (Dorrel *et al.* 2001). These gene clusters were subsequently characterized and used to classify strains

into classes: 35 classes had hitherto been defined for CPS (Poly *et al.* 2015) and 22 for LOS (Parker *et al.* 2005; Parker *et al.* 2008; Richards *et al.* 2013).

Structural variation of the CPS and LOS in *C. jejuni* is the result of multiple mechanisms, such as lateral gene transfer, gene inactivation by deletion or insertion of a base, single mutations causing the inactivation of a glycosyltransferases (e.g. heptosyltransferase I, sialyltransferase), and phase variation due to homopolymeric nucleotide runs of variable length (Parkhill *et al.* 2000; Gilbert *et al.* 2002).

Phase variation determines a structural inherent heterogeneity of *Campylobacter* LOS and LPS of other mucosal pathogens, such as *Neisseria* spp., in which a single bacterial strain can express a repertoire of LOS molecules (van Putten 1993; Parker *et al.* 2008; Guerry and Szymanski 2008). Parkhill *et al.* (2000) discovered that variations in the length of poly GC tracts caused slipped strand mispairing of a sequences of otherwise identical clones. Additionally, authors concluded that phase variation was associated mostly with genes involved in *C. jejuni* LOS biosynthesis. Linton *et al.* (2000a) confirmed phase variation of β-1,3-galactosyltransferase (encoded by *wlaN* gene), which resulted in expressing either $GM_1$ or $GM_2$ ganglioside mimicry in the LOS of *C. jejuni* NCTC 11168. Guerry *et al.* (2002) reported a shift in core OS structure of *C. jejuni* 81-176 (from $GM_2$ to $GM_3$ – mimicking epitopes) due to phase variation of *cgtA* gene expression, which encodes an N-acetylgalactosamine (GalNAc) transferase.

While the majority of studies on phase variability were focused on *C. jejuni*, high-frequency phase variation of flagellin expression in *C. coli* UA585 was discovered by Park *et al.* (2000). Authors suggested that a loss of flagellum due to mutations in a short poly T tract in the *flhA* gene may help bacteria to evade host immune response. Moreover, homopolymeric repeats have been detected in *C. coli* LOS and CPS gene regions (Richards *et al.* 2013). A variation in the number of L-α-D-Hep and/or PEtN residues was observed in *C. coli* isolates, however, no poly GC tracts were detected within LOS loci (Culebro *et al.* 2016). In this way, authors suggested that phenotypic variation in *C. coli* LOS composition may be determined by other factors, such as post-transcriptional regulation.

A significant strain-dependent heterogeneity in the electrophoretic mobility and $M_r$ of *C. coli* LOS was observed by Logan and Trust (1984). In accordance with this diversity of phenotypic chemotypes and similarly to *C. jejuni*, high gene diversity for the CPS and LOS loci was detected in *C. coli* (Lang *et al.* 2010). Aforementioned gene clusters have been characterized (Richards *et al.* 2013, Skarp-de Haan *et al.* 2014, Culebro *et al.* 2016): 11 *C. coli* LOS classes have been established. A diversity in LOS locus sizes (from 3 kbp to 18

kbp) among the Finnish *C. coli* isolates has been reported, implying that *C. coli* may have more, yet undiscovered, LOS classes (Culebro 2013; Culebro *et al.* 2016). Importantly, the genetic LOS classes displayed a host-specific distribution: the majority of human isolates pertained to LOS locus classes II, III, and IV, while swine was associated to LOS classes VI and X, and poultry was positively associated with class I (Culebro *et al.* 2016).

### 2.7.2 LOS sialylation

Sialic acid (Neu5Ac), a monosaccharide with a nine-carbon backbone, is widely distributed among vertebrate cells as a component of surfaces glycans, where it is employed in various cellular processes (e.g., cell adhesion and signaling). Some pathogenic bacteria can beneficially use sialic acid as a nutrient or as a protection against the host's innate immune response by synthesizing it *de novo* or scavenging directly from the host (Severi *et al.* 2007).

Many human pathogens, including *Escherichia coli* K1, *Haemophilus influenzae*, *Pasteurella multocida*, *Neisseria* spp., possess sialylated LPS. In most *C. jejuni* strains sialic acid is present as a constituent of the outer core OS (Moran *et al.* 1991b, 1997). Linton *et al.* (2000b) identified and characterized the multiple variants of *neuB* which encodes Neu5Ac synthetase in *C. jejuni*. The structure of sialyltransferase *cst-II*, the enzyme that transfers the sialic acid moiety, was elucidated by Chiu *et al.* (2004).

It was suggested that LOS sialylation decreases immunoreactivity and enhances serum resistance and invasiveness of *C. jejuni* in intestinal epithelial cells (Guerry *et al.* 2000; Louwen *et al.* 2008). In contrast, in the study of Ellström *et al.* (2013) only 23% of the *C. jejuni* strains isolated from patients with bacteremia contained LOS sialylation genes. Furthermore, no difference in serum resistance was observed between strains expressing sialic acid on their LOS and asialo-LOS strains (Ellström *et al.* 2013). Keo *et al.* (2011) proposed that *C. jejuni* CPS contributes more to complement resistance, while LOS protects against cationic antimicrobial peptides.

Nevertheless, certain classes of *C. jejuni* LOS (namely, A, B, and C) harboring sialyltransferase-encoding genes (*cst-II, cst-III*) are strongly associated with GBS, demonstrating that sialylation of the LOS is essential for the ganglioside mimicry (Gilbert *et al.* 2000, 2002; Godschalk *et al.* 2004). Two more LOS classes (R and M), possessing a Neu5Ac biosynthesis and transfer genes (*neuBCA*, *cst*) and thus potentially capable of inducing GBS, were described recently (Parker *et al.* 2008). Interestingly, three sialic acid orthologs were found not in LOS, but in the CPS cluster of *C. coli* H8 and 2553 (Richards

*et al.* 2013). Skarp-de Haan *et al.* (2014) detected sialic acid in the purified LOS of *C. coli* 76339 by chromatography analysis, corroborating genomic findings (Figure 4).

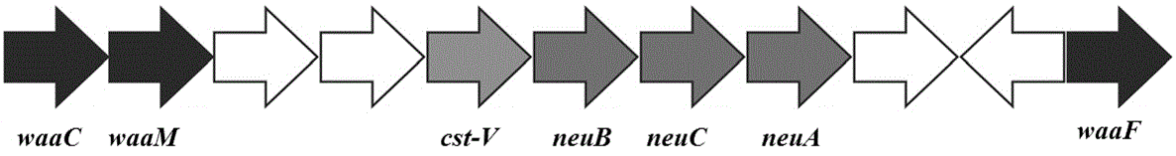



**Figure 4**. LOS locus of *C. coli* 76339. Black arrows indicate the conserved genes; *neuBCA*, the sialic acid biosynthesis genes; *cst-V*, putative sialyltransferase. Adapted from Skarp-de Haan *et al.* (2014).

Sialylated LPS and CPS help bacteria to evade the immune response, however, the presence of Neu5Ac may not be always beneficial in diverse microenvironments inside or outside the host (Severi *et al.* 2007). It is known that some pathogens, such as *Neisseria* spp., *H. influenzae* and *C. jejuni*, employ a rapid and reversible phase variation in order to modulate the structure of surface glycoconjugates (van der Woude and Bäumler 2004). Van Putten (1993) showed that sialylation of *N. gonorrhoeae* LPS controls bacterial passage across the mucosal barrier and the evasion of the host immune response in a phase-variable manner.

### 2.7.3 Growth conditions and phenotypic adaptation of LOS expression

The structure, composition, and content of bacterial endotoxins are modulated by growth conditions such as temperature, growth phase, and nutrients availability (Wilkinson 1996).

Thermal regulation of the fatty acid composition of LPS was observed in various enterobacterial species (*Salmonella* spp., *E. coli*, *Proteus mirabilis*, *Yersinia enterocolitica*): low growth temperature (12-15 °C) resulted in increased incorporation of unsaturated fatty acids into lipid A (Rottem *et al.* 1978; van Alphen *et al.* 1979; Wilkinson 1996). Kawahara *et al.* (2002) discovered that *Y. pestis* grown at 27 °C possessed predominantly tetraacylated lipid A with significantly higher L-Ara4N content, this lipid A was a stronger inducer of tumor necrosis factor. Knirel at al. (2005) reaffirmed these findings and suggested that a production of less immunogenic LPS at mammalian temperature (37 °C) may compromise the rapid immune response of a host.

Phenotypic variation of a LPS structures due to specific parameters of growth media may be reflected in the proportions of phosphate or PEtN groups, Kdo and heptoses, terminal sugars, amino acids, carbamoyl, and other non-stoichiometrical groups (Wilkinson 1996). Rosner *et al.* (1979) discovered that *E. coli* grown in low phosphate media possess two LPS fractions with a different degree of esterification in the fatty acyl groups. Tsai *et al.* (1983)

observed subtle variations in the SDS-PAGE profiles and in the galactose content of the *N. meningitidis* LPS extracted from cultures grown in different media under varied levels of aeration. McGee and Rest (1996) investigated LOS expression and serum resistance of *N. gonorrhoeae* strain F62 grown in different carbon sources and growth conditions. Authors noted that the presence of pyruvate and lactate in the growth medium significantly enhanced LOS sialylation, while a broth-grown bacteria expressed the highest sialyltransferase activity. Accordingly, pyruvate-grown *N. gonorrhoeae* was up to 16-fold more serum resistant than glucose-grown strains (McGee and Rest 1996).

Under certain growth conditions or in absence of selective pressure some Gram-negative bacteria may undergo irreversible smooth-to-rough (S-R) mutation. This type of LPS gradual shortening was used as a diagnostic marker for *Coxiella burnetii*, causative agent of Q fever (Lukáčová *et al.* 2007). Furthermore, it was shown, that *Salmonella* Anatum grown at lower temperature possess LPS lacking O-antigen with the higher *in vitro* phage-inactivating capacity (McConnell and Wright 1979).

*C. jejuni* and *C. coli* are thermotolerant and microaerophilic bacteria, their optimal growth conditions include a low oxygen tension (5% $O_2$, 10% $CO_2$, and 85% $N_2$) and relatively high temperature (42 °C). Garénaux *et al.* (2008) demonstrated that the oxidative stress response in *C. jejuni* is influenced by the growth temperature in a strain-dependent manner. Stintzi (2003) identified 336 genes (approximately 20% of *C. jejuni* genome) which were differentially expressed at 37 °C compared to 42 °C. Among them were the genes encoding the LOS biosynthesis and modification enzymes like UDP-glucose 4-epimerase (*galE*), phosphoheptose isomerase (*gmhA2*), Kdo transferase (*kdtB*), acylneuraminate cytidylyltransferase (*neuA2*), Neu5Ac synthetase (*neuB3*), putative ADP-heptose synthase (*waaE*), and a putative glycosyltransferase (*wlaE*) (Stintzi 2003).

Semchenko *et al.* (2010) discovered that *C. jejuni* strains of different origin grown at 37 °C and 42 °C possess heterogeneous LOS fractions: e.g., *C. jejuni* NCTC 11168 expressed the additional low-$M_r$ LOS form which was different in size and structure from the $GM_1$-mimicking LOS previously described by Corcoran and Moran (2007). The production of this low-$M_r$ LOS was temperature-dependent, a shift from 5% at 37 °C to 35% at 42 °C was observed (Figure 5). Authors excluded an influence of phase variation and suggested that the observed LOS diversity displayed by human and chicken isolates of *C. jejuni* grown at different temperatures may be associated with a stress response or adaptive processes occurring during the colonization of different hosts (Semchenko *et al.* 2010).

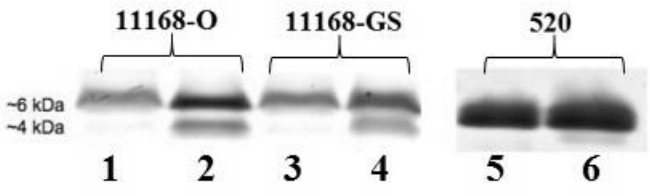



**Figure 5**. Silver-stained LOS of *C. jejuni* strains 11168-O, 11168-GS and 520 grown at 37 °C (lanes 1, 3, 5) and 42 °C (lanes 2, 4, 6). Adapted from Semchenko *et al.* (2010).

Corcoran and Moran (2007) reported about expression of additional polysaccharide fractions in *C. jejuni* NCTC 11168 influenced by growth conditions. One fraction developed the mid-$M_r$ bands on a gels stained with Alcian Blue and did not react with anti-$GM_1$ antibodies or cholera toxin, suggesting its attribution to capsular polysaccharides (Karlyshev 2000; Corcoran and Moran 2007). The second, previously undescribed, non-reactive, polysaccharide-related component was expressed at 37°C in broth cultures only. This component had a high-$M_r$ ladder-like band pattern on a silver stained gel similar to the neutral LPS of *C. jejuni* 81116 (Corcoran and Moran 2007).

### 2.8 LPS extraction, purification, and quantification.

A variety of methods have been developed for endotoxin/LPS extraction from whole cells (wet or dry) or cell-wall preparations from Gram-negative bacteria (Table 1). The phenol-water procedure of Palmer and Gerlough (1940) modified by Westphal *et al.* (1952, 1965) is the most widely used LPS extraction method due to its simplicity and efficiency: in a cooled biphasic phenol-water mixture (45:55, v/v) proteins dissolve in the phenol phase, while LPS, nucleic acids, and other polysaccharides remain in the aqueous phase.

However, several studies reported unexpected results in hot phenol-water R-LPS isolation – LOSs were found in an interphase and in a phenol phase (Kasai and Nowotny 1967). Thereafter, Galanos *et al.* (1969) established a new LOS extraction method utilizing phenol-chloroform-petroleum ether mixture (PCP), which is characterized by generally higher yields of water-soluble R-LPS and an exclusion of S-LPS forms. Lindberg and Holme (1972) evaluated several methods of LPS extraction from *Salmonella* Typhimurium: LPS obtained by the hot phenol-water method from mechanically disrupted γ-irradiated bacterial cells was more pure than LPS from acetone-dried or formaldehyde-fixed bacteria. Authors also confirmed that PCP-extraction is more suitable for R-LPS strains (Lindberg and Holme 1972).

**Table 1**. Methods of the endotoxin/LPS extraction employing the distinct treatments and agents, in chronological order. Adapted from Wilkinson (1996), Ridley *et al.* (2000).

| Treatment | Reference |
|---|---|
| 0.25 N Trichloroacetic acid, 4 °C for 3 h | Boivin *et al.* (1933) |
| 5% toluene, trypsin, 37 °C for 5 days, fractionation with ethanol | Raistrick and Topley (1934) |
| Diethylene glycol, 37 °C for 2 h, 0 °C for 2-3 d | Morgan (1937) |
| 2.5 M solution of urea, 38 °C for 9 h | Walker (1940) |
| 95% phenol, saline, ethanol precipitation | Palmer and Gerlough (1940) |
| 50% pyridine, 37 °C for 24 h | Goebel *et al.* (1945) |
| Water (filtrates of suspensions), 80 °C for 1 h | Roberts (1949) |
| 90% (w/v) phenol, 65-68 °C, 20-30 min. | Westphal *et al.* (1952) |
| 0.15 M NaCl, diethyl ether (1:2, by vol.), 12 °C | Ribi *et al.* (1961) |
| 5% cetyltrimethylammonium bromide, 5 °C for 10 min; pyridine-formic acid (76:49), 30 min | Nowotny *et al.* (1963) |
| Dimethyl sulphoxide (DMSO), 60 °C for 4 h | Adams (1967) |
| 89% aqueous phenol-chloroform-petroleum (2:5:8, by vol.), 5-20 °C for few min | Galanos *et al.* (1969) |
| 0.15 M NaCI-butanol (1:1), 0-4 °C for 15 min.; 0.5 M EDTA (pH 8.0-8.5), 37 °C for 15 min | Leive and Morrison (1972) |
| 90% (w/v) phenol, 70°C, 15 min, pretreatment with lysozyme (4 °C for 16 h), DNase, RNase | Johnson and Perry (1976) |
| 0.1 M EDTA, 2% SDS, 10 mM Tris, 37 °C, overnight. Ethanol precipitation. | Darveau and Hancock (1983) |
| 2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris (pH 6.8), 100°C for 10 min. | Hitchcock and Brown (1983) |
| Hot phenol-water method, gel filtration on a 2.5x100-cm Sephadex®G-75 column | Wu *et al.* (1987) |
| $MgCl_2$-Triton X-100 solution, 100°C for 10 min; 0.2 M EDTA (pH 8.0), 37 °C for 60 min | Uchida and Mizushima (1987) |
| Lyophilization of biomass. Water, 100 °C for 15 min. Proteinase K treatment. | Eidhin and Mouton (1993) |
| 96% (v/v) ethanol, methanol extraction | Nurminen and Vaara (1996) |
| 30 mM sodium triphosphate pentabasic, 1 h, supercritical carbon dioxide, 10 g/min, 90 °C | Rybka *et al.* (2008) |

Nowotny *et al.* (1963) concluded that a different species of Enterobacteriaceae require different LPS isolation procedures. Johnson and Perry (1976) improved the yield of the conventional phenol-water procedure (1.7-12.4 - fold) by preceding disruption of cells by grinding with glass beads or pretreating with lysozyme in presence of EDTA. Aforementioned method was adapted for LOS and further improved by Wu *et al.* (1987) by applying a gel filtration resulted in better yield (30-108% higher) and purity. Darveau and Hancock (1983) developed a LPS isolation technique employing a solubilization step with sodium dodecyl sulfate (SDS), which was effective in extracting high yields of both smooth and rough LPS.

Nevertheless, undesirable compounds such as proteins, RNA, and polysaccharides often contaminate LPS preparations obtained by the different isolation procedures. Essentially pure LPS requires the additional purification steps: ultracentrifugation, ethanol precipitation, enzymatic treatment with DNase, RNase, proteinase K, dialysis and/or gel filtration (Leive and Morrison 1972; Darveau and Hancock 1983; Wu *et al.* 1987). Muck *et al.* (1999) compared the traditional LPS purification methods, such as enzymatic digestion

and ultracentrifugation, and the separation by hydrophobic interaction chromatography (HIC): the latter technique produced LPS of superior purity with a minimal loss of product.

Logan and Trust (1984) applied the hot-phenol method for LOS extraction from *C. jejuni* and *C. coli*: resulted LOS yield was ~ 0.8% of the *Campylobacter* dry weight, and the lyophilized LOS was considerably less soluble than reference *E. coli* LPS. Perez-Perez and Blaser (1985) found that the LPS extraction procedure of Darveau and Hancock (1983) was more suitable for serum resistant *C. fetus* strains, while the phenol-water method had greater yields in serum-sensitive *C. jejuni* and *C. fetus* strains; overall LPS yields ranged from 0.9 to 8.3%. Blake and Russel (1993) emphasized the role of technical factors in the isolation of *C. jejuni* LOS by comparing the results of the PCP method of Galanos *et al.* (1969) and the rapid isolation-staining method of Al-Hendy *et al.* (1991).

Determination of LPS in the extracted fractions is traditionally based on the detection of the lipid A, heptose and/or Kdo (Vigdahl 1967; Osborn *et al.* 1972; Darveau and Hancock 1983; Perez-Perez and Blaser 1985; Wu *et al.* 1987). Kdo can be identified by gas-liquid chromatography, mass spectrometry, or by a colorimetric assay with thiobarbituric acid (Waravdekar and Saslaw 1959; Weissbach and Hurwitz 1959). However, the thiobarbituric acid assay is not applicable for certain bacteria, including *V. cholera, H. influenza*, *B. pertussis*, *C. coli* and *C. jejuni*, which contain only one Kdo at the C-4 or C-5 position, impeding the periodic acid digestion (Brade et. 1983; Carof *et al.* 1987; Lodowska *et al.* 2013). In order to overcome this obstacle, Lee and Tsai (1999) developed a new method of bacterial LPS measurement – the purpald assay, in which the unsubstituted terminal vicinal glycol (UTVG) groups of the Kdo and heptoses are oxidized by periodate, producing formaldehyde measurable spectrophotometrically via reaction with the purpald reagent.

Another commonly used method for LPS quantification is the turbidimetric Limulus amoebocyte lysate (LAL) assay which is 3 to 300 times more sensitive than the rabbit pyrogen test (Wachtel and Tsuji 1977). Gu *et al.* (1995) applied quantitative densitometry of silver-stained SDS-PAGE gels for measuring the levels of released and cell-bound LOSs from *H. influenzae* strains. Authors concluded that this method was 100-fold more sensitive than the Kdo assay but less sensitive than LAL assay.

### 2.9 Innate immunity and Toll-like receptors.

The human immune system is responsible for the recognition and subsequent elimination of different pathogenic entities via acute inflammation. The innate immune system response is triggered by microbial invasion or by the presence of endotoxins. The

main cell-effectors are phagocytes: dendritic cells (DCs), macrophages, and neutrophils. However, non-specialized cells such as epithelial and endothelial cells may also contribute to innate immunity (Takeuchi and Akira 2010). The innate immune response is important not only for pathogen clearance, but also for the activation of acquired immunity employing T- and B-lymphocytes (Medzhitov and Janeway 1997a).

The concept of immune recognition was proposed by Janeway (1989): he coined the term "pattern recognition receptors" (PRRs), describing the receptors on antigen-presenting cells (APC) which sense a microbial presence and induce a second signaling. PRRs are capable to detect the conserved structures of microorganisms, i.e. pathogen-associated molecular patterns (PAMPs). The recognizing of PAMPs by PRRs upregulates the transcription of genes responsible for inflammatory response, affecting the expression of various pro-inflammatory mediators, e.g. cytokines, interferons, chemokines (Medzhitov and Janeway 1997b, 2002). These mediators cause a complex cascade of events, such as exudation of fluids, increased vessel permeability, and attraction of leukocytes, resulting in phagocytosis and clearance of a microbes (Heumann and Roger 2002).

The family of Toll-like receptors (TLRs) is one of four PRRs families found in the innate immune system of mammals. TLRs are responsible for microbial presence detection outside the cell and in intracellular endosomes and lysosomes (Akira *et al.* 2006). The term "Toll" originally referred to a protein with a single transmembrane domain determining a dorsal-ventral orientation of the *Drosophila* embryo (Anderson *et al.* 1985). Lemaitre *et al.* (1996) demonstrated that Toll was crucial for the response of *Drosophila* to fungal infection. Later, a homologue of the *Drosophila* Toll receptor was discovered in mammals (Medzhitov *et al.* 1997).

TLRs consist of an extracellular domain with leucine-rich repeats and a cytosolic domain containing a TOLL/interleukin-1 receptor (TIR). To date 10 human TLRs have been identified, different TLRs recognize and specifically bind the different PAMPs and self-components (Table 2). TLR-specific ligands represent, for example, microbial lipids such as LPS, and nucleic acids from bacteria and viruses. Upon binding, PAMPs provoke the dimerization of two domains of TLRs, which in turn activates a different signaling pathways depending on the TIR domain-containing adaptor molecules recruited to receptors (O'Neill and Bowie 2007). In general, TLR signaling is organized via two distinct pathways employing MyD88 and TRIF adaptor molecules.

**Table 2**. Human TLRs and their ligands. Adapted from Pandey *et al.* (2015).

| Receptor | Main ligands | Associated pathogens |
|---|---|---|
| TLR1 | Triacylated lipoproteins | Bacteria, spirochetes, mycoplasma |
| TLR2 | Lipoproteins, peptidoglycan, LPS (some species) | Bacteria, viruses, parasites, self |
| TLR3 | dsRNA | Viruses |
| TLR4 | LPS (major species), respiratory syncytial virus (RSV) fusion protein | Gram-negative bacteria, RSV |
| TLR5 | Flagellin | Bacteria with flagella |
| TLR6 | Diacylated lipoproteins | Bacteria, viruses |
| TLR7 | ssRNA | Viruses, bacteria, self |
| TLR8 | ssRNA | Viruses |
| TLR9 | CpG motifs (DNA) | Bacteria |
| TLR10 | Unknown | - |

### 2.10 LPS recognition and TLR4 activation.

LPS acts as the endotoxin because it binds the CD14/TLR4/MD2 receptor complex in APCs, such as macrophages, monocytes, dendritic cells and B cells, which activates a host defense through secretion of reactive oxygen, nitric oxide, pro-inflammatory cytokines, and eicosanoids (Figure 6, A). Cytokines, particularly tumor-necrosis factor (TNF), interleukin (IL)-1 and IL-6, are prime mediators of the endotoxin activity, they act directly or via hormonal or paracrine system (Tracey and Cerami 1992). It should be noted, that the uncontrolled, massive release of kinins and eicosanoids is dangerous, as it may contribute to a pathological process and distort the balance between pro- and anti-inflammatory pathways causing hypotension, disseminated intravascular coagulation, septic shock, and death (Hodgson 2006).

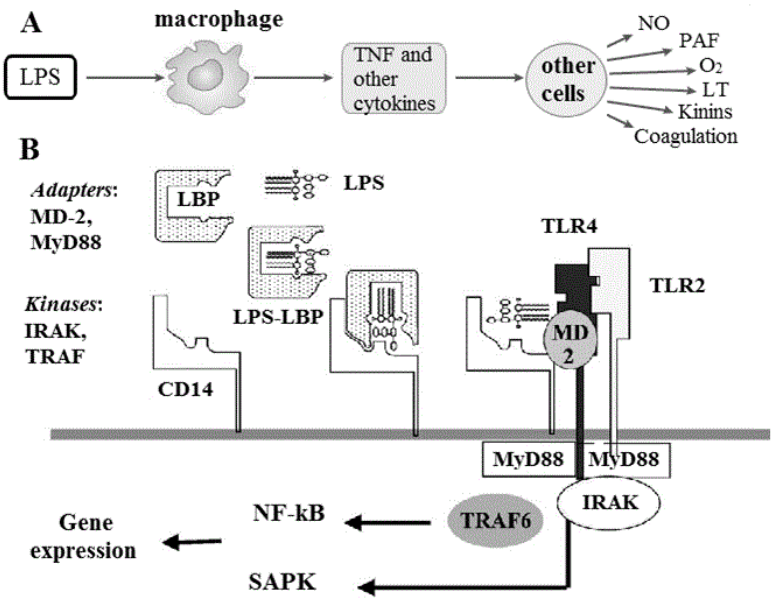



**Figure 6**. LPS-induced innate immune response (A), LPS recognition and a core pathway of TLR4 signaling (B). TNF, tumor-necrosis factor; NO, nitric oxide; PAF, platelet-activating factor; LT, lymphotoxin; SAPK, stress-activated protein kinase; IRAK, interleukin-1 (IL-1) receptor-associated kinase; TRAF6, TNF receptor-associated factor 6. Adapted from Beutler and Rietschel (2003); Heumann and Roger (2002) with a permission from the publisher.

Chow *et al.* (1999) demonstrated that TLR4 is engaged in LPS signaling and following activation of a transcription factor NF-κB. The important role of TLR4 was confirmed in TLR4-knockout mice which were unresponsive to LPS (Poltorak *et al.* 1998). Besides TLR4, recognition of LPS requires a participation of other molecules (Figure 6, B): LPS-binding protein (LBP), plasma membrane glycoprotein – the cluster differentiation antigen 14 (CD14), and an extracellular protein called myeloid differentiation factor 2, MD2 (Tobias *et al.* 1986; Wright *et al.* 1990; Shimazu *et al.* 1999).

LBP is a 60-kD glycoprotein synthesized by hepatocytes in the liver, LBP expression increases in response to endotoxin (Tobias *et al.* 1986; Schumann *et al.* 1990). The importance of LBP is determined by the amphiphilic nature of LPS molecules: in aqueous solution they form micelles poorly recognizable by monocytes and macrophages (Wright *et al.* 1989). LBP binds the LPS monomers through the lipid A moiety (Mathison *et al.* 1992; Taylor *et al.* 1995) and transfers them to CD14, which is present at the surface of the myelomonocytic cells (mCD14) or circulates in the serum in soluble form (sCD14) (Gegner *et al.* 1995; Yu and Wright 1996).

CD14 acts as a functional receptor for LPS, it drastically increases the sensitivity of cells to lipid A, allowing to bind picomolar concentrations of endotoxin (Lee *et al.* 1992). Haziot *et al.* (1996) discovered that CD14-deficient mice, in spite of expression of the other proteins necessary for LPS recognition, were resistant to endotoxic shock. In contrast, Huber *et al.* (2006) stated that R-LPS (LOS) can activate cells independently from the CD14. Structurally CD14 is a dimer, which contains a hydrophobic "pocket" binding the acyl chains of the lipid A (Kim *et al.* 2005). CD14 lacks an endoplasmic domain, thus the transmembrane proteins, TLR4 and MD2, are needed for a further signal transduction (Akira 2003).

CD14 transfers the LPS molecules to the TLR4-MD2 receptor complex; MD2 is attached to the TLR4 ectodomain and it is crucial for LPS signaling (Shimazu *et al.* 1999; Gangloff and Gay 2004). The number, position and length of the lipid A fatty acids affect MD2 conformation, hence, influencing TLR4 activation (Seydel *et al.* 2000; Hajjar *et al.* 2002). For example, a lipid A with five acyl chains is 100-fold less active than the "canonical" variant, while Eritoran, a synthetic analogue of lipid A with four acyl chains, completely lack agonistic activity and acts as TLR4 antagonist (Teghanemt *et al.* 2005). The crystallographic studies on TLR4, MD2, and LPS interaction revealed that two TLR4-MD2-LPS complexes form a symmetrical m-shaped homodimer (Park *et al.* 2009).

Stimulation with LPS initiates TLR4 signaling through recruitment of the adapter molecule MyD88 and the IL-1 receptor-associated kinase (IRAK) (Muzio *et al.* 1998).

MyD88, myeloid differentiation factor 88, is the Toll-like cytoplasmic adapter, which binds to TLR4 via TIR domain adaptor protein (TIRAP) (Kagan and Medzhitov 2006). Following interaction of MyD88 with IRAK, a serine/threonine kinase with an N-terminal death domain (Takeuchi and Akira 2010), activates the NF-κB via TRAF6 (Figure 6, B). Apart from MyD88-dependent signaling, TLR4 triggers a TRIF-dependent pathway resulting in the activation of IRF3 transcription factor (Yamamoto *et al.* 2003). Nevertheless, the MyD88 gene was shown to be critical for TLR signaling: MyD88-deficient mice were unresponsive to LPS (Adachi *et al.* 1998; Kawai *et al.* 1999).

### 2.11 Innate immune response to *Campylobacter*

In the intestine, *Campylobacter* adhere to and invade the intestinal epithelial cells, inducing a pro-inflammatory response in the host tissues (Janssen *et al.* 2008). In order to evade the innate immunity system, bacteria employ different strategies. Watson and Galan (2005) discovered that *C. jejuni* flagellin poorly stimulates TLR5 and pro-inflammatory cytokine production. Furthermore, it has been shown that DNA from *C. jejuni* weakly activates TLR9 due to low GC content (Dalpke *et al.* 2006).

Nonetheless, innate immunity is crucial for host defense. The essential role of TLR pathways was determined in MyD88-deficient mice, which were prone to *Campylobacter* infection and colonization (Watson *et al.* 2007). Previous studies showed that *C. jejuni* infection of human monocytic cells initiates NF-κB translocation (Mellits *et al.* 2002; Jones *et al.* 2003); NF-κB-dependent mechanism of *C. jejuni* clearance was proven in knockout mice (Fox *et al.* 2004). A prominent role of the intracellular PRR, NOD1, in innate response to *C. jejuni* was elucidated by Zilbauer *et al.* (2007), authors suggested that activation of both TLRs and NOD-like receptors by different PAMPs can modulate invasion and epithelial innate responses.

*C. jejuni* CPS can stimulate IL-6 secretion from intestinal epithelial cells through TLR2 in a MyD88-independent manner (Friis *et al.* 2009). High levels of NF-κB activation by lysed bacteria via human TLR1/2/6 and TLR4 and chicken TLR2t2/16 and TLR4 were reported by de Zoete *et al.* (2010). Interestingly, viable *C. coli* activated TLR4 moderately, while no response was detected for viable *C. jejuni* strains (de Zoete *et al.* 2010). Hu *et al.* (2006) observed *in vitro* maturation of dendritic cells (DCs) infected with viable and dead *Campylobacter* cells, followed by the activation of multiple cytokines of both innate and specific immune systems. Notably, purified LOS from *C. jejuni* strain 81-176 used in the study induced high levels of TNF, gamma interferon, and interleukins, implying a key role of LOS in the stimulation of cytokines in DCs (Hu *et al.* 2006).

Variations in *Campylobacter* LOS structure and composition influence TLR4 activation. According to van Mourik *et al.* (2010) inactivation of the genes encoding synthesis of GlcN3N precursor enhances the TLR4-MD2-mediated NF-κB response and susceptibility to antimicrobial peptides (AMPs). In contrast, removal of PEtN from *C. jejuni* lipid A resulted in attenuated TLR4-MD2 activation and decreased resistance to mammalian and avian AMPs (Cullen *et al.* 2013). Kuijf *et al.* (2010) reported that sialylation of *C. jejuni* LOS increases TLR4 and DCs activation, as well as B cell proliferation. Recently, a cumulative effect of *C. jejuni* LOS modifications, namely, sialylation, phosphorylation and abundance of ester linkages, on TNF induction and TLR4 signaling was observed (Stephenson *et al.* 2013).

# EXPERIMENTAL RESEARCH

## 3 AIMS OF THE STUDY

The main aim of this thesis was to investigate whether different growth conditions (i.e. growth medium and temperature) affect *C. coli* LOS expression and its immunogenicity. The objectives were:

1. To compare electrophoretic patterns of LOS extracted from *Campylobacter coli* grown under different conditions.
2. To evaluate the effect of different growth conditions on human Toll-like receptor 4 (TLR4) response using the HEK-Blue™ hTLR4 cells.
3. To highlight possible method-based biases in studying *Campylobacter* LOS-host interaction.

## 4 MATERIALS AND METHODS

### 4.1 Bacterial strains and growth conditions

Eight *Campylobacter coli* strains were selected for the present study from the laboratory collection (Table 3). Pure cultures were obtained from -70 °C stock and routinely grown at 37 ºC under microaerobic conditions (85% $N_2$, 10% $CO_2$, 5% $H_2$) on NBA agar comprised of the nutrient broth № 2 (CM0067, Oxoid Ltd. Basingstoke, UK) and 1.5% agar, supplemented with 5% (v/v) of defibrinated horse blood, unless stated otherwise.

**Table 3**. Description of *C. coli* strains selected for a study.

| Strain number | Strain | Source | Year | Country | LOS class | Reference |
|---|---|---|---|---|---|---|
| 38 | DSM-24199 | Pig | - | Denmark | VIII | Campynet collection |
| 45 | FB6470 | Human | 1996 | Finland | X | Kärenlampi *et al.* (2007) |
| 51 | 76339 | Human | 2006 | Finland | IX | Skarp-de Haan *et al.* (2014) |
| 73 | 2946 | Poultry | 1999 | Finland | II | Kärenlampi *et al.* (2007) |
| 138 | E107.3 | Pig | 2009 | Finland | X | Olkkola *et al.* (2010) |
| 151 | E163.2 | Pig | 2009 | Finland | II | Juntunen *et al.* (2010, 2011) |
| 137 | E90.1 | Pig | 2009 | Finland | II | Olkkola *et al.* (2010) |
| 177 | A35.1 | Pig | 2007 | Finland | IV | Juntunen *et al.* (2010, 2011) |

Effect of growth conditions on LOS expression was assessed by cultivating *C. coli* strains at different temperatures (37, 39, and 42 °C, corresponding to the human, swine, and avian host body temperature, respectively) for 24 hours in presence/absence of defibrinated horse blood (NBA/NA agar), 5% (v/v), and presence/absence of N-acetylneuraminic acid – Neu5Ac (Sigma-Aldrich, St. Louis, MA, USA), 50 μm/ml. *C. coli* strains were grown under different conditions three times independently (three biological replicates).

### 4.2 LOS extraction

During the present investigation *C. coli* LOSs were extracted by different methods which are described below.

#### 4.2.1 Fast crude LOS extraction

NA/NBA plates with *C. coli* strains grown at 37 ºC were washed with 1 ml Dulbecco's Phosphate Buffered Saline (DPBS, Sigma-Aldrich Corporation, St. Louis, MA, USA). Bacterial suspensions were adjusted by $OD_{600}$ measurement to 0.5 and treated proteinase K (0.6 μg/μl) (Thermo Fisher Scientific, Waltham, MA, USA) at 55°C for 1 hour. Samples were inactivated by boiling for 10 min. Then, LOS samples were run in 15% acrylamide gel and visualized by silver staining.

#### 4.2.2 Mini-phenol-water LOS extraction

*C. coli* biomass from NA and NBA plates was collected by washing with endotoxin-free DPBS. Bacterial suspensions were centrifuged at 5000 g for 5 min, pellets were washed 3 times with DPBS and finally resuspended in 1.5 ml DPBS, and stored at -70 °C until further use.

Afterwards, 750 μl aliquots of bacterial mass samples were centrifuged at 5000 g for 5 min, supernatants were discarded, and pellets were resuspended in 750 μl PCR water. LOSs were extracted using mini-phenol-water method of Prendergast *et al.* (2001), with some modifications. Briefly, 750 μl pre-heated (60 °C) 90% (v/v) aqueous phenol was added to each sample, tubes were vortexed for 1 min and incubated at 65 °C for 15 min, with vortexing for 30 sec at 2 min intervals. Tubes then were cooled on ice and centrifuged at 15000 g for 3 min. The upper clear aqueous phase containing the LOS was transferred to a new tube. Then, LOS was re-extracted from the phenol phase by adding 300 μl of sterile water.

Residual phenol from the LOS phase was removed by layering 0.5 ml diethyl ether, vortexing, centrifuging (15000 g, 3 min, 4 °C) and discarding the ether phase 3 times. Remaining diethyl ether was removed from LOS samples by evaporation for 1 hour. Extracted LOS were stored at -20 °C until further use.

#### 4.2.3 Hot phenol-water LOS extraction

*C. coli* biomass was obtained as previously described. The pellets were resuspended in 10 ml buffer containing 10 mM Tris-HCL and 5 mM EDTA (pH 7.4), and digested with lysozyme (1 mg/ml), RNase A (50 μg/ml), DNase I (25 μg/ml), and proteinase K (100

µg/ml). Bacterial lysates were then subjected to LOS extraction employing the hot phenol-water method (Westphal *et al.* 1952), essentially as described above. After diethyl ether extraction the LOS samples of two biological repetitions were mixed with two volumes of cold absolute ethanol with 0.375 M sodium acetate pH 5.4 and incubated overnight at -20 °C. Upon ethanol precipitation samples were centrifuged at 16000 g for 40 min, pellets were washed with 70% cold ethanol and centrifuged again under the same conditions. Dried pellets were resuspended in 1 ml sterile endotoxin-free water and were stored at 4 °C until further use.

### 4.3 LOS lyophilization

LOS samples extracted by the hot phenol-water method were lyophilized using DryWinner 3 freeze-dryer (Heto Holten, Denmark) following the manufacturer's instructions (-53 °C, ~0.1 hPa, overnight).

### 4.4 Agarose gel electrophoresis

In order to detect possible DNA/RNA contamination LOS samples were subjected to electrophoresis in a 1.5% (w/v) SeaKem® LE Agarose gel (Lonza, Basel, Switzerland) stained with 1 mg/ml of ethidium bromide, in 1 X TAE buffer for one hour at 110 V, gels were viewed under UV light and photographed by the AlphaImager IS-2200 (Alpha Innotech Corp., San Leandro, CA, USA). Molecular weight marker GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used as a reference.

### 4.5 LOS concentration measurement

Quantification of LOS was performed by the purpald assay (Lee and Tsai 1999). In brief, 10 µl of 36 mM $NaIO_4$ was added to 10 µl of LOS samples and incubated for 25 min at room temperature. Then, 10 µl of 136 mM purpald reagent (Sigma-Aldrich, St. Louis, MA, USA) was added. After incubation for 20 min at room temperature, 10 µl of 64 mM $NaIO_4$ was added and samples were further incubated for 20 min. Finally, samples were measured at 550 nm with NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and LPS of *E. coli* 0111:B4 (Sigma-Aldrich, St. Louis, MA, USA) was used as a standard.

### 4.6 SDS-PAGE of LOS

The concentration of the LOS samples was adjusted to 0.3 µg/µl in 20 µl prior to electrophoresis. Then, 20 µl of Laemmli sample buffer (Bio-Rad Lab. Inc., Hercules, CA, USA) with 4% 2-mercaptoethanol was added (1:1), and 13 µl of mixture was subjected to

15% (v/v) sodium dodecyl suphate-polyacrylamide gel electrophoresis (SDS-PAGE), which was conducted at 20 mA for 2 h. LPS of *E. coli* 0111:B4 (0.3 μg/μl) was used as a control. Gels were silver stained (Tsai and Frasch 1982), and images were captured and analyzed using the GS-800™ calibrated densitometer and PDQuest 2-D analysis software, version 7.3 (Bio-Rad Lab. Inc., Hercules, CA, USA).

### 4.7 Detection of sialic acid

NBA plates with *C. coli* and *C. jejuni* 81-176 (positive control) biomass were washed with DPBS. Then, 200 μl aliquots of each samples were adjusted by $OD_{600}$ measurement to 0.5, and proteinase K (0.6 μg/μl) was added. Afterwards, tubes were incubated at 55°C for 1 hour, followed by boiling for 10 min. Aliquots (20 μl) were transferred to two 1.5 ml Eppendorf tubes containing either 5 μl of sterile endotoxin-free water (negative control) or neuraminidase from *Clostridium perfringens* (Sigma-Aldrich, St. Louis, MA, USA). Upon overnight incubation at 37 ºC samples were loaded to SDS-PAGE.

### 4.8 Cell cultures

HEK-Blue™ hTLR4 cells and HEK-Blue™ Null2 cells (InvivoGen, San Diego, CA, USA) were maintained and subcultured in DMEM (Life Technology, Carlsbad, CA, USA) according to manufacturer's instructions. The growth media were renewed as necessary; cells were passaged when a 70-80% confluency was reached.

Infection of HEK-Blue™ hTLR4 and HEK-Blue™ Null2 cells with LOS was done in triplicate (3 technical repetitions). Plates with 25000 cells/well were prepared and incubated for 18 hours at 37 °C, 5% $CO_2$. Upon incubation, cells were infected with LOS samples at a final concentration of 1 ng/ml and incubated for 18 hours again. LPS of *E. coli* 0111:B4 (0.0625 μg/μl) was used as positive control.

NF-κB-induced activity of secreted embryonic alkaline phosphatase (SEAP) was assessed using the QUANTI-Blue™ colorimetric assay (InvivoGen, San Diego, CA, USA), in accordance with the manufacturer's instructions. Absorbance at 620 nm was read by Multiskan™ FC microplate photometer (Thermo Fisher Scientific, Waltham, MA, USA).

### 4.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6 for Windows (San Diego, CA, USA). One-way ANOVA with Tukey-Kramer post-test (cut-off 0.05) was applied for a group comparison, for pairwise comparison paired and unpaired two-tailed t-

tests with Welch correction were used. Error bars in the graphs in figures represent either Standard Error of the Mean (SEM), or Standard Deviation (SD).

## 5 RESULTS

### 5.1 LOS mobility patterns

LOSs from *C. coli* strains 38, 45, 51, 73, 138, and 151 grown under different conditions (37 °C, 39 °C, 42 °C / NA and NBA), and *C. jejuni* strain 81-176 grown at 37 °C on NBA were run on SDS-PAGE. Figure 7 shows the LOSs extracted with mini-phenol-water method, Figure 8 demonstrates the LOSs of six *C. coli* strains extracted with hot-phenol method, and Figure 9 – the pattern from crude extracts. All tested LOS samples extracted with both methods resolved as a one band of low Mr (~4 kDa). The observed differences in electrophoretic mobility of the LOS samples were strain-dependent. A different intensity of the bands or their absence (Figure 7, A, lanes 8-11) on the SDS-PAGE gels was unexpected, as all LOS samples were standardized prior to electrophoresis.

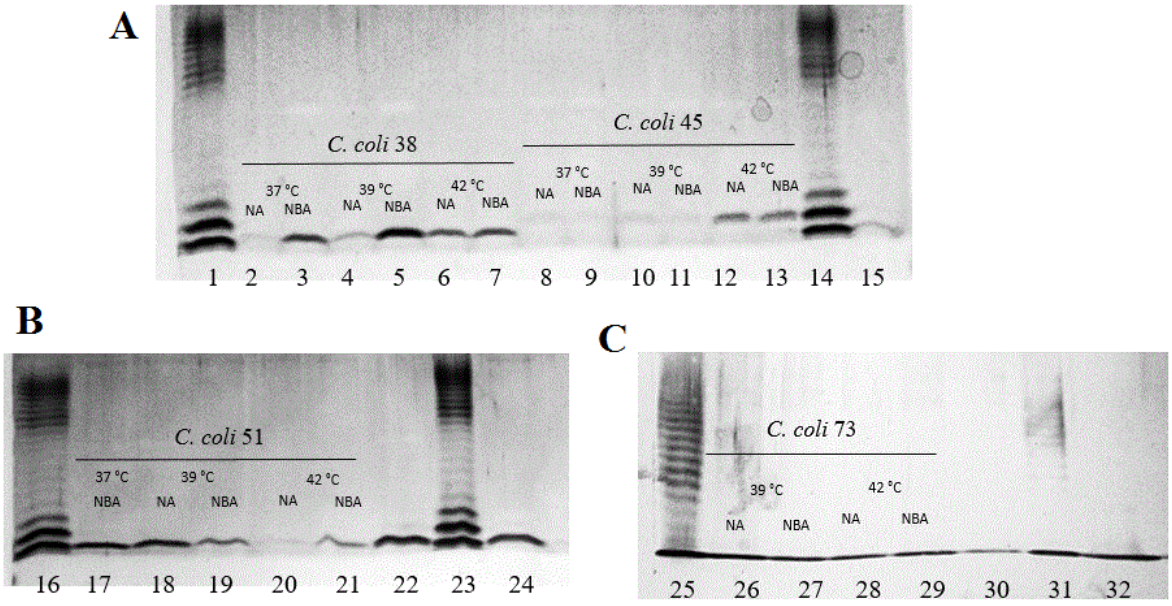



**Figure 7.** Silver stained SDS-PAGE gels of miniphenol-water-extracted LOSs from *C. coli* strains grown under various conditions. Lanes 1, 14, 16, 23, 25, and 31 contain the purified LPS from *E. coli* 0111:B4 as a positive control. Lanes 2-7 contain LOS samples of *C. coli* 38 grown on NA agar (2, 4, 6) and NBA agar (3, 5, 7) at 37°C (2, 3), 39°C (4, 5) and 42 °C (6, 7). Same pattern of LOS samples applies to *C. coli* 45 (lanes 8-13), *C. coli* 51 (15, 17-21) and *C. coli* 73 (22, 24, 26-29). Lanes 30 and 32 contain LOS of *C. jejuni* 81-176. LOS samples were standardized to 0.3 µg/µl by the purpald assay.

Furthermore, SDS-PAGE analysis revealed that LOSs of *C. coli* strains 45 and 138 were absent in two biological repetitions (Figure 8, lanes 6-9, 21-24, and 37-44). Moreover, 24 LOS samples of 3$^{rd}$ biological repetition adjusted to 0.3 µg/µl by the purpald assay were not detected on SDS-PAGE gels (Appendix I).

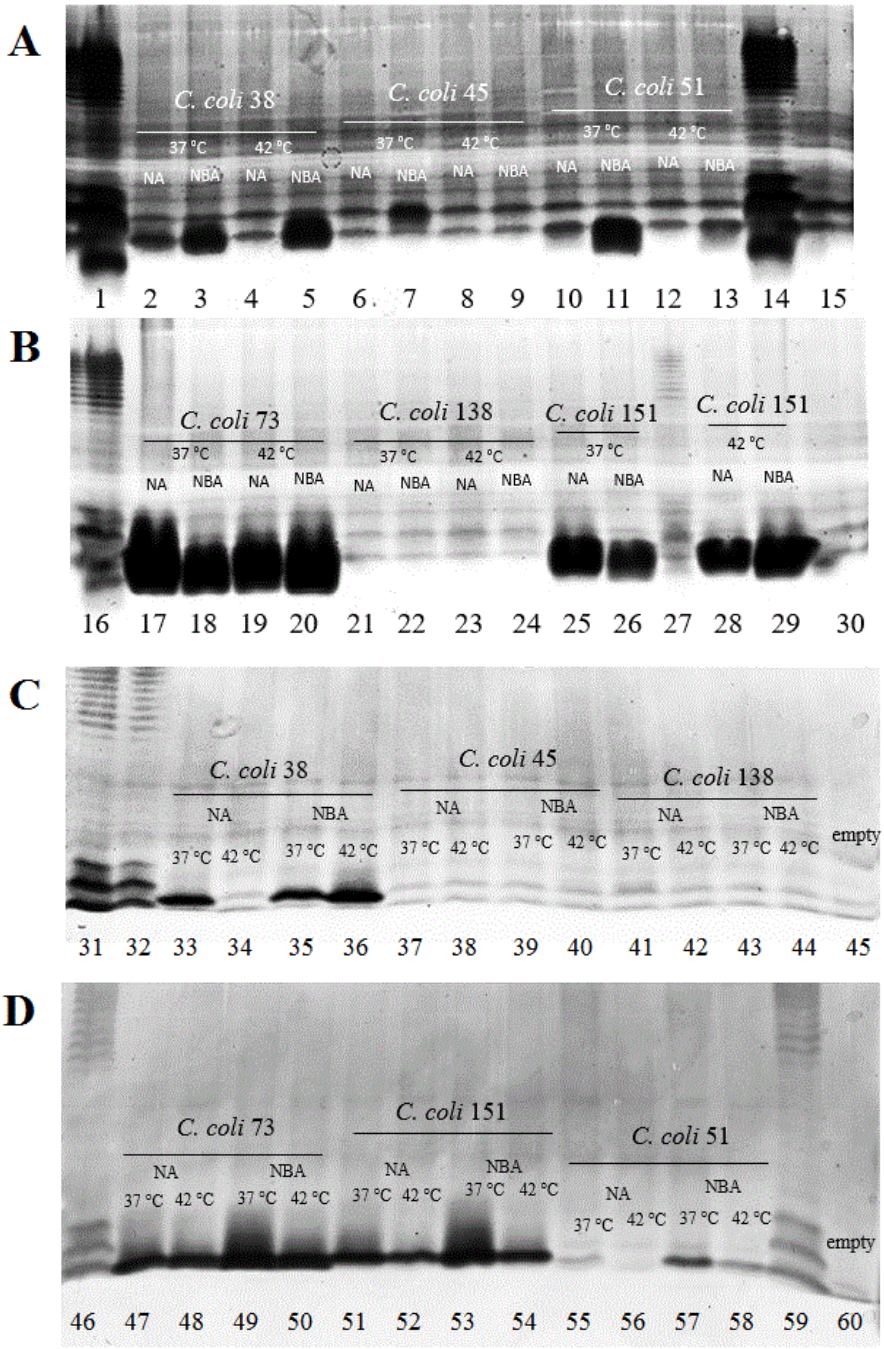



**Figure 8.** Silver stained SDS-PAGE gels of the phenol-water-extracted LOS from *C. coli* biomass grown under different conditions, 1st rep. samples (A, B) and 2nd rep. (C, D). Strains and growth conditions tested are indicated above the bands. Lanes 1, 14, 16 27, 31, 32, 46, and 59 contain the purified LPS from *E. coli* 0111:B4 as a positive control. Lanes 15, 30, 45, and 60 are empty, negative control. LOS samples were precipitated in ethanol prior to freeze-drying, except the samples in lanes 34, 37, 38, 39, 43, 44, and 56 which were freeze-dried only. LOS concentration was measured by the purpald assay. Samples were standardized to 0.3 µg/µl prior to electrophoresis.

The crude-extracted LOS preparations of six *C. coli* strains grown on NBA and NA media at 37 °C were subjected to SDS-PAGE (Figure 9). As it was observed previously in this study, $M_r$ of isolated LOS samples varied between the strains tested, but not between the growth conditions (Figure 9, A).

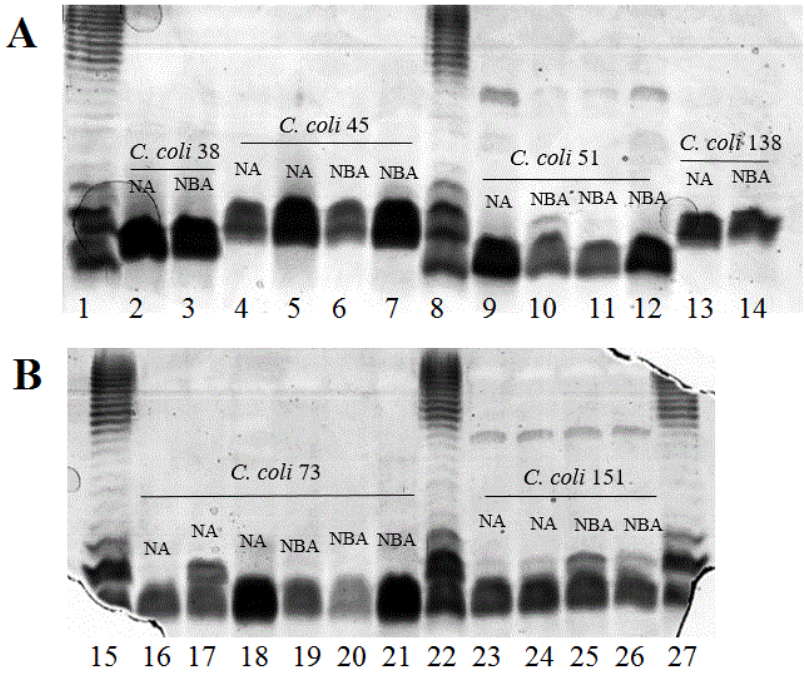



**Figure 9.** Silver staining of crude LOS samples of *C. coli* strains 38, 45, 51, 73, 138, and 151 (digested cell lysates). Strains were grown on NA and NBA at 37 °C, obtained biomass was standardized by $OD_{600}$ measurement and treated with proteinase K. Lanes 1, 8, 15, 22, and 27 contain the purified LPS from *E. coli* 0111:B4 (0.3 µg/µl). Lanes 2, 3, 5, 7, 9, 12, 18, and 21 contain the crude LOS of one repetition.

### 5.2 Standardization by weight

Due to the observed discrepancies between the estimated and actual LOS quantities, electrophoretic analysis of LOS banding patterns was repeated. The LOS samples of 3rd biological repetition were re-lyophilized, weighed and resuspended in endotoxin-free water to a concentration of 2-4 mg/ml. However, detection of LOS in SDS-PAGE was still not possible in all the cases (Figure 10, A). No significant differences in electrophoretic mobility of the studied LOS samples were detected (Figure 10, B).

Accuracy of the both methods of LOS concentration measurement applied in the present study, weighing and the purpald assay, was evaluated. The lyophilized LOS samples of *C. coli* strains 73 and 45 (3rd rep., 37 °C, NA) were adjusted to the concentrations used in the purpald assay for a standard curve construction (2-0.125 µg/µl). These LOS samples and the purified LPS from *E. coli* 0111:B4 were loaded to SDS-PAGE and analyzed by the purpald assay (Figure 11). While a dilution pattern of *C. coli* 73 LOS was clearly distinguishable on the SDS-PAGE gel, the resuspended freeze-dried LOS preparations of *C. coli* strain 45 did not yield a bands (Figure 11, A). Moreover, at least 60-fold overestimation of a LOS concentration by the purpald assay was observed. Both *C. coli* 73 and 45 LOS at concentrations of 2, 1 and 0.5 µg/µl caused a saturation of the reagents used in the purpald assay, while LOS samples of 0.25 µg/µl and 0.125 µg/µl were beyond the range of the standard curve (Figure 11, B).

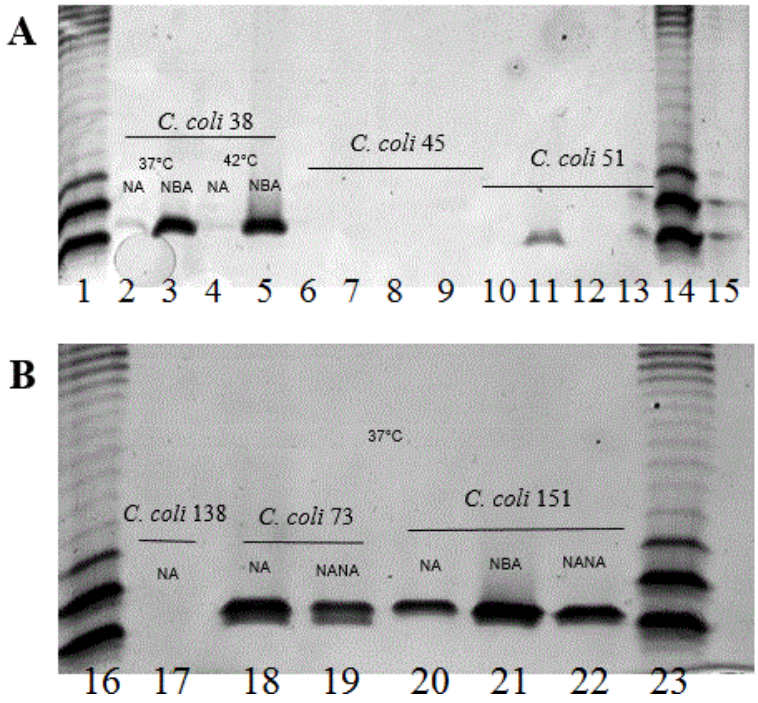



**Figure 10.** Silver stained SDS-PAGE gels of the phenol-extracted and re-lyophilized *C. coli* LOS samples, 3rd rep. (A), and LOS samples of *C. coli* strains 73 and 151 grown in presence of Neu5Ac at 37 °C (B). All lyophilized LOS samples were weighed and adjusted to 2 µg/µl prior to electrophoresis. The strains and conditions tested are indicated above the bands. Lanes 1, 14, 16, and 23 contain the purified LPS from E. *coli* 0111:B4 (0.3 µg/µl) as a positive control.

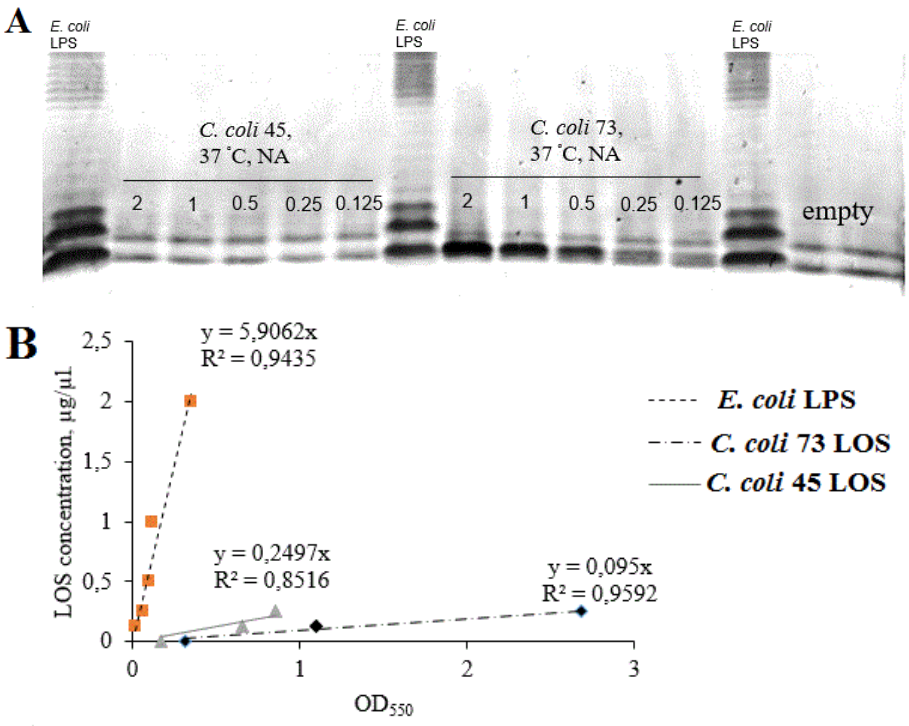



**Figure 11.** Silver stained SDS-PAGE gel of *C. coli* strains 45 and 73 LOS (3rd rep.), freeze-dried, weighed and diluted (A); the purpald assay of the same LOS samples and LPS from *E. coli* 0111:B4, latter was used for a standard curve (B). Numbers above the bands indicate the LOS concentration, µg/µl.

### 5.3 Neuraminidase treatment

The crude-extracted LOS samples of *C. coli* strains 51, 137 and 177 were examined for the presence of sialic acid, and *C. jejuni* strain 81-176 was used as a positive control. These three *C. coli* strains were selected since they showed the presence of a glycosyltransferse GT-42 family homologs (Culebro *et al.* 2016). Cleavage of the sialic acid moiety by neuraminidase resulted in a shift in the control LOS band only (Figure 12, lane 13). In this way, it can be suggested, that *C. coli* strains 51, 137 and 177 do not possess sialylated LOS (Figure 12, lanes 2, 3, 5, 6, 8, and 9).

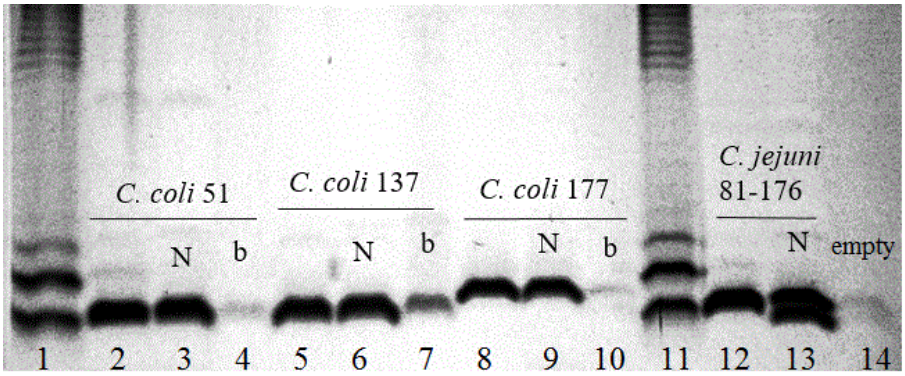



**Figure 12.** Silver staining of *C. coli* 51, 137, 177, and *C. jejuni* 81-176 crude LOS samples (digested cell lysates). Strains were grown on NBA at 37 °C, obtained biomass was standardized by $OD_{600}$ measurement and treated with proteinase K. The LOS samples treated with neuromidase from *C. perfringens* are denoted as "N" (lanes 3, 6, 10, and 13), "b" - LOSs isolated from strains grown in liquid culture (lanes 4, 7, 10). Lanes 1 and 11 contain the purified LPS from *E. coli* 0111:B4 (0.3 µg/µl).

### 5.4 TLR4 activation

#### 5.4.1 Effect of LOS concentration

*C. coli* LOS samples isolated by the hot phenol-water method were free from DNA contamination (Appendix II). HEK-293 cells stably transfected with human TLR4-MD2-CD14 (HEK-Blue™ hTLR4) and parental HEK-Blue™ Null2 cells were stimulated with 1 ng/ml of the LOSs of six *C. coli* strains grown under different conditions. The NF-κB induction in HEK-Blue™ Null2 cells treated with *C. coli* LOS was not different from untreated cells ($OD_{620}$, 0.053±0.001). Unexpectedly, a majority of the tested LOS samples poorly activated a TLR4 response (Figure 13). Observed differences in levels of NF-κB activation among LOSs of two biological repetitions were not statistically different ($p > 0.05$). Considerable variations in TLR4 response triggered by the LOS samples of the same *C. coli* strains, as well as between biological repetitions reflected incorrect estimation of LOS concentration. In this way, it was not possible to evaluate the effect of the growth conditions on immunogenicity of the LOS samples standardized by the purpald assay.

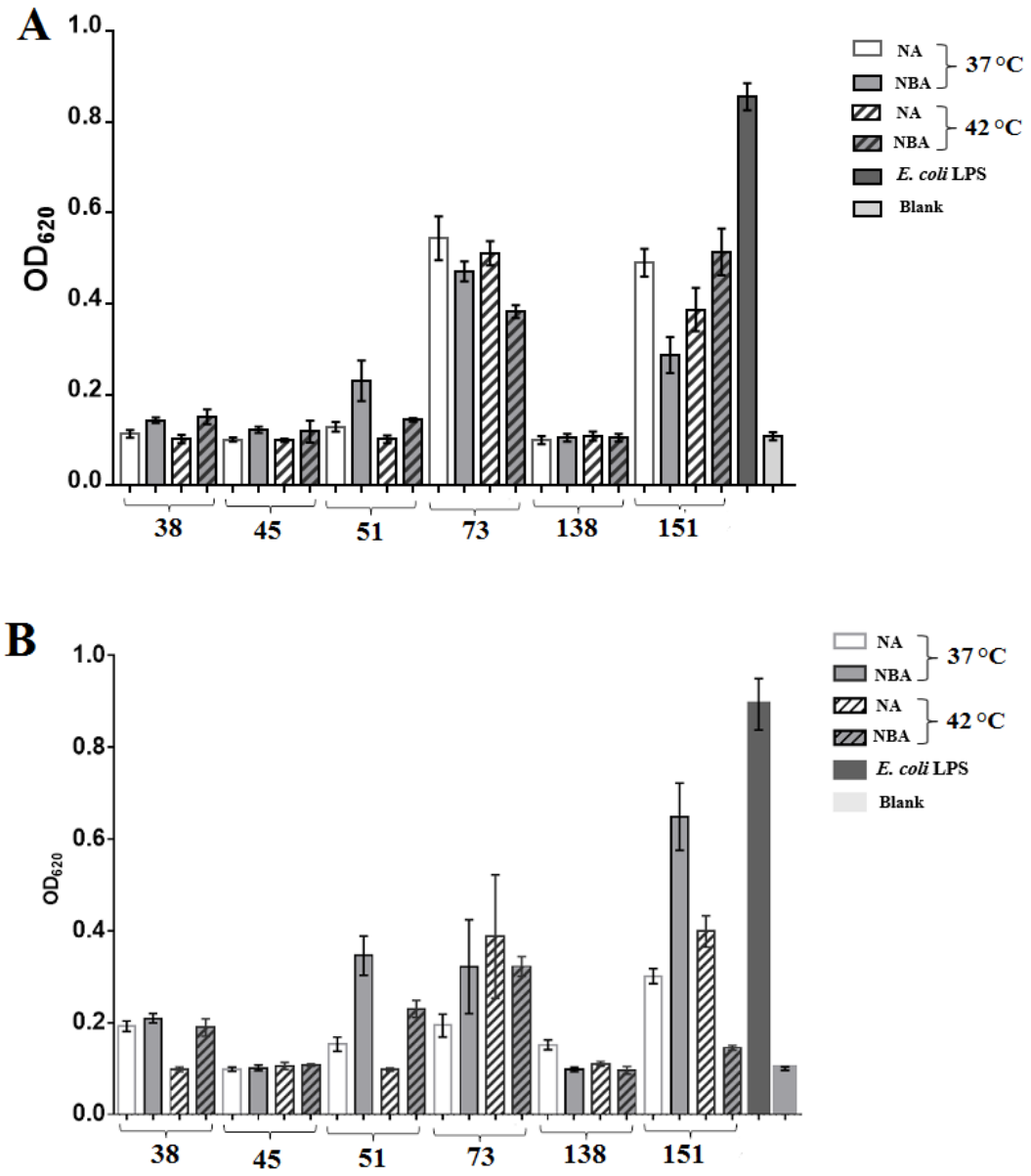



**Figure 13.** Human TLR4 activation by *C. coli* LOS (1 ng/ml): samples of the 1st biological repetition (A) and 2nd biological repetition (B). Data represent average of three technical replicates. TLR4 activation was assessed colorimetrically using a SEAP reporter gene placed under the control of an NF-κB-inducible promoter. LPS from *E. coli* 0111:B4 was used as the positive control. Error bars show ± SEM. Numbers under X-axis indicate the strain number.

### 5.4.2 TLR4 stimulation with crude LOS

The crude-extracted LOS samples of six *C. coli* strains induced a high TLR4 response (Figure 14, B). Importantly, HEK-Blue™ Null2 cells were not triggered, which indicated an absence of signaling by TLR4-unspecific NF-κB-agonists (Figure 14, A). The effect of different growth medium on LOS immunogenicity was assessed only for *C. coli* strain 73, as LOS samples of this strain only were present in 3 independent biological replicates. It can be suggested that a growth medium did not affect LOS agonistic activity of *C. coli* strain 73, $p > 0.05$ (Figure 15).

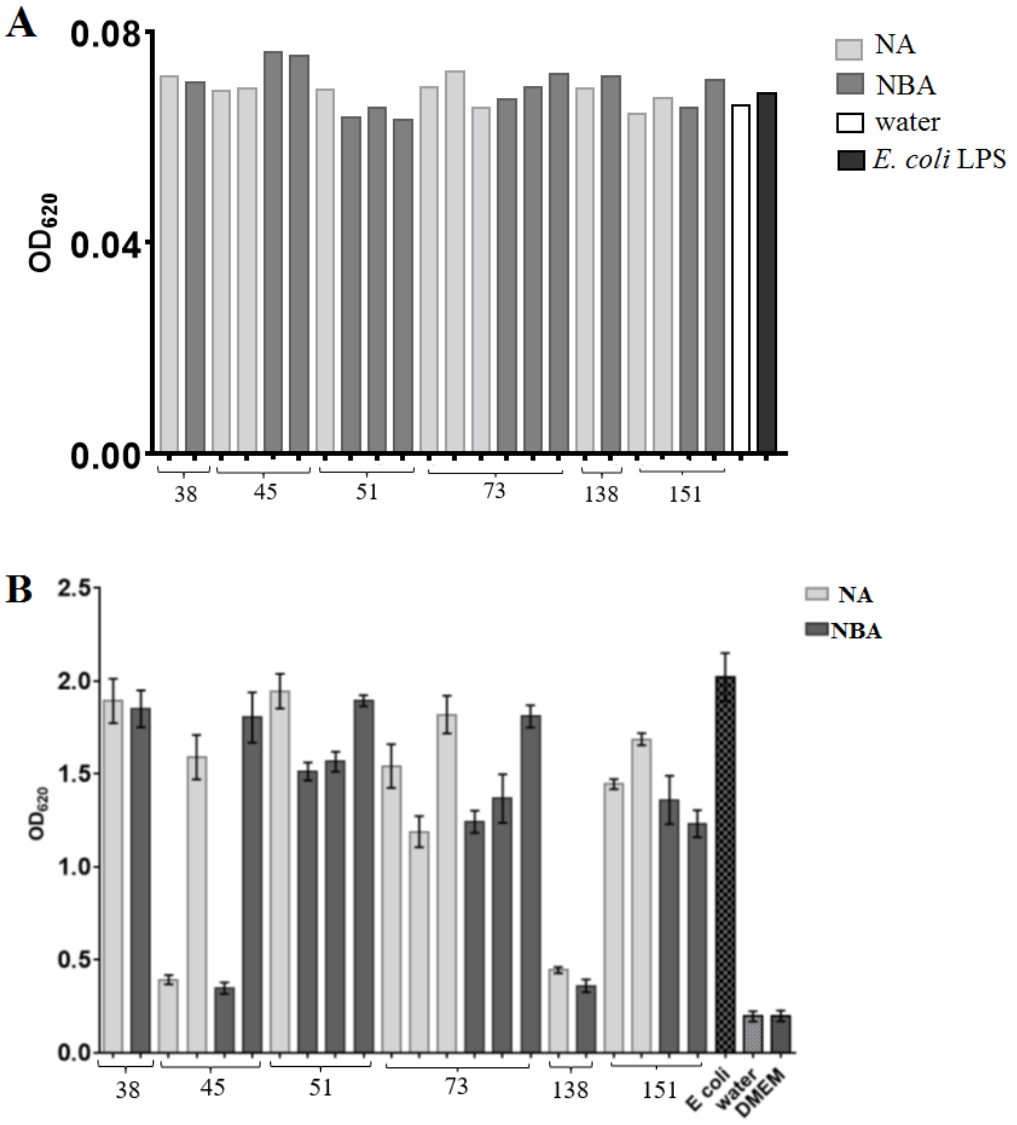



**Figure 14.** HEK-Blue™ Null2 cells (A) and TLR4 activation (B) by the crude-extracted *C. coli* LOS, average data of three technical repetitions. The strains are indicated below. Error bars show ± SEM. Bacteria were grown at 37°C and standardized to appr. $4\times10^8$ cells by $OD_{600}$ prior to LOS extraction. Note, that only LOS samples of *C. coli* 73 represent 3 independent biological replicates for both conditions.

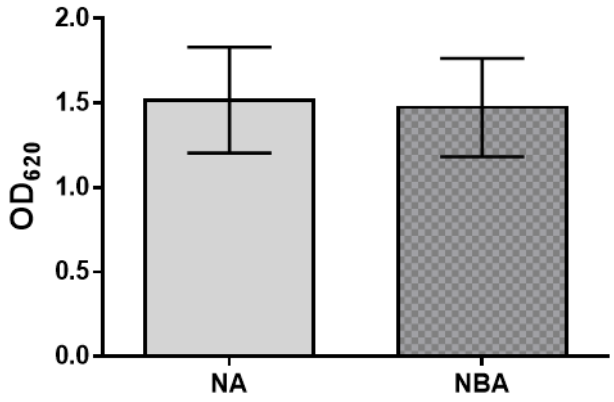



**Figure 15.** Effect of a growth medium on TLR4 activation by the crude-extracted LOS of *C. coli* 73. Error bars show ± SD. Data passed normality test, correlation coefficient r = 0.7, one-tailed $p = 0.01$.

## 6 DISCUSSION

The LPS is an indispensable component of outer membrane of Gram-negative bacteria, contributing to its structural integrity. The LPS of the established food-borne pathogens, *C. jejuni* and *C. coli* lacks the O-antigen and therefore is often referred to as LOS. A number of studies emphasized the role of *Campylobacter* LOS in GBS development, as well as in biofilm formation, resistance to complement and AMPs, intraepithelial survival and host colonization (Moran *et al.* 1996; Guerry 2000; Allos 2001; Naito *et al.* 2010). Various genetic mechanisms determine a hyper variability of *Campylobacter* cell surface glycoconjugates, and differential gene expression at different growth conditions also contributes to a phenotypic variation of LOS structures (Dorrel *et al.* 2001; Semchenko *et al.* 2010). In the present work the effect of different growth conditions on *C. coli* LOS expression was assessed. However, evaluation of TLR4 agonistic activity was hampered by the applied LOS isolation and quantitation methods which may reflect a common methodological issue.

Due to vast structural heterogeneity of bacterial endotoxins a dozens of extraction methods have been devised in order to conform with the variable physical properties of LPS. Thus, there is no universal "golden standard" for LPS isolation. In practice, a choice of a particular extraction technique depends on experimental aims, LPS type (rough or smooth), available materials and equipment, among others. However, it is well-known that the different methods of LPS extraction yield products of distinct quality and quantity (Nowotny *et al.* 1963; Fukushi *et al.* 1964; Blake and Russel 1993; Delahooke *et al.* 1995; Ridley *et al.* 2000). The proposed methods based on a mild treatment (heat alone, chelating agents) rely on week association of LPS with the outer membrane and thus they are not always effective and have not been widely applied (Wilkinson 1996).

The fast crude LPS extraction method employed in the present study utilized proteinase K, which decreases protein content in whole-cell lysates containing LOS and other glycans. The LOS samples extracted by the mini-phenol-water method of Prendergast *et al.* (2001) were directly analyzed by SDS-PAGE. The quality of visualized LOS bands of the both extraction methods was in accordance with data from the original publications (Hitchcock and Brown 1983; Prendergast *et al.* 2001). For example, as it was observed before in *Salmonella* LPS, the staining patterns of the whole-cell lysates varied between the strains, most probably reflecting the biochemical composition of the chemotypes. Visualization of LOSs extracted by the mini-phenol-water method revealed a significant difference in the intensity of LOS bands on silver-stained gels, which was initially attributed

to technical mistakes. However, a following TLR4 activation assays revealed a more complex nature of this problem. Nevertheless, it can be concluded that the crude LPS/LOS extraction and the mini-phenol-water method are simple and rapid methods suitable for LOS visualization by silver staining.

The hot phenol-water method of Westphal *et al.* (1952) is the widely used extraction procedure which is characterized by a high LPS yield but also by higher amounts of protein and/or nucleic acids contamination (de Castro *et al.* 2010). To enhance LOS yield and purity, biomass of the *C. coli* strains grown under the different conditions was enzymatically digested (with lysozyme, RNase A, DNase I and proteinase K) and then subjected to hot phenol-water extraction. Isolated LOS fractions were precipitated in cold absolute ethanol applying a modified method of Darveau and Hancock (1983) or lyophilized; subsequent agarose gel electrophoresis of purified LOS samples revealed a negligible contamination with nucleic acids.

Despite undertaken measures, overall yield of hot-phenol extracted LOS was rather low in this study. It was shown previously, that the overnight treatment of R-LPSs of *Salmonella* spp. and *E. coli* K-12 with 70% or absolute ethanol containing $MgCl_2$ at 4 °C is sufficient for LOS precipitation (Kato *et al.* 1990). However, upon ethanol precipitation and centrifugation, a LOS pellet was not visually detected. Poor yield or an absence of the hot-phenol-extracted LOS have been reported before (Kasai and Nowotny 1967; Brade and Galanos 1982; Perez-Perez and Blaser 1985; Uchida and Mizushima 1987; Moran *et al.* 1992). LOS fraction of high yield and purity was extracted in a number of studies by the PCP method employing an aqueous phenol-chloroform-light petroleum mixture (Galanos *et al.* 1969; Lindberg and Holme 1972; Moran *et al.* 1991b).

Nonetheless, both PCP and hot-phenol methods selectively separate LPS based on its solubility in the organic solvent(s), which is a major drawback, as solubility varies between different LPS forms. Indeed, the limitations in the extraction methods sometimes may affect an investigation: Blake and Russel (1993) reported about non-detectable *C. jejuni* LPS isolated by the combined PCP-proteinase K method. Accordingly, it was suggested that certain LPS/LOS extraction methods are more suitable for certain bacterial species (Nowotny *et al.* 1963) or even strains (Perez-Perez and Blaser 1985; Ridley *et al.* 2000). It can be speculated that some amount of LOS in this study was retained in the phenol phase and lost during hot-phenol extraction.

An effect on the different growth conditions on LOS expression in the tested *C. coli* strains was assessed by the SDS-PAGE analysis. As SDS-PAGE separates the LPS fractions,

this method is widely employed to study LPS heterogeneity; in the combination with a silver staining it provides a good selectivity and sensitivity (Tsai and Frasch 1982). The single bands at the bottom of a gel is a typical representation of LOS molecules, which due to a low molecular mass migrate faster than LPS. In contrast, the LPS from *E. coli* 0111:B4 used in this study, produced the polydisperse, so-called ladder-like, banding pattern due to the presence of molecules with a different number of repeating units in the O-antigen. The fastest moving band represent a complete core of LPS; the second fastest band - the core plus one repeating unit; and so forth (Hitchcock and Brown 1983). Importantly, the molecular weight of an LPS and its components cannot be estimated applying standard weight markers for proteins (Russell and Johnson 1975), thus, it is common to estimate $M_r$ by comparing the relative mobility of the unknown LPS band on SDS-PAGE gel with the corresponding unit of the LPS with the published structure, such as *Salmonella* Typhimurium or *E. coli* LPSs (Wilkinson 1996). Nevertheless, the chemical compositions of the LOSs from nine *C. coli* isolates have been proposed and the molecular weights of their components are known (Culebro *et al.* 2016). The $M_r$ of the *C. coli* LOSs tested in the present work were estimated based on the aforementioned findings; LOSs were approximately 4 kDa.

Semchenko *et al.* (2010) investigated an influence of different growth conditions on *C. jejuni* LOS expression: silver-stained gels revealed the unique mobility patterns of LOSs of different strains and the temperature-dependent production of two LOS forms. In the present work all tested LOSs obtained from the *C. coli* strains grown under different conditions resolved as a single band, with a unique, strain-dependent values of the electrophoretic mobility. The latter fact reflects a structural diversity between and within the LOS classes. Accordingly, the similar phenotypic diversity of the phenol-extracted (Logan and Trust 1984) and crude (Blaser *et al.* 1986) *C. coli* LOS has been reported previously. As the concentration of the LPS positively correlates with the intensity of the bands (Tsai 1986), observed variations in intensity of the studied *C. coli* LOS samples reflected the improper standardization of the LOS concentration by the purpald assay and lyophilization/dilution. However, for the assessment of the molecular weight of LOS and its possible structural changes caused by a growth condition, normalization of the quantity of the LOS molecules in the studied samples is not crucial. It was demonstrated before that a loss of one heptose unit resulted in the different electrophoretic mobility of the studied R-LPS, furthermore, analogous strain-to-strain differences in the mobility were observed in the LPS samples extracted by the different methods (Hitchcock and Brown 1983; Sugiyama *et al.* 1990; Semchenko *et al.* 2010). Strain-dependent mobility of LOSs was also observed in the present

study. In contrast, analysis of LOS mobility patterns revealed that different growth conditions do not affect phenotypic LOS expression in the tested *C. coli* strains.

The concentrations of all phenol-extracted and lyophilized LOS samples in the present study were measured by the purpald assay. Dilutions of a commercial preparation of LPS from *E. coli* 0111:B4 were made for a standard curve construction. It should be pointed out, that according to the published structures of inner cores, *C. coli* LOS and *E. coli* LPS possess similar number of the unsubstituted terminal vicinal glycol (UTVG) groups: 4 in *E. coli* strain 0111:B4 (2 at Kdo + 2 at heptoses) and 3-4 in *C. coli* (Jansson *et al.* 1981; Raetz 1990; Aspinall 1993a; Culebro *et al.* 2016). Thus, theoretically, the concentrations of these LPS/LOS determined by the purpald assay should be comparable to each other. However, the limitations of the purpald assay were emphasized by its inventors, Lee and Tsai (1999): the actual number of UTVG groups in the sample and the predicted number of Kdo+heptose units are not essentially the same, as LPS/LOS preparations are structurally heterogeneous, while the number of L-α-D-Hep and degree of sialylation may also vary. For example, Beer *et al.* (1986) detected 3-fold higher amount of Kdo and heptose in *C. jejuni* LOS than Logan and Trust (1984) before.

Notably, a significant overestimation of LOS quantity by the purpald assay was observed in the present work. The concentration of the extracted LOS samples which were precipitated in cold ethanol was within a range of the standard curve, 0.125-2 μg/μl. In contrast, the LOS samples obtained by the mini-water-phenol method and the freeze-dried LOS samples from hot-phenol extraction required a dilution, 1:50 and 1:1000, respectively. Furthermore, upon standardization by the purpald assay, twenty-four LOS samples (lyophilized and then diluted) of the six *C. coli* strains and several ethanol-precipitated LOS samples did not evoke TLR4 response and, accordingly, were not detected on the silver-stained SDS-PAGE gels. Taking into account the high sensitivity of silver staining (limit of detection – 5 ng of R-LPS), an absence of LOS bands on gels disproves the fact that prior to electrophoresis *C. coli* LOS samples were normalized to 0.3 μg/μl (6 μg per well). Apart from excessive dilution, such phenomenon can be explained as unsuccessful extraction with hot phenol.

Importantly, the amount of *E. coli* 0111:B4 LPS (0.3 μg/μl) used in the purpald assay and in SDS-PAGE analysis yielded intense bands on gel and triggered a considerable TLR4 response. In this way, aforementioned overestimation of LOS quantity observed in this study was caused most probably by a certain contamination interfering with the measurement. It can be suggested that the ethanol precipitation applied to some extracted samples selectively

separated the LOS fraction (although with a probable loss of endotoxin), reducing false-positive quantification. Lee and Tsai (1999) noted that the samples analyzed by the purpald assay should not contain ethylene glycol, linear monosaccharides, Tris buffer or glycerol, since $NaIO_4$ will react with UTVG groups present in these molecules, producing formaldehyde and thus contributing to the measured absorbance values.

Even though it can be claimed that the LOS samples obtained in this work where free from the aforementioned impurities, the contamination with a capsular polysaccharide (CPS) residues cannot be excluded. It has been demonstrated that stationary phase cells of *C. jejuni* grown on solid media had increased CPS production (Corcoran and Moran 2007). Previously, the purpald assay was adapted for quantification of bacterial CPS since it contains glycol groups (including UTVG), e.g. in ribitol, galactose, arabinitol, glycerol, and sialic acid moieties (Lee and Frasch 2001). Michael *et al.* (2002) solved the structure of CPS of *C. jejuni* 11168 which included β-D-Rib (ribose), β-D-GalNAc, glucuronic acid and D-*glycero*-α-L-*gluco*-heptopyranose. The ability to integrate various complex heptoses in *C. jejuni* CPS was demonstrated (Karlyshev *et al.* 2005b). Interestingly, a similar water-soluble antigenic polysaccharide of high $M_r$ has been found in phenol-water extracts of *C. coli* serotype 0:30 in a study of Aspinall *et al.* (1993d). This polysaccharide possessed an unusual, teichoic acid-like structure comprised of a poly-ribitol phosphate and 6-deoxy-*talo*-heptose (Aspinall *et al.* 1993d). In fact, eight CPS classes have been identified in *C. coli* by genomic analysis (Richards *et al.* 2013). Two putative CPS biosynthesis proteins and the sialyltransferase associated with the CPS locus (*cst-I*) were found in *C. coli* 76339 (Skarp-de Haan *et al.* 2014), the strain that was used in this study.

Unfortunately, the hot phenol-water method, unlike another commonly used extraction methods, preferentially extracts the LPS and capsular antigens. The CPS has been found in the aqueous phase together with endotoxin fraction (Lindberg and Holme 1972). Karlyshev *et al.* (2005b) and Michael *et al.* (2002) isolated the CPS fraction by the hot water-phenol method: the aqueous phase was lyophilized, dissolved and subjected to ultracentrifugation which yielded a pellet containing LOS and supernatant containing CPS. However, it was reported that the mixtures of LPS/LOS and CPS is difficult to purify: separation of LPS and CPS was not achieved by repeated ultracentrifugation (de Castro *et al.* 2010). Moran *et al.* (1991b) purified the LOS of *C. jejuni* by centrifugation at 105,000 g (4 °C, 4 h) and gel chromatography in order to eliminate impurities (CPS, oligosaccharides, free sialic acid) from the preparations. Repeated ultracentrifugation prior to the purpald assay was applied to purify *C. jejuni* LOS in the study of van Mourik *et al.* (2010). Such treatment was not used

in the present study, thus, it can be hypothesized that inaccurate estimation of LOS concentration by the purpald assay was due to contamination of the extracted *C. coli* LOS samples with CPS or sialic acid residues possessing UTVG groups. In summary, it can be concluded that a correct and reliable LPS/LOS quantification using the purpald assay requires a high degree of purity of the tested samples and the standards.

Because the purpald assay was not suitable for quantification of the actual amount of LOS in the preparations obtained in this study, an attempt was made to adjust LOS concentration according to the weights of lyophilized LOS samples. It should be noted that lyophilization of the extracted endotoxin fractions (regardless of the isolation procedure used) is the most common method for a final or temporary LPS/LOS recovery. In a number of studies, the weight of freeze-dried LPS was measured using a high-precision balance. Weighing/dilution and subsequent SDS-PAGE as a control measure were employed for the standardization of *Campylobacter* LOS by Kuijf *et al.* (2010) and Stephenson *et al.* (2013). In this study, the LOS samples of the six *C. coli* strains were re-lyophilized: each samples yielded a white powder/foam which was weighed and resuspended in endotoxin-free water. However, following SDS-PAGE and silver staining revealed an absence of LOS-specific bands on gel in most of aforementioned samples. Therefore, it was not possible to use weighing and dilution as a method of standardization of a LOS concentration. Presumably, the lyophilized preparations contained both LOS and an undetermined contaminant(s). Moreover, different environmental conditions (humidity) and different ability of LOS structures to absorb water could also contribute to the observed discrepancies. These data suggest that standardization of LOS concentration by weight may lead to incorrect results.

An accuracy of the LOS standardization by weight and by the purpald assay was evaluated using the phenol-extracted LOS samples of *C. coli* strains 45 and 73: LOS extracts of only one strain yielded bands in accordance with a dilution and weight used. In contrast, the concentrations of all LOS samples estimated by the purpald assay were beyond the range of the standard curve (60-fold higher absorbance and complete saturation). This observation further supports a hypothesis of incorrect estimation of LOS amount by the both methods.

Proper quantification and a purity control are crucial for an investigation of biological activity of endotoxins (Tirsoaga *et al.* 2007). The importance of LPS re-purification, particularly, of the elimination of a protein contamination, was shown before in a TNF stimulation studies (Manthey and Vogel 1994). A removal of lipoprotein contaminants from the commercial R-LPS preparations resulted in the diminished TLR2 activation, which implied that this receptor is not responsible for a LPS signaling in most species (Hirschfeld

*et al.* 2000). Accordingly, an influence of the extraction methods on a biological potency of endotoxins has been reported, indicating an insufficient LPS purification in many studies (Morrison 1983; Tirsoaga *et al.* 2007). Noteworthy, according to the Caroff and Karibian (2003) the impurities, such as, lipoproteins, nucleic acids and phospholipids, were also found in the commercial LPSs.

It can be stated that the phenol-extracted LOS samples obtained in the current work were essentially free from DNA, RNA and protein contamination. In a study of Delahooke *et al.* (1995) protein contamination was found in all LPS preparations extracted by the different methods, notably, the extremely low levels of proteins were detected in phenol-extracted LPS samples; following proteinase K treatment completely eliminated the presence of proteins. The human TLR4 (hTLR4) response was evaluated in this study using the HEK-Blue™ hTLR4 cells which express the hTLR4, the MD2/CD14 co-receptor genes and a secreted embryonic alkaline phosphatase (SEAP) reporter gene: the stimulation with LOS activates NF-κB and induces the production of SEAP. As it was mentioned before, not all phenol-extracted LOS samples activated the hTLR4 in this work, furthermore, it was not possible to assess an impact of the growth conditions on NF-κB induction due to unsuccessful normalization of LOS concentration. This explains the controversial data obtained after the stimulation of the hTLR4-expressing cells with the phenol-extracted LOSs.

In this way, an improper standardization of LOS concentration was a major limitation in this study which hindered the evaluation of a TLR4 response. As LPS/LOS is only bacterial ligand of hTLR4, possible contaminants should not affect the assay. However, hTLR4 reporter cells used in this study express the endogenous level of TLR3, TLR5 and NOD1. In order to detect possible influence of the non-specific ligands, HEK-Blue™ Null2 cells which do not express hTLR4 were used as a control. No activation of HEK-Blue™ Null2 cells was seen in this work upon stimulation with the crude and phenol-extracted LOS samples, which implies an absence of signaling through TLR3, TLR5, and NOD1 by RNA, flagellin, and peptidoglycan, respectively. Hence, a stimulation of the HEK-Blue™ reporter cells with the crude LOS preparations in this study was methodologically appropriate. However, as a composition of all LOS samples was by no means certain, the usage of other reporter systems, e.g., macrophages or mononuclear monocytes, would be impossible. Accordingly, Delahooke *et al.* (1995) proposed that the different methods of LPS extraction and contamination with CPS were responsible for the unexpected levels of TNF induction observed in their study. The interaction of the *Campylobacter* CPS with the host innate

immune system is poorly studied, *C. jejuni* CPS was shown to reduce cytokine production in dendritic cells (Rose *et al.* 2012), to activate TLR2 (Friis *et al.* 2009) and to modulate TLR4 response (Maue *et al.* 2013), however, latter is uncertain due to often contamination (Phongsisay *et al.* 2015). Nevertheless, it can be concluded, that the elimination of CPS and other impurities is essential for studies on biological activity of *Campylobacter* LOS.

In order to evaluate an effect of the growth conditions on TLR4 activation by *C. coli* LOS, a different approach, unbiased by the purpald assay and weighing, was adopted. Bacterial suspensions were standardized by $OD_{600}$ measurement prior to the fast crude LOS extraction. Obtained whole-cell lysates were analyzed by SDS-PAGE and then used in TLR4 activation assay: notably, all samples produced the prominent bands on a gel and exhibited high agonistic activity. Subsequent statistical analysis revealed that crude LOS extracts obtained from *C. coli* strain 73 grown on different media had the similar levels of NF-κB activation. This finding corroborates SDS-PAGE analysis of the same crude LOS samples, as well as phenol-extracted LOSs.

## 7 CONCLUSIONS

In conclusion, different growth conditions did not affect LOS mobility patterns of the tested C. *coli* strains which implies no differences in LOS composition. In contrast, strain-dependent LOS size variation was detected. The overestimation of LOS quantity by the purpald assay observed in this study was caused most probably by the considerable contamination of the LOS samples with capsule polysaccharide (CPS) fraction. Thus, further studies on occurrence of CPS in *C. coli* are needed. Additionally, it can be suggested that correct standardization of LOS concentration by the purpald assay and by weighing would require essentially pure LOS samples. Notably, crude LPS extraction was suitable for LOS visualization by silver staining and for immune response evaluation using TLR4-expressing cells. Overall, proper research on biological activity of endotoxins requires a non-biased LPS quantitation and correct standardization of concentration, while the determined composition of endotoxins and absence of impurities may be crucial for non-specific immunological assays.

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## Appendix I

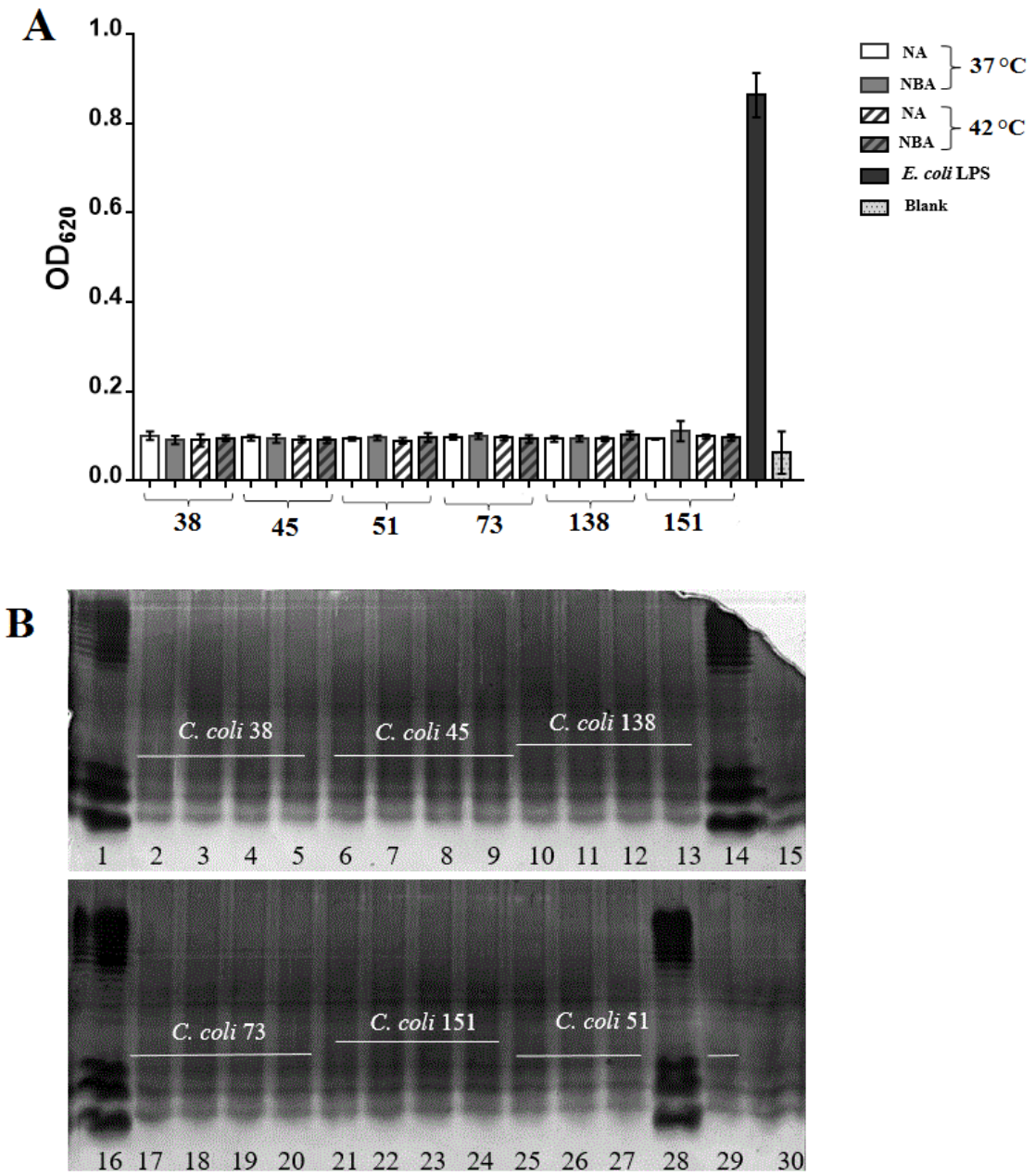



**Figure 16.** TLR4 activation (A) and silver stained SDS-PAGE gels (B) of the hot phenol-water-extracted LOS from *C. coli* biomass grown under different conditions, 3rd repetition samples. Samples were not precipitated in ethanol. Lanes 1, 14, 26, and 28 contain the purified LPS from *E. coli* 0111:B4 as a positive control. Lanes 2-5 contain samples of *C. coli* 38, *C. coli* 45 (lanes 6-9), *C. coli* 51 (25-27, 29), *C. coli* 73 (17-20), *C. coli* 138 (lanes 10-13), *C. coli* 151 (21-24). Lanes 15 and 30 - negative control. Concentration of LOSs was standardized to 0.3 µg/µl.

## Appendix II

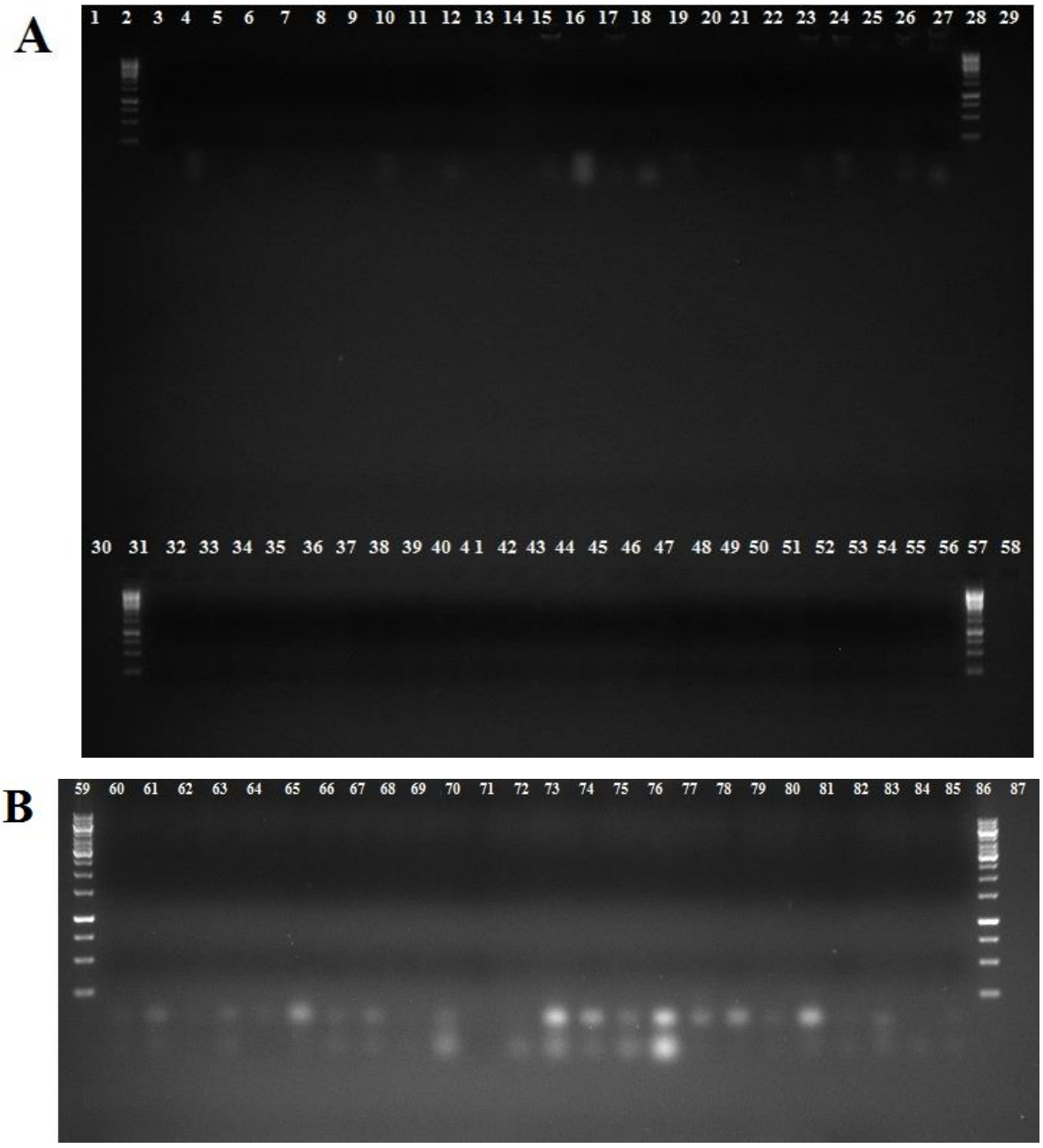



**Figure 17.** Agarose gel electrophoresis of the LOSs extracted by hot phenol-water method from six *C. coli* strains grown under different conditions. Lanes 3-26 contain samples of *C. coli* LOSs of 2nd biological repetition, lanes 32-55 – of the 3rd, lanes 60-84 – of the 1st. Lanes 27, 56, and 85 contain the LPS from *E. coli* 0111:B4. Lanes 1, 29, 30, 58, and 87 - negative control. Lanes 2, 28, 31, 57, 59, and 86 contain the molecular weight marker GeneRuler 1 kb DNA Ladder.