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Enterohaemorrhagic *E. coli* O103:H25 modulates expression of LEE-encoded virulence genes in response to direct cell-cell contact with the gut commensal *Bacteroides thetaiotaomicron*

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) is a foodborne pathogen of the colon that causes disease with varying severity. Symptoms range from mild diarrhea to hemolytic uremic syndrome (HUS), which at worst can be fatal. In 2006 there was a severe outbreak of EHEC serotype O103:H7 NIPH-11060424 in Norway, with an especially high occurrence of HUS indicating high virulence.

When EHEC enters the intestine it comes in contact with the endogenous commensal microbiota, which causes interactions between commensals and pathogen. These interactions affect EHEC's gene regulation, and thus impact how the pathogen reacts in the intestine. The commensal microbiota usually plays an important role in the body's defenses against pathogenic microorganisms, but it appears that some pathogenic, such as EHEC, have found ways to exploit the endogenous intestinal microbiota to promote virulence.

In earlier work there has been detected an elevated gene expression in EHEC's Locus of enterocyte effaccement(LEE) pathogenicity island (PAI), when it was co-cultured in the presence of the gut commensal *Bacteroides thetaiotaomicron (B. theta).* Some findings indicated that it might be adhesion between the species that was responsible for the increased gene expression, and this thesis was therefore devoted to examine if physical cell to cell contact could be the reason for the elevated expression in the adhesion related genes.

Among the methods used was comparative quantitative PCR analysis of samples of EHEC co-cultures with mainly *B. theta* as a secondary species, under various conditions.

The results from the experiments strengthened the cell-cell contact hypothesis, but also illustrated how different EHEC serotypes can react differently to interspecies contact and culture conditions.

Sammendrag

Enterohemorrhagisk *Eschericha. coli* (EHEC) er en matbåren tykktarmspatogen som forårsaker sykdom med varierende alvorlighetsgrad. Symptomene kan variere fra mild diaré til hemolytisk uremisk syndrom (HUS), som i verste fall kan være dødelig. I 2006 var det et alvorlig utbrudd av EHEC serotype O103:H7 NIPH-11060424 i Norge, med en spesielt høy forekomst av HUS som tydet på høy virulens.

Når EHEC entrer tarmen kommer den i kontakt med den endogene kommensale mikrobiotaen, som fører til interaksjoner mellom kommensaler og patogen. Disse interaksjonene påvirker EHECs genregulering, og påvirker således hvordan EHEC reagerer i tarmen. Den kommensale mikrobiotaen spiller vanligvis en viktig rolle i kroppens forsvar mot sykdomsfremkallende mikroorganismer, men det ser ut til at noen patogene, slik som EHEC, har funnet måter å utnytte den endogene mikrobiotaen for å fremme virulens.
I tidligere arbeider har det blitt oppdaget et forhøyet genuttrykk i EHECs Locus of enterocyte effaccement (LEE) patogenitetsøy (PAI), når den ble dyrket i samkultur med den kommensale tarmbakterien *Bacteroides thetaiotaomicron (B. theta).* Noen funn indikerte at det kan være adhesjon mellom artene som er ansvarlig for denne økningen i genuttrykk, og denne oppgaven ble derfor viet til å undersøke om fysisk celle til celle kontakt kan være årsaken til økningen av ekspresjon i disse genene som er forbundet med adhesjon.

Blant metodene som ble benyttet var komparativ kvantitativ PCR analyse av prøver med EHEC i samkultur med hovedsakelig *B. theta* som sekundær art, under forskjellige betingelser.
Resultatene fra forsøkene styrket hypotesen om celle til celle kontakt, men illustrerte også hvordan ulike EHEC serotyper kan reagere forskjellig på kontakt med andre arter og dyrkningsforhold.

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Abbreviations

AE lesions- attachment and effacement lesion

BA- blood agar

BSA– bovine serum albumin

CFU- colony forming units

CI- confidence interval

EHEC- enterohemorrhagic *Escherichia coli*

F-actin- filamentous actin

FBS- foetal bovine serum

FITC- fluorescein isothiocyanate

Gb3- Globotriaosylceramide

HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

LEE- Locus of enterocyte effacement

LPS- lipopolysaccharide

MSB- menadione sodium bisulfite

OD- optical density

PAI- pathogenicity island

PI- propidium iodide

SD- standard deviation

TEM- transmission electron microscopy

T3SS- Type three secretion system

Part 1- Introduction

Pathogenic *E. coli*

In the field of microbiology, there is no other organism that is as well studied as *Escherichia coli*. It was first discovered and described in 1885 by the German-Austrian pediatrician and professor Theodor Escherich (hence the name Escherichia). *E. coli* belong to the family of *Enterobacteriaceae* and is a Gram-negative, flagellated (motile) facultative anaerobic rod that has its natural habitat in the intestine of mammals [1]. *E. coli* was for a long time recognized as a common, highly abundant part of the intestinal microbiota, that could cause disease if inoculated into extra-intestinal tissue [2]. The discovery of its potential as a pathogen was, however, much later described by Neter et al [2] who created the term "Enteropathogenic *E. coli"* (EPEC) for all *E. coli* that had the ability to cause bowel disease [2]. Diarrheagenic *E. coli* strains are currently divided into five major pathogroups. The groups are: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E.coli* (EAEC), enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC). The pathotypes are classified according to their virulence traits and the mechanisms involved in disease development[1, 3]. The different pathotypes may also be classified into serogroups and serotypes, based on expression of various antigens in the lipopolysaccharide (LPS) (O antigen) and on the flagella (H antigen). The term serogroup entails classification based on the O antigens, while serotypes include both O and H antigens.

EHEC, with its exceptionally low infectious dose (<100 cells) [1] has a reputation for being the deadliest of the *E. coli* pathotypes. EHEC causes disease in humans that varies in severity, where children normally are most severely affected. Symptoms vary from mild diarrhea to bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS), that can evolve to renal failure, chronic damage to the kidneys and in the worst case, death [1]. It is often referred to as "the hamburger bug", because of the first reported outbreak of EHEC O157:H7 in 1982 in the USA where people became sick after consuming undercooked hamburgers. Ground beef is a big potential source for EHEC outbreaks as ruminants and mainly cattle are the main reservoirs for the bacteria. EHEC can also be transmitted by fecally contaminated drinking-water, from person to person and through direct contact with farm animals (Zoonosis) [4].

Of all the EHEC serotypes, O157:H7 is the most studied. In the USA, it is also the most recognized serotype since it has an annual infection rate estimated to approximately 73 000, including 2 168 registered hospitalizations and 61 deaths [5]. Other EHEC serotypes are estimated to cause approximately 37 000 infections per year, including 1 084 hospitalizations and 30 deaths registered per year [1, 5]. The cost of EHEC infections in the USA is estimated to be 607 million USD every year for O157:H7 alone [6]. According to the annual epidemiological report done by the European Centre for Disease Prevention and Control [7], there is between 3 000-4 000 reported cases of EHEC infection per year in Europe (2006-2009), with a varying prevalence of HUS (242 cases in 2009). Over fifty percent of the HUS cases (52%) were linked to serotype O157:H7. The largest EHEC outbreak experienced in Europe was in 2011 in Germany, where a total of 3 816 cases were reported, including 845 HUS cases of which 54 were fatal [8]. The outbreak strain was later characterized as an Stx encoding EAEC *serotype* O104:H4. The development of disease was atypical for EHEC, as the patients that were most severely affected and developed HUS were adults [8].

In Norway, the number of confirmed cases of EHEC infections varies from year to year, and the annual infection rate is heavily influenced by outbreaks. The number of registered cases varied from 50 in 2006 to 26 and 22 in 2007-2008 and 108 in 2009 [7]. The best known EHEC outbreak in Norway was in 2006, where the source of infection was traced back to fermented cured mutton sausage. The outbreak involved a total of 17 reported patients, where 10 developed HUS and 1 died [9]. The strain was identified as EHEC O103:H25, and the high rate of HUS development indicated an extraordinary high virulence potential [10]. The high economic costs, the risk for fatality and serious chronic repercussions makes research on EHEC infection, and especially mechanisms and factors related to virulence, highly relevant.

Virulence factors of EHEC

EHEC has several properties that contribute to its high virulence, e.g. fimbriated surface for effective adhesion[1], and tolerance for low pH that allows it to survive passage through the acidic environment in the stomach [11]. There is, however, two features that defines the

pathotype: First, the ability to produce Shiga toxin (Stx) and, secondly, the ability to form attaching and effacing (AE) lesions in the colonization process[1].

Shiga toxin

The production of Stx is traditionally considered as the main virulence factor of EHEC[1, 12, 13], and it is the production of this highly potent toxin that can cause development of HUS [4]. Stx is secreted by EHEC, but the toxin genes are acquired from a Stx encoding bacteriophage λ [12] When the phage infects *E. coli* it will enter either lytic or lysogenic life cycle. When entering the lysogenic cycle, the phage integrates itself into the host genome, creating a prophage, where it resides within the bacteria without causing any harm [14]. The production of Stx however, is associated with induction of the lytic cycle [13].

Stx has the structure of a holotoxin of the AB family of protein toxins. It consists of a single polypeptide subunit A and five identical B subunits. The B subunits are responsible for binding to the glycolipid globotriaosylceramide (Gb_3) receptor expressed on the surface of the target cell. The A subunit is the enzymatically active part of the toxin, which cleaves ribosomal RNA, resulting in disruption of protein synthesis [1, 12, 13]

There are two subgroups of Shiga toxin, Stx1 and Stx2. Stx1 is nearly identical to the "original" Shiga toxin produced by *Shigella dysenteriae*, while Stx2 shares approximately a 55% homology with Stx1 [12]. Certain types of Stx2 can be 40-400 times [15] more potent than Stx1, and Stx2 is therefore the main contributor to the high virulence of EHEC.

Stx is a nephrotoxin and it has been reported that it can be responsible for induction of apoptosis (programmed cell death) in many eukaryotic cell types, even if the precise pathway for this induction is unknown[13]. In addition, it has recently been suggested that the Shiga toxin influence tissue tropism of EHEC, by enhancing EHEC colonization of the colonic epithelium [16].

Colonization process

The colonization process of EHEC can roughly be divided into four steps after it passes through the gastrointestinal (GI) tract and enters the colon (Fig. 1). The first step is the migration towards the epithelial layer, where motility (flagella) are important for passing

through two mucus layers where the outer layer is heavily populated with the endogenous microbiota, and the inner layer is free from bacteria and more dense [17]. The second step is initial adhesion and occurs when EHEC encounters a suitable epithelial cell to colonize. In the initial stage of adhesion, adhesins such as fimbriae, flagella, outer membrane proteins and type three secretion system (T3SS) binds to the surface of the enterocyte and anchors the bacterium [18][19]. The early adhesion is a facilitator for subsequent stronger adhesion of the bacterium to the epithelial cell, which is the third step in EHEC's colonization process. The tight adhesion is made possible by EHECs T3SS and involves the formation of attaching and effacing (AE) lesions [1]. The fourth step is the pedestal formation by actin filament reorganization and accumulation [1, 20], where the pedestal is suggested to work as an "anchor" for the bacterium (by cupping it) [19].

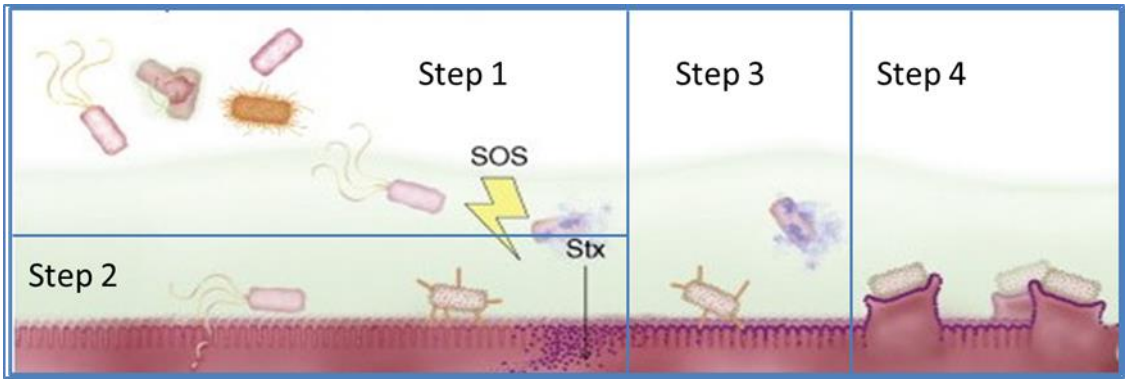

Figure 1. Schematic representation of EHEC colonizing the colon epithelium modified from Tree et al [18].

Attaching and effacing lesions

The formation of attaching and effacing lesions is imperative to EHEC's colonization process of the human colon. An AE lesion is characterized by loss of microvilli in the area where the bacterium attaches intimately to the mammalian cell membrane [21]. The genes involved in formation of AE lesions are encoded in the locus of enterocyte effacement (LEE) pathogenicity island (PAI). The LEE PAI is essential for disease in EHEC and it encodes adhesins, chaperones, translocators and other effectors with different tasks (e.g. modulation of host cytoskeleton) [22]. LEE PAI has five operons, and all five operons are important for

optimal attachment to epithelial cells [1]. It also encodes EHEC´s T3SS which is responsible for transporting LEE encoded (and some non-LEE encoded) effector proteins and chaperones into the epithelial cells [23].

The T3SS structure resembles a needle in both function and appearance (Fig. 2). It is comprised of a basal structure that anchors the needle to both the periplasmic membrane and the outer membrane of the bacterium. The basal structure encompasses a ring structure, inserted through both membranes, and a central channel that resembles a pipe. This periplasmic channel is constructed of the highly conserved lipoprotein EscJ [24][23].

The needle is shaped as an elongated tube and is composed of numerous copies of the EspA protein. EspA is also important for early colonization of the intestinal epithelium. It creates a filamentous structure that will coat the bacterial surface and facilitate initial attachment to the epithelium [25]. The needle tip is comprised of the proteins EspD and EspB which sense the presence of eukaryotic cells. The last component is the translocon, which uses the needle tip as a base and is responsible for creating a pore in the eukaryotic cell membrane. The pore allows flow of effector proteins into the host cell. EspA is also an important protein in the translocon [25].

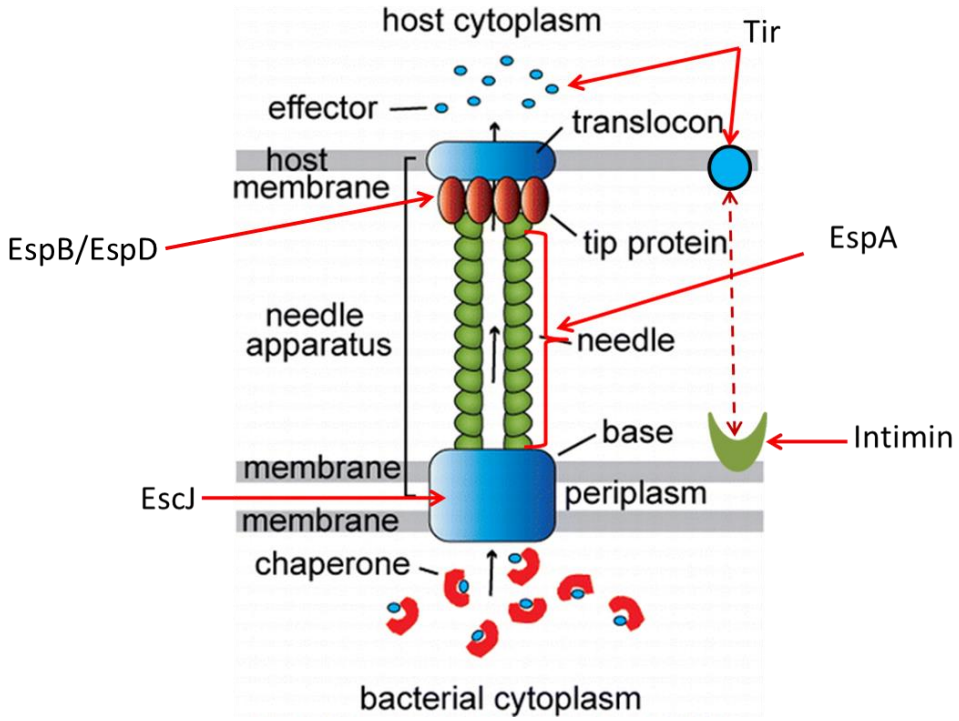

Figure 2. A schematic representation of a T3SS system modified from Chatterjee et al [23].

The Translocated intimin receptor (Tir) is involved in intimate binding of EHEC to host cells. Tir is encoded by the gene called *espE* in EHEC (it is however almost always only referred to with its general name Tir, which it will be in this thesis as well. During the formation of an AE lesions, Tir is injected into the epithelial cell, translocated to the cell surface and forms a receptor for intimin mediated bacterial binding to the host cell (described below). The ability to introduce its own receptor for binding ensures tight adhesion and further cell contact for continued and efficient secretion of virulence proteins from the pathogen to the host cells [1, 20, 23]. Intimate contact between EHEC and host cells is necessary for one of EHEC's most recognizable pathogenicity traits; the pedestal formation. There are many different proteins involved in pedestal formation and some of them are more important than others.
Intimin is a LEE PAI- encoded adhesin expressed on the bacterial cell surface. Intimin is important for both early adhesion and the subsequent tight adhesion. The early adhesion is due to the ability of intimin-γ to bind to nucleolin on the epithelial surface. The early adhesion ensures contact that allows the T3SS to insert effector proteins such as Tir into the host cell cytoplasm [19]. There are currently five different types of intimin classified as: α, β, γ, δ and ε [26, 27]. Structural differences and the presence of an carboxyl group at the C-

terminus of intimin determines the tissue tropism of EHEC (what location in the GI tract it ends up colonizing) [28].

Table 1. LEE-encoded proteins examined in this thesis

Trivial name	Protein	Gene	Role in AE lesion formation	Location in LEE operon
Intimin	Eea	*eae*	Adhesin, binds to Tir	LEE5
Translocated intimin receptor	EspE/Tir	*espE/tir*	Adhesion, receptor for intimin	LEE5
***E. coli* secreted protein A**	EspA	*espA*	Early adhesion-protein coating the bacterium, structure in needle tip	LEE4
	EscJ	e*scJ*	T3SS protein, forms channel through periplasm of bacterium	LEE2

Gut microbiota

The human gastrointestinal tract houses a vast number of bacteria, some archaea, eukaryotes and viruses [29]. The colon is the part of the bowels with the highest density of microbes and it is estimated to contain > 10^{12} organisms/gram of intestinal content [30]. There is a wide diversity of more than 1000 different microbial species colonizing the human colon [29]. The species composition and diversity varies greatly from person to person [29, 31]. The diversity is dependent on multiple factors such as status of health, diet, the environment the individual lives in, has grown up in and the development of the microbiota during early years of life which is influenced by factors such as mode of delivery and breastfeeding versus formula feeding [32]. Even if there is a considerable difference in the microbial composition, there are patterns that normally recur. In healthy adults, the phyla Bacteroidetes and Firmicutes normally dominate, while Proteobacteria, Actinobacteria and Verrucomicrobia occur in smaller amounts [29, 33].

The microbiota that normally resides in the colon is often referred to as the commensal microbiota, which is a term referring to the type of symbiosis where one species benefits from the symbiosis, while the other is neither harmed nor has any benefit from the

interaction. The term “commensal” was applied when the importance of the gut microbiota still was not discovered. The term has remained although it is now known that the host-microbiota interaction is a mutualistic type of symbiosis, and essential for our health [34, 35]. The human intestinal microbiota is so important for our health, that it has been called “the extra organ of the human body” [33]. Aside from providing protection against pathogenic microorganisms, the microbiota extracts nutrients from the diet that otherwise are unavailable for us and produces vitamins and important fatty acids (short chained fatty acids- SCFA). It also contributes to retain normal immune function. Imbalance in the composition of the microbiota (dysbiosis) has been associated with a number of diseases [33, 35].

EHEC and the commensal microbiota

EHEC is a foodborne pathogen, which means that it is transmitted through food. It enters the digestive system orally, and travels through the gastrointestinal tract before it colonizes the colon. When EHEC enters the colon, it not only interact with the colon epithelium and mucus layer but also with the gut microbiota [34, 36, 37].

That the commensal microbiota provides the body with protection from pathogenic bacteria is not an entirely new concept, but it is mostly during the last decade that the molecular mechanisms behind this protection has begun to be understood [38]. The protection provided by the microbiota provides a direct inhibition by competition for limited nutrients, and an indirect protection where the commensals increase the resistance against pathogens by enhancing the hosts intestinal immunity [34, 38]. The microbiota increase the immune mediated colonization resistance by triggering development of immune cells and by stimulating production of pro-inflammatory agents and antimicrobial factors [38]. The commensal microbiota exists in a state of tolerance amongst themselves and with the host. When new bacteria enter, this tolerance does not apply to them and the subsequent interaction will alter the gene expression of both the newcomers and the resident microbiota. An example is *Enterococcus faecalis*, that demonstrates enhanced expression of virulence genes in the presence of pathogenic *E. coli* [39]. As the diversity of the gut microbiota can vary excessively from person to person, its composition could influence how an infection unfolds in different individuals [38].

Bacteroides thetaiotaomicron

Bacteroides thetaiotaomicron (B. theta) is an obligate anaerobic, gram negative, fimbriated, non-motile bacterium that occurs in high abundance in the human large intestine [36]. It has an extensive glycobiome and it is considered important for digestion of carbohydrates, especially starch [37, 40]. The ability of *B. theta* to degrade carbohydrates that are un-degradable by most other commensal bacteria and by the host, stabilizes the symbiosis between different species in the colonic microbiota and makes it important for maintaining digestive health both in humans and animals [41]. *B. theta* offers the commensal microbiota a secured access to digestible/absorbable carbohydrates [36] and it can switch to hydrolyzing host derived glycans (e.g. mucus derived polysaccharides) when dietary polysaccharides are unavailable [36, 40-42]. In addition to its positive influence on stable nutrient access, it also has the ability to activate production of the antimicrobial peptide Angionin, by paneth cells, that specifically targets pathogenic bacteria, but not commensals[43]. Angionin also inhibit inflammation responses that can have a negative impact on the gut microbiota (dysbiosis).[44]

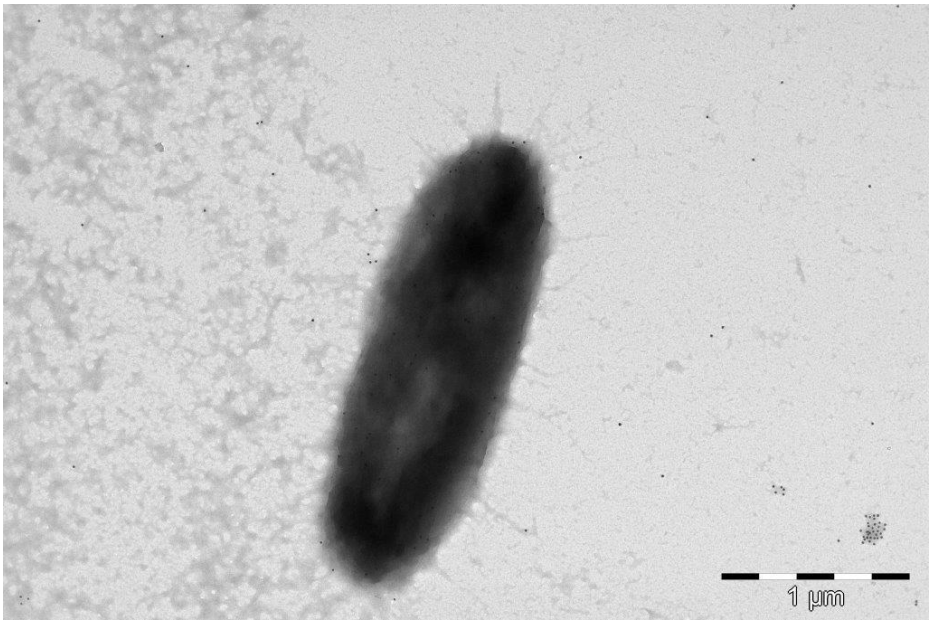

Figure 3. TEM image of *B. theta* (Iversen et al. unpublished results) .

All features mentioned above, paints the picture of a benevolent symbiont but *B. theta* can, however, also act as an opportunistic pathogen[36]. There are emerging reports about the "pathogenic side" of *B. theta* where its virulence potential is speculated to be comparable to the pathogenic species *Bacteroides fragilis* [45]. *B. theta* has been shown to play a role in development of perforated, gangrenous appendicitis [36], and it has also been shown to interact with certain blood proteins in a way that enhances inflammation [45]. In addition, it has been reported to induce colitis in mice genetically susceptible to inflammatory bowels disease (IBD) [46]. Together these reports suggest that the benevolence of *B. theta* is questionable.

In previous work by Iversen et al [47] it has been shown that *B. theta* influences the virulence of EHEC O103:H25 by stimulating up-regulation of LEE genes when co-cultured with the pathogen [5]. This regulation has been proven not to be specific to serotype O103:H25 and *B. theta*, as it also occurs in serotype O157:H7. In addition, the closely related *B. fragilis* and *E. faecalis* (a member of the phylum firmicutes), have also been shown to induce up-regulation of LEE when co-cultured with EHEC O103:H25 [47] and O157:H7 [6]. Since some of the LEE genes are essential for adhesion and colonization of the gastrointestinal tract, these interactions could potentially influence the severity of disease [20].

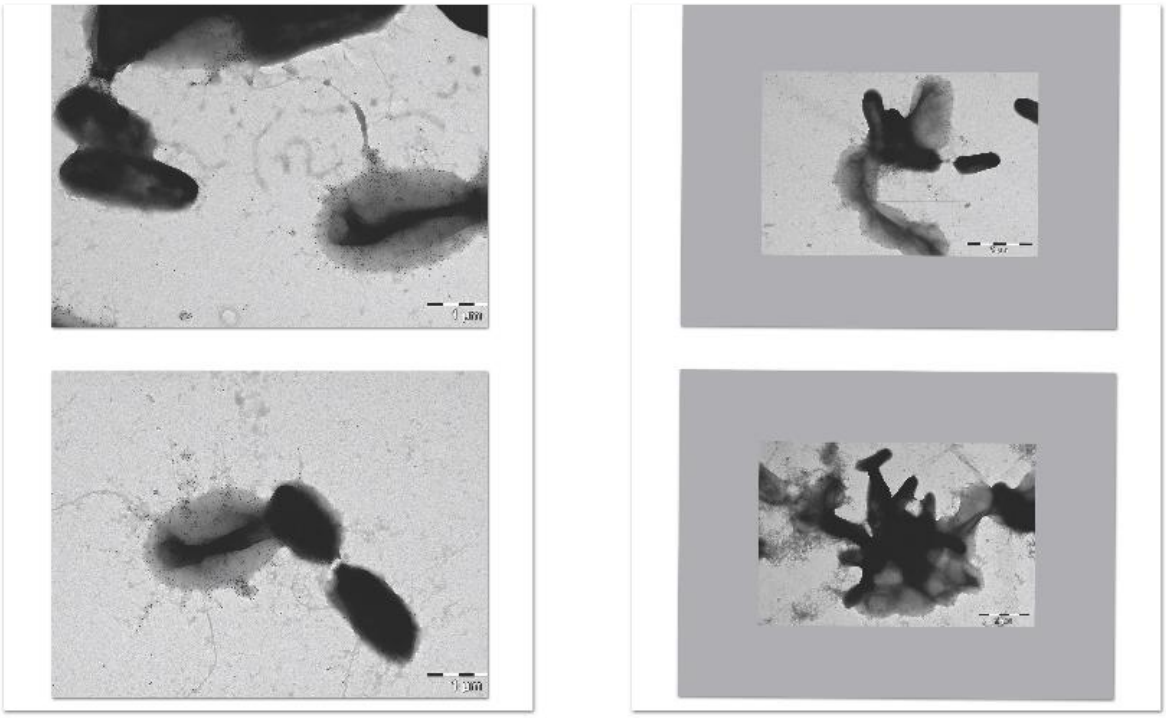

Figure 4. TEM pictures taken of EHEC NIPH-11060424 grown in co-culture with *B. theta*. The photos to the left were taken with a scale of 1µm and the right there was a 2µm scale. The larger bacteria (light grey) is immune-gold labeled EHEC cells, while the smaller and darker cells are *B. theta*. The figure illustrates different levels of clustering between the species (Iversen et al. unpublished results).

It is not known if the up-regulation of LEE genes provides EHEC with more efficient adhesion to host cells, and what attribute of *B. theta* that is responsible for the up-regulation of LEE genes. Iversen et al [47] reported an increased expression of LEE genes when EHEC was co-cultured with *B. theta*. The up-regulation was not observed when EHEC was cultured in spent medium from *B. theta.* Transmission electron microscopy (TEM) analysis of EHEC co-cultured with *B. theta* reveals an intimate contact between the two species (Fig. 4).

Together these results suggest that the elevation in LEE expression is dependent on direct physical contact between the species, rather than diffusion of biochemical compounds produced by *B. theta*. However, Curtis et al [48], explained the up-regulation of LEE

expression in EHEC co-cultures with *B. theta* as a response to metabolites produced by *B. theta* (especially succinate).

The primary aim of this thesis was to increase our knowledge about what factors causes the elevation in virulence gene expression in EHEC when it interacts with *B. theta.* This knowledge will provide insight on how virulence regulation might work *in vivo* and may contribute to development of novel strategies to prevent disease.

Part 2- Materials and Methods

Bacterial strains

Bacterial strains used in this project are shown in Table 2.

Table 2. Bacterial strains used in the experiments.

Bacterial strain	Characteristic
***Escherichia coli* O157:H7 EDL933**	Type strain
***Escherichia coli* O103:H25 NIPH-11060424**	outbreak strain from 2006
***Enterococcus faecalis* DSM 20478**	Type Strain
***Bacteroides thetaiotaomicron* CCUG 10774 (VPI 5482)**	Type strain
***Lactobacillus acidophilus* DSM 20079**	Type strain

E. faecalis, B. theta, L. acidophilus, EHEC EDL933 are type strains (lab strains) ordered from a manufacturer. *E. coli* O103:H25 NIPH-11060424 is the reference strain for the Norwegian outbreak in 2006 (described in[10] and [49]) isolated from fecal matter from an afflicted patient. This work builds on the study by Iversen et al [47], which focused on EHEC O103:H25 NIPH-11060424. Therefore, this strain was also used here. O103:H25 is a rare serotype, and it was therefore also interesting to investigate the effects of co-culturing on the well-known serotype O157:H7. Thus, all co-culturing experiments were done with both EHEC NIPH-11060424 and EDL933.Initially, *E. faecalis* was not included the project. The plan was to use *L. acidophilus* in addition to *B. theta* in co-cultures with the EHEC strains. It was however discovered quite early that *L. acidophilus* had an inhibitory effect on the growth of *E. coli*

when co-cultured, and *L. acidophilus* was replaced by *E. faecalis*. *E. faecalis* did not inhibit growth of EHEC.

For results regarding *L. acidophilus* see appendix 5.

Gene expression recorded by quantitative PCR in co-cultures under differing conditions

Conditions for culturing

The bacteria were spread on agar plates from frozen stock cultures (-80°C). EHEC was either plated on Blood Agar (BA) or Luria-Bertani (LB) agar, and incubated aerobically for 24 h at 37°C. *B. theta* was plated on BA and incubated anaerobically for 48 h at 37°C. *E. faecalis* was grown on BA for 24-48 h anaerobically at 37°C. All anaerobic experiments were done in an anaerobic workstation (Whitley A35 Anaerobic Workstation, Don Whitley scientific, West Yorkshire, UK).

All cultures were performed in modified Bacto™ Brain Heart Infusion (mBHI)(Beckton Dickinson and company, Sparks, USA) broth. The modification of the BHI broth, as described in Eley et al [50], entailed an addition of 5 g yeast extract/L (Oxoid Ltd, Basingstoke, UK) to BHI 37 g/L stock. After sterilization and chilling, 10 mg/L Menadione sodium bisulfite (MSB) (synthetic Vitamin K3) and 5 mg/L Haemin were added to the BHI media.

Overnight cultures (ONC) were made by inoculating single colonies from agar into culture broth.

Co-cultures were inoculated with bacterial suspensions according to table 3. Monocultures were run in parallel with co-culturing to ensure growth and to be used as control samples.

After 3.5 h growth or at OD_{600} 0.5, 500 µl of the cultures were harvested, immediately mixed with 500 µl ice cold (stored at -20°C) Methanol (CH_3OH, MeOH) and freezed at -80°C. Samples were kept at -80°C for a maximum of 14 days before RNA was extracted.

Table 3. Overview of co-culturing experiments.

Co-culture conditions	Bacterial species/strain	Second species	Ratio between EHEC and second species
Anaerobic	NIPH-11060424	*B. theta*	1:100[b]
		E. faecalis	
	EDL933	*B. theta*	
		E. faecalis	
Anaerobic, dead *B. theta*	NIPH-11060424	*B. theta*	1:100
			1:10000[c]
	EDL933	*B. theta*	1:100
			1:10000
Aerobic	NIPH-11060424	*B. theta*	1:100
			1:10000
	EDL933	*B. theta*	1:100
			1:10000
Dialysis tube Anaerobic	NIPH-11060424	*B. theta* (DT)[a]	1:1000[d]
	EDL933	*B. theta* (DT)	1:1000

[a)] DT- indicates which culture was inside the dialysis tubing.
[c)] indicates 5 µl EHEC+ 500 µl of secondary species
[c)] indicates 5 µl EHEC+ 50 ml of secondary species
[d)] indicates 5 µl EHEC + 5 ml of secondary species

10 mg/L of MSB (instead of 1 mg/L ([50]), was mistakenly added to the BHI stock solution. Since the elevated levels of MSB did not affect growth of either *B. theta* or EHEC significantly, the ten times higher concentration of MSB was used in all experiments.

Menadione sodium Bisulfite stability

It was observed that the mBHI changed color during storage, which does not apply to normal BHI. Laboratory experiments to investigate the shelf life of the modified medium (data not shown) and literature research revealed that Menadione sodium bisulfite (MSB) has low

stability and that its stability is decreased when subjected to light, heat, humidity, some salts and variations in pH [51, 52]. Therefore, MSB was since added immediately before use in the samples.

Aerobic co-cultures

Since no growth of the *B. theta* was expected in the aerobic cultures, two different ratios of EHEC: *B. theta* was tested to ensure a sufficient level of *B. theta* for co-culturing experiments. The EHEC: *B. theta* ratios were 1:100, as in the anaerobic co-culturing experiments and 1:10 000 (Table 3). In practice, to obtain the 1:10 000 ratio 5 µl of EHEC ONC was incubated with spun cells from 50 ml of *B. theta* ONC.

To test if the growth of EHEC was inhibited by *B. theta,* EHEC from the co-cultures were plated onto BA and incubated aerobically at 37°C for 24h, and CFU/ml was determined. Since the species ratio did not influence growth severely, both ratios were used for aerobic co-culturing experiments, but also for co-culturing experiments with dead *B. theta*.

Dialysis co-culture

For dialysis co-culture, a Spectra/por molecular porous membrane tube (Spectrum Laboratories, Inc. Rancho Dominguez CA, USA) was filled with 5 ml of overnight culture of *B. theta.* The tube was submerged in 50 ml of mBHI inoculated with 5 µl of either EHEC EDL933 or NIPH-11060424 overnight culture. The dialysis co-culture was otherwise conducted as described for aerobic culturing.

To prevent contamination by *B. theta* into the EHEC compartment, the entrance of the dialysis tube used to add *B. theta* was never in contact with the inside of the flask or with the growth medium. To monitor potential escape or contamination of *B. theta* from the membrane, the EHEC culture was plated on Bacteroides Bile Esculin (BBE) agar, which is selective for the *Bacteroides fragilis* group (which includes *B. theta*).

Heat inactivation of *B. theta*

To determine the lowest possible temperature for *B. theta* inactivation/killing, the heat tolerance of *B. theta* was tested.

500 µl of ONC of *B. theta* was heat treated in a water bath at 50°C, 55°C and 60°C, for either 15 or 30 min. The samples were treated in glass test tubes for optimal heat transfer. 100 µl of the heat-treated samples were inoculated onto blood agar (BA) and incubated anaerobically for 48 h at 37°C. As a positive control for growth, untreated ONC, that was aerated for the same time interval as the heat treated samples, were also inoculated onto BA and thereafter treated similarly as the heat-treated samples. After 48 h, the presence of bacterial growth was determined. The treatment ensuring 100% bacterial killing in the shortest time and lowest temperature was used in the co-culturing experiment with dead cells.

The mono- and co-culturing experiments were repeated three times with technical duplicates throughout all of the experimental steps.

For results see Appendix 6.

Co- culture with dead cells

For co-culturing, two volumes of cells were used, 500 µl and 50 ml. The 50 ml of ONC of *B. theta* was centrifuged at 1000g for 5 min in a 50 ml falcon-tube, and approximately 45.5 ml of supernatant was siphoned off. The pellet was then re-suspended in the remaining 500 µl of supernatant.

After re-suspension, *B. theta* was heat treated at 55°C for 30 min (according to the results from the heat inactivation tests, appendix 6). To ensure that *B. theta* was heat killed the bacteria were plated onto BA and inoculated into 50 ml mBHI and incubated anaerobically at 37°C for 48 h.

The co- cultures were otherwise performed as described in “conditions for culturing”.

It has been shown that centrifugal speed forces can cause damage to bacterial cells. Especially the surface of the cells are affected by too heavy/massive cell compaction [53]. It has been shown that centrifugation at 15000 x g reduces the viability of *E.coli* more than centrifugation at 5000 x g [54]. General cell surface damage due to centrifugal speed forces has been shown in various studies(e.g. Peterson et al [53]). To avoid damage to the cell surface, cells were pelleted at low speed centrifugation (1000 x g for 5 min). The cells were

centrifuged prior to heat treatment, since they probably would be more fragile after heat treatment.

Primer design

All the primers used for NIPH-11060424 were from Iversen et al [50] (Table 4).

Table 4. Primers used for qPCR

		Primer sequences			
Strain	**Gene**	**Forward (5' to 3')**	**Reverse (5' to 3')**	**Slope[a]**	**% Eff[b]**
NIPH-11060424	*gapA*	AGGTCTGATGACCACCGTTC	AACGGTCAGGTCAACTACGG	-3.3	99.7
NIPH-11060424	*espA*	CGCTTGAGCTGAAATAGCTG	AACGCTGAGTGATTCTGTGC	-3.4	95
NIPH-11060424	*escJ*	TAGCACCATCGGTCATTCAG	ACATATTACCCGTCCTGTCCTG	-3.2	84
NIPH-11060424	*tir*	ATCAACAGCTTCCAGCGTTC	TTGTAGGATCATCCGGTTCC	-3.2	96
NIPH-11060424 + EDL933	*eae*	ACATTATGGAACGGCAGAGG	AAGCGGGAGTCAATGTAACG	-3.1	88
EDL933*	*Tir*	TCAACTTCCAGCCTTCGTTC	TGCAAGCGCCTGTACAATAC	-3,37	98,1
EDL933*	*escJ*	TAGCACCATCGGTCATTCAG	TCCTGTCCTGAGGATGACTTG	-3,3	98,8
EDL933*	*espA*	AGCCAAACTTCCTCAAGACG	ACCAGCGCTTAAATCACCAC	-3,2	93,8

[a] Slope was calculated from the regression line in the standard curve
[b] Efficiency was calculated using the slope of the regression line in the standard curve
* Primers specifically designed for this work. The other were designed for Iversen et al [47]

The primers for EDL933 were designed using the software primer3Plus [55, 56]. The specificity of each primer was tested *in silico* with the translated nucleotide database (Blastn) function on NCBI [57]

The primers used for EDL933 are listed in table 4. All primers were screened for not intended binding to DNA from *E. faecalis* and *B. theta* to make sure that the primer specifically detected EHEC sequences.

Because of some irregularities in the *in silico* screening of binding capacity and high costs of qPCR reagents, a regular PCR was performed to test the primer binding capability.

Isolation of genomic DNA

Isolation of genomic DNA (gDNA) was done according to a protocol by Pospiech and Neumann [58], with some modifications. Deviations from the cited protocol entailed 16100 x g centrifugation of the ONC instead of 3000 x g, and centrifugation 15600 x g for 25 min instead of 4500 x g for 15 min to form a more defined DNA pellet. The DNA was also re-suspended in milliQ water instead of TE-buffer.

After purification all DNA samples were tested for quantity and purity using the NanoDrop 1000 (Thermo Fischer Scientific).

PCR

The PCR reactions using Thermo Scientific DyNAzyme II DNA polymerase were performed according to manufacturer's instructions (Finnzymes, Vantaa, Finland).

The thermocycler was programmed as follows: initial denaturation at 94°C for 2 min, denaturation at 94°C for 30 seconds. Annealing at 55°C for 30 seconds followed by extension for 2 min at 72°C and a final extension at 72°C for 5 min

Denaturation-extension was run for 30 cycles, and PCR product was stored at -20°C.

Agarose gel electrophoresis

PCR products were separated on 1.0% SeaKem®LE agarose gels (Lonza, Rockland ME, USA) in TAE buffer. DNA bands were visualized using a Gel Logic 200 imaging system (Kodak) (full protocol in appendix 2).

RNA isolation and treatment

RNA was extracted using Purelink RNA mini kit (Life technologies, Carlsbad, California) according to the manufacturer's instructions except for in step 1, where the speed on the

centrifuge was adjusted from 500 x *g* to 1000 x *g* for 5 min, to generate more compact and defined cell pellet.

The RNA quantity was measured using a NanoDrop 1000 spectrophotometer. The DNA was removed using the Invitrogen Turbo DNA-free™ kit (Life technologies, Carlsbad, California) according to the manufacturer's instructions with the following modifications done for Iversen et al [47]: 10 µl 10xDnase buffer and 2 µl TURBO DNase were added to 90 µl RNA regardless of RNA concentration. The samples were incubated in 37°C water for 10 min, followed by addition of further 2 µl of DNase and then incubated an additional 30 min at 37°C. 10 µl of DNase-inactivation agent was added and the samples were incubated at room temperature for two min followed by centrifugation for 90 seconds at 15800 x g to pellet agglutinated DNase and inactivation agent. The supernatant (75 µl) was transferred to a new tube, the RNA was precipitated with a mixture of 187.5 µl 100% EtOH and 7.5 µl 3M NaAc (pH5,2) and incubated at -80°C for 1 h (or overnight at -20°C). The samples were centrifuged at maximum speed (16100 x g) for 30 min at 4°C and washed with 80% EtOH before RNA was dissolved in 20 µl RNase free water. The purified RNA was stored at -80°C.

After a completed DNase treatment, the quantity (A_{260}) and purity ($A_{260/280}$, $A_{260/230}$) of the mRNA preparations were measured using NanoDrop 1000.

RNA integrity

All the steps prior to qPCR were done according to the *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments (the MIQE guidelines) [59]. RNA integrity number (RIN) is one of the essential criteria in MIQE, and it informs about the intactness of the mRNA [60]. The integrity of the RNA was determined with an Agilent 2100 bio-analyzer, according to the manufacturer's instructions. Only RIN values ≥8 were accepted and further used.

Synthesis of cDNA

The copy DNA (cDNA) synthesis was done with high capacity cDNA reverse transcription kit with Ambion® SUPERase• In™ RNase Inhibitor from Applied Biosystems (Life technologies, Carlsbad, California) according to the manufacturer's instructions.

Quantitative PCR

Standard curves for primers were prepared to ensure that the primers had a satisfying binding efficiency to the template genes.

The standard curves were prepared using five dilutions of genomic DNA (1:5, 1:25, 1:125, 1:625 and 1:3125) The primers were diluted 1:20 in milliQ water. The master mix contained: 12.5 µl SyBR green, 1 µl forward primer, 1 µl reverse primer and 5.5 µl H2O per reaction.

All dilutions were loaded on the MicroAmp™ 48 well reaction plate for qPCR in 3 technical parallels. Each well was loaded with 20 µl master mix and 5 µl of the different dilutions of gDNA. Each plate contained at least two negative controls.

The comparative quantitation of mRNA levels for LEE genes was done with cDNA. The gene expression levels were related to the expression of *gapA* (glyceraldehyde-3-phosphate dehydrogenase), which is stably expressed in NIPH-11060424 [47]. The cDNA was diluted 1:100 before use. It was assured that the final concentration of cDNA was 5 ng/µl in all samples. To ensure that the RNA that was used as a template for cDNA synthesis was not contaminated by DNA (the DNase treatment was successful), reverse transcription tests were run on each batch of RNA using a selected set of primers. If cDNA was detected in the RT negative control samples, it indicated presence of contaminating DNA. In such cases, the cDNA was remade with new RNA or the same RNA after an additional DNase treatment.

For comparative quantitation, the master mix was made as described for preparation of standard curves.

All qPCR samples were run in three technical replicates for each primer pair. Duplicates of negative control samples without cDNA, and reverse transcription (RT) negative control samples were included in each run. The MicroAmp™ 48 well reaction plate wells were loaded with 5 µl of diluted cDNA, milliQ water in the negative controls without cDNA, and diluted RNA in the RT negative control wells.

Both standard curve preparations and comparative quantitation were done on a StepOne system from Applied Biosystems. The thermal cycling conditions were as followed: 95°C for 10 min and 40 cycles of 60°C for 15 seconds and 95°C for 1 min. A melt curve analysis was performed for each sample to confirm amplification of specific transcripts.

Treatment of data

The results from the qPCR was analyzed with the Pfaffl method/software [61].

Fluorescein Actin Staining- FAS assay

To examine if up-regulation of adhesion associated genes detected by qPCR would affect EHEC's attachment to eukaryotic cells, the level of pedestal formation in HeLa cells after exposure to EHEC NIPH-11060424 cells with and without the co-presence of *B. theta* was fluorescently visualized and determined by the following procedure:

HeLa cells were grown overnight in HyClone Minimum Essential Medium with Earle's Balanced Salts (MEM/EBSS) supplied with Fetal Bovine serum and antibiotics (streptomycin and penicillin) to 80% confluence on 22x22 mm glass coverslips treated with Poly-D-lysine [1] as described in Sitterley [62] (appendix 2). The coverslips were then moved to new wells and washed 3 times with PBS pH 7.4 (Gibco™, Paisley, Scotland) before 3 ml of fresh MEM/EBSS without antibiotics was added. Bacterial ONC suspensions were diluted 1:100 for EHEC and 1:10 for *B. theta* in mBHI and added to the coverslips as shown in table 4.

All experiments were performed in three biological triplicates.

The samples were incubated for 3 h in 37°C at 5% CO_2. After incubation, the samples were washed three times with PBS 3 to remove non-adhering bacteria and fixed in 4.0% formaldehyde in PBS for 10 min. The cells were washed once with PBS and permeabilized with 0.1% Triton X-100 in PBS for 5 min, for an optimal penetration of the dyes/stains. For actin staining, the coverslips were washed three times and soaked in 5 µg/ml Fluorescein isothiocyanate (FITC)-labeled phalloidin in PBS for 20 min in darkness. To remove non-absorbed FITC, the coverslips were washed thoroughly 3 times in PBS.

Table 5. Combinations of bacterial inoculums and control media added to HeLa cells.

Component 1	Component 2
10 µl EHEC	10 µl *B. theta*
10 µl EHEC	10 µl mBHI
10 µl *B. theta*	10 µl mBHI
10 µl mBHI (neg. control)	10 µl mBHI (neg. control)

The EHEC cells were visualized using 100 µl of the BacTRace Goat anti-*E. coli* O103 antibodies (KPL, Gaitersburg MD, USA) diluted 1:500 in HEPES Buffer containing 0.5% Bovine Serum Albumin (BSA) for 1 h, washed 3 x 5 min in PBS, followed by incubation with the secondary antibody Alexa fluor® 594 Donkey Anti-goat (H+L) antibody (Thermo scientific, Rockford, USA) diluted 1:500 in 0.5% BSA in HEPES Buffer for 1 h. The cells were washed for 3 x 5 min in PBS, and mounted directly using 25 µl of Mowiol Coverslip Mounting Solution for Fluorescence Microscopy (Mowiol®4-88, Polysciences, Inc., Warrington PA, USA) containing the anti-quenching agent *p*-phenylenediamine (PPD) according to the manufacturer's instructions. For practical reasons, the microscopy was done the following day, and the coverslips were therefore stored overnight in the dark at 4°C.

The samples were analyzed by fluorescence microscopy (Inverted fluorescence microscope, Olympus IX81). For each sample, the cells were counted from 10 randomly selected fields of vision and the proportions of pedestal forming cells were calculated as cells with pedestals/total amount of cells. Students unpaired T-test was used to determine the statistical relevance of the data.

Aggregation assay

Measuring of co-aggregation between EHEC and *B. theta,* EHEC and heat inactivated *B. theta* and EHEC and *E. faecalis* was done by measuring changes in optical density, as described

previously by Collado et al. and Handley et al [63-65], but with small adjustments to fit the experimental systems used in this thesis.

In brief, bacterial overnight cultures were washed once in phosphate buffered saline (PBS 130 mM NaCl, 10 mM Na_2HPO_4) pH 7.2and then re-suspended in the same buffer. Dead *B. theta* was heat treated as described in "heat inactivation of *B. theta*" To achieve an approximately equal amount of bacteria in the solutions, a standardization of bacterial concentration was done to an absorbance (A_{600}) of OD 0.5 ± 0.05.

The bacterial suspensions were mixed 1:1 (500 µl each) by gentle vortexing, and incubated aerobically in room temperature for 24 h in a cuvette used for spectrophotometry. As controls, the OD was simultaneously measured in monocultures of EHEC and *B. theta* (auto-aggregation). OD was measured at 0, 1, 2, 3, 4, 6 and 21 h, without agitating the solutions. The calculation of co-aggregation was calculated as follows:

Auto- Aggregation: $[1-(A_{bacteria,t=n} / A_{bacteria,t=0})] \times 100$
Coaggregation: $[(A_{mix,t=0} - mix_{,t=n})/mix_{,t=0}] \times 100$

Part 3- Results

Gene expression in co-culture experiments

Physical contact between EHEC and *B. theta* influences expression of Lee encoded genes

Previous data by Iversen et al [47] suggest that co-culturing of EHEC with *B. theta* results in increased expression of LEE-encoded genes. In contrast, when EHEC was grown in the presence of spent medium from *B. theta* such effect was not observed. These results suggested that direct interspecies contact could be involved in the up-regulation of LEE encoded genes. To examine this further, dialysis culturing was performed. *B. theta* was cultured within a dialysis tube which was surrounded by a pure EHEC culture. The dialysis tubing used here was permeable to bacterial derived products below 12000-14000 Dalton (the size of small proteins) and molecules such as succinate ($C_4H_4O_4^{-2}$), acetate ($CH_3CO_2^-$), lactate ($C_3H_6O_3$) and fumarate ($C_4H_2O_4^{-2}$) which are all in the size range of 50-150 Dalton.

Quantitative analysis of expression levels of LEE-encoded genes revealed either a down-regulation or unchanged expression levels in the dialysis cultures compared to those in monocultures. Notably, the dialysis co-culture samples with the highest LEE gene expression levels, correlated with higher levels of *B. theta* contamination ("escaping") outside the dialysis membrane (data not shown). All dialysis cultures demonstrated escaping of *B. theta* from the dialysis tube at a level of $\leq 1x10^3$ CFU/ml. In comparison, a mixed co-culture contains approximately $1x10^8$ *B. theta*/ml.

B. theta induces increased expression of adherence-associated genes in NIPH-11060424 under aerobic conditions

B. theta, as an obligate anaerobe, does not grow in aerobe conditions. It has a severely lowered and altered metabolism when it is exposed to oxygen. Succinate production, as an example, is very high in anaerobic conditions but halts completely when exposed to O_2[66]. To investigate if the up-regulation of LEE encoded genes influenced by the oxygen level, EHEC was co-cultured with *B. theta* under aerobic conditions. Co-cultures under aerobic conditions is a suitable method to examine whether LEE expression is altered because of *B. theta's* traditional anaerobic metabolic products (succinate, acetate, fumarate, pyruvate etc.)[66], or if the elevation is caused by something that is independent of metabolic products (such as cell-to-cell contact). When two bacterial species are grown together and the initial ratio of inoculum is very skewed, the bacterium with the lowest amount of cells can be inhibited. The mean of EHEC growth in the 1:100 ratio was $1.02x10^9$ CFU/ml, while the mean of the 1:10 000 ratio was $1.91x10^8$ CFU/ml after 3.5 h. These growth rates do not differ significantly from the growth rate of the EHEC monoculture and suggest that the growth was not severely affected by the presence of *B. theta*. To compensate for the inability of *B. theta* to proliferate under aerobic conditions [66], two quantities of *B. theta* was used in the aerobic co-culturing experiments (See table 3).

Table 6. Comparison of expression levels of LEE-encoded genes in EHEC EDL933 and EHEC NIPH-11060424 under different culture conditions.

Gene	Co-culture conditions	Fold change qPCR EDL933	Fold change qPCR NIPH-11060424
tir	Co- culture *B. theta*		1,37
eae	Co- culture *B. theta*		1,68
espA	Co- culture *B. theta*		1,35
escJ	Co- culture *B. theta*		1,88
tir	Co- culture *E. faecalis*	-1,23	**4,93**
eae	Co- culture *E. faecalis*	-1,14	**6,18**
espA	Co- culture *E. faecalis*	-1,42	**15,78**
escJ	Co- culture *E. faecalis*	-1,48	**12,06**
tir	Dialysis culture *B. theta*	-1,41	1,56
eae	Dialysis culture *B. theta*	-1,36	1,01
espA	Dialysis culture *B. theta*	1,02	1,38
escJ	Dialysis culture *B. theta*	-1,63	0,76
tir	Aerobic EHEC: *B. theta* (1:100)	1,05	2,28
eae	Aerobic EHEC: *B. theta* (1:100)	1,48	3,75
espA	Aerobic EHEC: *B. theta* (1:100)	0,93	1,96
escJ	Aerobe 1:100	2,08	3,32
tir	Aerobe 1:10 000	**1,05**	**31,94**
eae	Aerobe 1:10 000	**1,07**	**68,48**
espA	Aerobe 1:10 000	**1,34**	**132,73**
escJ	Aerobe 1:10 000	**1,65**	**22,22**
tir	Dead *B. theta* 1:100	-1,46	-1,33
eae	Dead *B. theta* 1:100	-1,42	-1,26
espA	Dead *B. theta* 1:100	1,52	-1,47
escJ	Dead *B. theta* 1:100	-1,16	-1,02
tir	Dead *B. theta* 1:10000	-1,15	1,13
eae	Dead *B. theta* 1:10000	-1,06	-1,07
espA	Dead *B. theta* 1:10000	-1,35	1,2
escJ	Dead *B. theta* 1:10000	-1,25	1,25

Significant results are shown in bold.

Quantitative analysis of expression levels of LEE encoded genes from EHEC grown in aerobic co-culture with *B. theta* showed an increased expression level of all LEE-encoded genes. The adherence-associated genes, *espA* and *eae,* demonstrated dramatically increased expression levels, (132- and 68- fold change, respectively) compared to a monoculture (Table 6). In contrast, the same genes had no elevation in EHEC O157:H7 EDL933.

Co-culturing EHEC with the same ratios of heat-killed *B. theta* cells did not result in significantly increased expression levels of the four LEE genes tested (Table 6).

Co-culturing with *E. faecalis* affect expression of LEE-endoded genes in EHEC

In addition to *B. theta*, both *B. fragilis* and *Clostridium perfringens* was co-cultured with EHEC NIPH-11060424 in Iversen et al [47]. While co-cultures with *B. fragilis* and *B. theta* increased LEE gene expression, *C. perfringens* did not. To investigate if the increase in virulence gene expression was restricted to the phylum Bacteroidete*s,* we co-cultured EHEC NIPH-11060424 and EDL933 with *E. faecalis*, a member of the phylum Firmicutes. The two EHEC strains differed considerably, in the response to co-culturing with *E. faecalis;* NIPH-11060424 showed a >10 fold increase in the expression level of T3SS structural genes, and *tir* and *eae* demonstrated >5 fold increased expression levels. In EDL933, however, all the tested genes were down-regulated.

The qPCR results suggests that the two EHEC strains examined in this thesis might interact differently with other bacteria. The results do also suggest that LEE up-regulation does not occur without cell-cell contact in NIPH-11060424.

Aggregation

The results from the dialysis cultures and results from Iversen et al [47] and unpublished results, indicate that there is a physical interaction (aggregation) between *B. theta* and EHEC that causes elevation in LEE gene expression in co-cultures. Analysis by light microscopy demonstrated co-aggregation between *B. theta* and both EHEC strains, and auto-aggregation of *B. theta.* (Fig. 5).

When bacterial cells form aggregates/clusters in a liquid solution it will cause a quicker sedimentation rate of the bacteria. The aggregation of a bacterial suspension containing *B. theta* and EHEC was monitored by measuring absorbance/optical density in the liquid solution at specific time points [65].

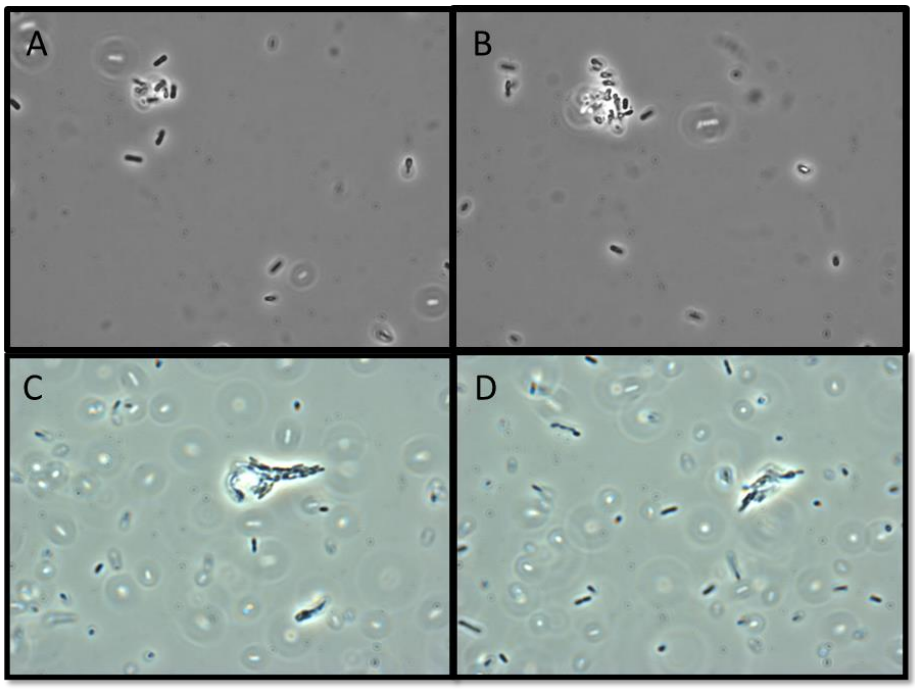

Figure 5. A. Light microscopy of a bacterial suspension containing EDL933 + *B. theta.* B. Light microscopy of a bacterial suspension containing NIPH-11060424 + *B. theta.* C+D. light microscopy of auto-aggregation of *B. theta*. 1000X magnification in all the pictures.

The measurements of absorbance revealed a very strong auto-aggregation of living *B. theta* cells. The heat-treated *B. theta*, however, demonstrated a lower auto-aggregation ratio and co-aggregation ratio with the EHEC strain (Fig. 6).

The co-aggregation between EHEC and both heat-treated and untreated *B. theta,* did not exceed the calculated average of the auto-aggregation of the two species. This makes it difficult to draw any firm conclusions from the co-aggregation experiments.

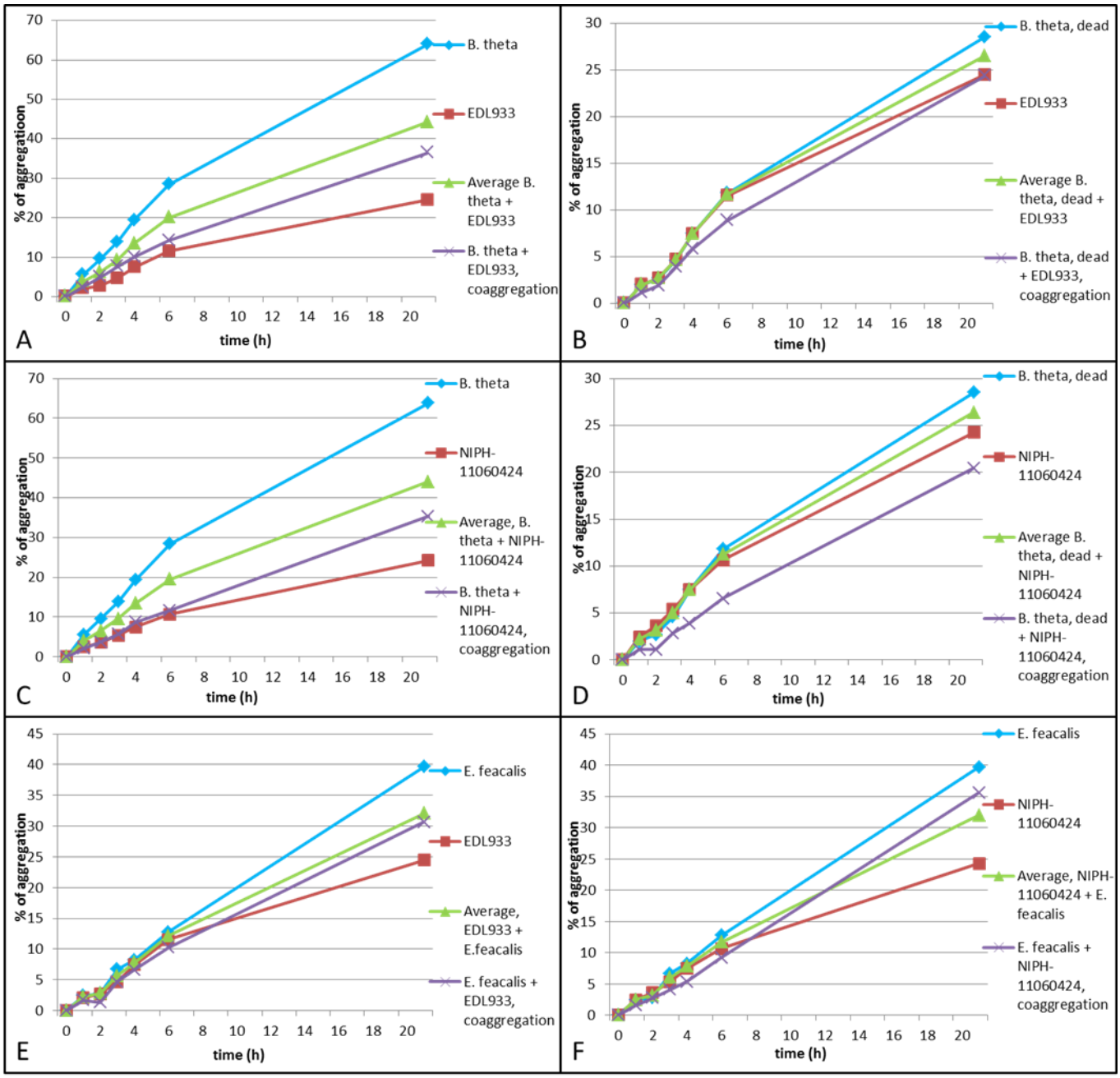

Figure 6. The graphs show the percent of co-aggregation, auto-aggregation and the average of the two auto-aggregations calculated mathematically. A. Live *B. theta* and EDL933, B. Dead *B. theta* and EDL933, C. Live *B. theta* and NIPH-11060424, D. Dead *B. theta* and NIPH-11060424, E. *E. faecalis* and EDL933, F. *E. faecalis* and NIPH-11060242.

EHEC mixed with *E. faecalis* on the other hand, demonstrated stronger co-aggregation than the calculated average of the auto-aggregation of the species combined, proving occurrence

of co-aggregation. The co-aggregation after 21 h was somewhat stronger for NIPH-11060424 (35.5%) compared to EDL933 (30.6% with).

There was only done one repetition of the aggregation assay with *E. faecalis*. It would have been interesting with three repetitions, to be able to draw any conclusions further than that there is co-aggregation between *E. faecalis* and EHEC.

The results from the aggregation assay revealed a strong auto-aggregation in *B. theta*, but the method for measuring co-aggregation proved to be inaccurate because of large variation in auto-aggregation between species.

FAS

Presence of *B. theta* does not influence adherence and pedestal formation by EHEC

To investigate if the increased expression of LEE-encoded genes in co-culture with *B. theta* influences EHECs adherence to target cells and pedestal formation HeLa cells were infected with EHEC NIPH-11060424 alone or with a mixture of EHEC and *B. theta*. HeLa cells were chosen for this experiment since they have earlier been used to study the mechanisms of EHEC infection such as adherence and pedestal formation [48, 67].

EHEC infected HeLa cells were stained with phalloidin FITCH for visualization of actin accumulation (pedestal formation) and analyzed by fluorescece microscopy. Ratios of the total amount of adherent EHEC cells per total amount of HeLa cells were counted. The results revealed no significant difference in the number of cells with pedestals after infection with EHEC alone or by a mixture of EHEC and *B. theta*. Neither was there a significant difference in number of cells infected with EHEC or the total number of adhering EHEC (Figure 7).

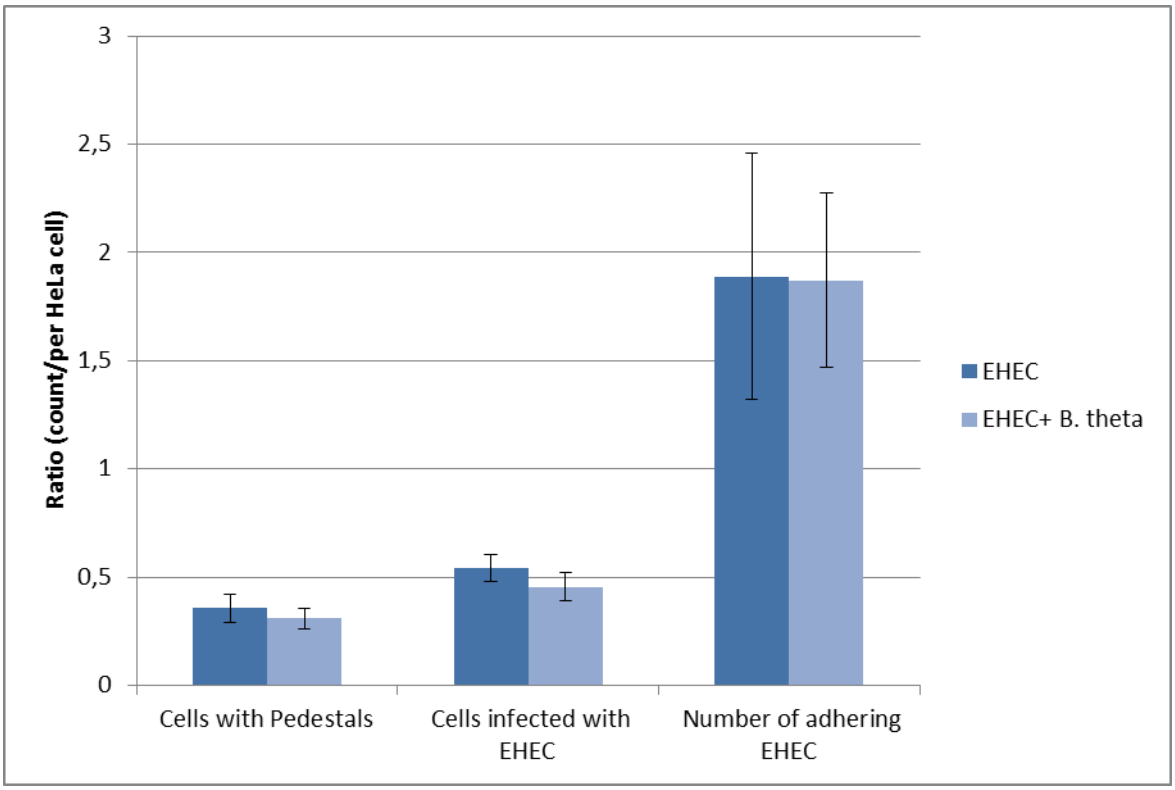

Figure 7. Ratios of counts of pedestals, amount of EHEC infected cells and total adhering EHEC per total amount of HeLa cells with added EHEC NIPH-11060424 alone and with *B. theta.*

These results suggest that the presence of *B. theta* has no influence on EHEC's adherence to HeLa cells under the conditions the experiment was conducted.

Figure 8 illustrates how pedestals appear on the same locations as EHEC is attached to the cells, proving pedestal formation to be caused by EHEC adhesion.

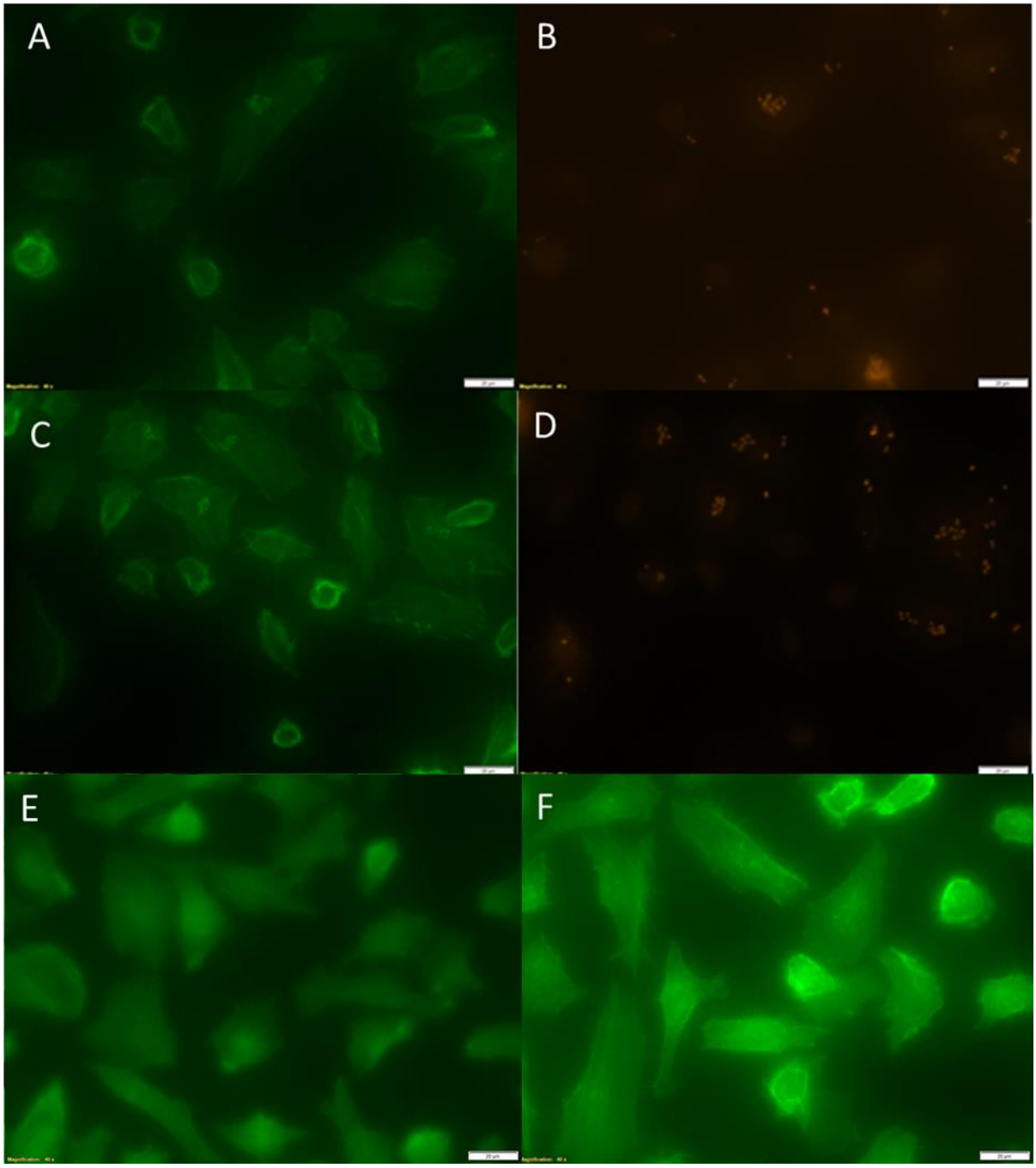

Figure 8. A. FITC staining of HeLa cells infected with EHEC B. Visualization of immunofluorescently stained EHEC (Orange color) (same field of vision as A) C. FITCH staining of HeLa cells infected with EHEC and *B. theta* D. Visualization of immunofluorescently stained EHEC (same field of vision as C) E. FITCH staining of HeLa cells without bacteria (negative control). F. FITCH stained HeLa cells with *B. theta.* All microscopy was done at 400X magnification.

When the FAS assay was performed the first time, both an incubation time of 3 h and 6 h were tested, as both time periods were suggested as viable options by Knutton et al [67]. When the two incubation times were tested, there seemed to be no noticeable difference in pedestal formation, and since pedestal formation was observed after 3 h incubation in equal degree compared to 6 h, the shorter incubation was chosen for practical reasons. When pedestals were counted, it became evident that number of pedestals did not coincide with number of adherent EHEC. This made pedestal formation an inaccurate measurement of the amount of infection, and it became necessary to count total number of adherent EHEC.

B. theta influences health of HeLa cells

When HeLa cells are in distress, or have been in distress that lead to apoptosis, they undergo rearrangement/shriveling of the actin cytoskeleton, that gives the cells a circular shape (resembling apoptosis)[68].

When control samples, incubated with *B. theta* alone, were examined we observed an increased frequency of HeLa cells with actin cytoskeleton rearrangements (Fig. 9).

Because of this visual observation, the proportion of apoptotic/distressed HeLa cells in the negative control and in HeLa cells infected with *B. theta* cells alone was determined.

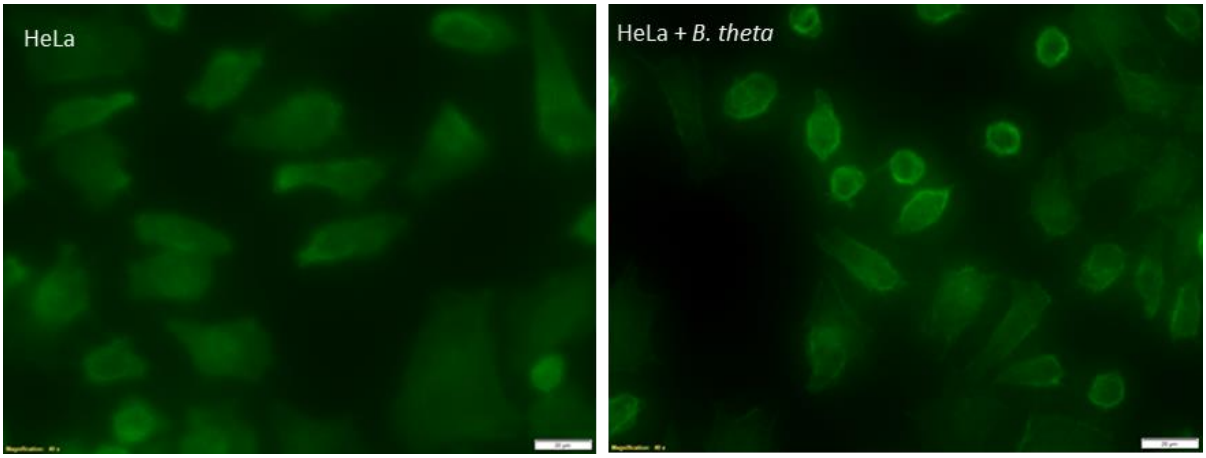

Figure 9. The images show an example of HeLa cell culture with no added bacteria (to the left) and cell culture inoculated with *B. theta* (to the right) both at 400X magnification.

Quantitative analysis showed over a doubling of distressed/apoptotic cells in the cell cultures inoculated with *B. theta* (Table 7).

The large overlapping standard deviations shows that more counts was necessary, to ensure statistical relevance. This only gives an indication of the possibility of *B. theta* having a negative effect (acting as a pathogen) on HeLa cells under aerobic conditions.

The results from the FAS assay suggest that *B. theta* has no influence on EHEC adhesion, but that it might cause damage, resembling apoptosis, to HeLa cells.

Table 7. Quantification of distressed/apoptotic HeLa cells

Cell counts	*B. theta*	Negative control
Total	347	342
Distressed	98	45
Mean per field of vision	17,35	17,1
Mean distressed	4,9	2,25
Distressed %	28,24	13,6
Standard deviation %	12,54	9,32

Part 4- Discussion

The results presented in this thesis, reinforce that direct interspecies cell-cell contact between EHEC and *B. theta* leads to increased expression of LEE-encoded genes. Elevated LEE gene expression was also achieved with a member of the phylum Firmicutes, *E. faecalis,* showing that increased expression in these genes where not limited to co-cultures with Bacteroidetes. The results also illustrated that reactions to chemical compunds and other bacterial species could differ between EHEC serotypes, as EDL933 showed no elevation in LEE genes where NIPH-11060424 did. Aggregation between *B. theta* and EHEC was difficult to determine, and no increased adherence to HeLa cells was observed when with *B. theta* present.

The results from both the dialysis co-culture and the aerobic co-cultures in NIPH-11060424 seem to support the findings by Iversen et al [47], and indicate that physical contact is important for elevated expression levels of LEE encoded genes in co-cultures with *B. theta*. In the dialysis co-cultures, there was a dense *B. theta* culture inside the dialysis tubing. This saturated culture would initially contain a high concentration of metabolites produced by *B. theta*, and the production of metabolites would also continue after the dialysis tubing was submerged into the fresh broth with EHEC. The permeability of the dialysis tubing ensures that the metabolic landscape EHEC grew in was changed in the same manner that it would in co-culture. However, in contrast to co-cultures where both species were present in the same suspension no elevated expression levels of LEE encoded genes was observed when the species where prevented from direct physical contact.

One could perhaps argue that the levels of succinate and other metabolites produced in a co-culture could be lower inside the dialysis tube. In addition, direct cell -cell contact with EHEC, could also stimulate *B. theta* to produce/release more metabolites that could affect expression of LEE-encoded genes. However, the large amount of *B. theta* saturated inoculum inside the dialysis tube and preformed metabolites in the inoculum should compensate for a potentially lesser metabolic production caused by lack of contact.

Aerobic culturing conditions

B. theta is an obligate anaerobe [36].The definition of an obligate anaerobe is that it cannot grow when oxygen is present, and many obligates also die due to oxygen "poisoning". According to Ning Pan and James Imlay's review [66] on Oxygen's influence on *B. theta*, this does not apply to *B. theta*, since it can resume growth after aeration for 48 h [66]. When the experiments were performed, *B. theta* was observed to be viable after 8 h and 24 h incubation in aerobic conditions at room temperature (Iversen et al, unpublished results). Even if *B. theta* can tolerate oxygen, it will stop replication, the metabolism will slow down dramatically and become incomplete [66]. Oxygen or O_2^- blocks the enzyme fumarase, which is necessary for succinate production. The oxygen is believed to most likely damage the iron-sulfur cluster in fumarase by oxidative stress (as all ferric compounds normally is sensitive to oxygen), and thereby disabling the conversion of fumarate into succinate ($C_4H_2O_4^{2-} \rightarrow C_4H_4O_4^{2-}$) [66].

The damage to fumarase results in *B. theta* not being able to produce succinate when it is transferred to an environment containing oxygen [66]. Acetate and Pyruvate continue to be produced at moderate levels, while secretion of succinate stops completely [48] [66].

The oxygen-dependent inhibition of fumarase activity can be reversedby the addition of a sufficient amount of fumarate to the growth medium [66]. However, pure fumarate was not added to the growth medium in this study, so one could assume that the fumarasewas inactive. The medium used in this thesis was however not exactly the same as the media used previously in the study that examined *B. theta*'s metabolic activity under aerobic and anaerobic conditions[66]. In their experiments, BHI was also used, but theirs was not modified. In this thesis, opposing to Pan et Imlay[66], there was added hemin ($C_{34}H_{32}Cl_4$) and menadione sodium bisulfite ($C_{11}H_9NaO_5S$), which both can be assumed to be free from fumarate and not interfering in this pathway. MSB in solution will be degraded to menadione. Menadione demonstrate different degradation pathways under aerobic and anaerobic conditions. Under anaerobic conditions, hydrogen peroxide is a byproduct of menadione degradation [52]. Hydrogen peroxide can damage the iron-sulfur cluster in fumarase (in the same manner as oxygen) [66]. This can have an inhibiting effect on succinate production under anaerobic cultures. The fact that menadione has different end products in aerobic and anaerobic conditions may have an impact on how the bacteria reacts to the culture-media.

Another substance that is added under the modification of the culture media is yeast extract. It is difficult to know the constituents of yeast extract, especially since the manufacturer cannot confirm or deny if it contains fumarate. However, according to the chapter "Design and formulation of Microbial culture media"[69] in the section for yeast extract published in 1970 and written by Oxoid, which is the same producer as the yeast extract used in this experiment, there is no fumarate in yeast extract.This coincides with the method used for yeast extract production: The baker's or brewer's yeast (*Saccaromyces cerevisia*) are grown to saturation, then washed and then auto-lysed. The lysed material is then dried at an appropriate temperature to preserve certain components sensitive to temperature, e.g. vitamins [69]. This process would ensure that the only fumarate that could possibly be present in yeast extract, would be from intracellular intermediates in the TCA

cycle and the quantities would therefore be minuscule and insignificant in the finished product.

Curtis et al [48] reported that it is *B. theta*'s metabolites, especially succinate, that causes elevation in LEE genes. Since no fumarate was added to the media in the aerobic cultures, it can be assumed that succinate was not produced, because of markedly slowed metabolism in *B. theta*. Since oxygen disrupts metabolic pathways, far less metabolites are produced. The increase in gene expression is therefore likely caused by something other than metabolites, which support the hypothesis that the increase in LEE gene expression levels likely is a result of interspecies cell to cell contact.

Effect of preparatory treatment on *B. theta*

To examine whether cells with no metabolic activity would influence expression of LEE encoded genes EHEC was cultured in presence of heat-killed *B. theta*. The results from the aggregation assay with heat treated *B. theta* showed a lower co-aggregation and auto-aggregation than in the live cells. Even if precautions were taken when cells were heat killed by using low velocity centrifugation and low temperature, cell surface structures could have been damaged. The pellet of centrifuged and heat treated cells was visually different (data not shown) from the pellet of live cells. While the live cells had a defined pellet, the pellet of dead cells was looser and the cells in the pellet appeared as if it was enveloped in an viscous extracellular matrix, which indicates cell lysis. This, in combination with the results from the co-cultures with dead cells, suggests that the heat treatment and centrifugation might have had a damaging effect on the surface of the cells. Thus, it is difficult to draw any conclusions from the aggregation assays with dead *B. theta* since surface structures potentially involved in cell-cell contact with EHEC could have been destroyed or not functional.

Difference in LEE gene expression between two EHEC strains

The co-cultures of EHEC EDL933 and *B. theta* showed no elevation of LEE encoded gene expression in any of the co-cultures. These results, contradicts the previous reports by Curtis et al [48] and Njoroge et al [70] which reported that EHEC EDL933 display increased

expression of LEE-encoded genes when co-cultured with *B. theta* and *E. faecalis.* These contradictory results can probably be explained by differences in culture conditions.

Both medium and incubation time in the previously published co-culture studies with EDL933 were different from the conditions used in our study; These studied had 6 h incubation time, instead of 3.5 h (as used in our study) and the bacteria were cultured in Dulbecco's modified Eagles medium (DMEM) instead of mBHI.

Surprisingly, EHEC NIPH-11060424 and EDL933 in the aerobic co-cultures, in addition the co-culturing experiments with *E. faecalis* demonstrated that differences in adherence associated gene expression between strains can be very distinct. While EDL933 had no upregulation in LEE encoded genes in any of the co-cultures, NIPH-11060424 responded to large cell matters and had an especially high expression in the 1:10 000 ratio under aerobic co-cultures.

The anaerobic NIPH-11060424 co-cultures with *B. theta* demonstrated only a very low increase (not significant) in LEE gene expression levels. The same experiment was done by Iversen et al [47], where the same LEE genes had 5-11 fold up-regulation. The only condition that differed between the two experiments was the ten-fold higher MSB concentration used in this work. This indicates that MSB may have an inhibitory effect on expression of LEE-encoded genes. Whether or not MSB had an inhibitory effect (eg. dialysis cultures) in all co-cultures samples is not tested here, but it is possible that the results would be different with a lower MSB concentration.

MSB belongs to the chemical family of quinones, which has been suggested to influence the activity of membrane proteins [71-73]. Ando et al [71] speculate that quinones affect the formation of T3SS. They are, however, stating that it might enhance T3SS maturation and not inhibiting it. If membrane proteins are modulated by quinones, and cell-cell contact (which is dependent on surface proteins) is necessary for up-regulation of LEE-encoded genes in co-culture menadione might affect these interactions.

If there would have been time, the experiments would preferably be re-done with the same MSB content as in Iversen et al [74]. It would be especially interesting to see how the MSB content affect the two different EHEC serotypes.

Aggregation assay

The first thing that became apparent during the aggregation assay, was the high level of auto-aggregation in *B. theta*. This is interesting, because according to Blake et al [75] there is not supposed to be any auto-aggregation in *B. theta,* opposed to the closely related *B. fragilis*. One can argue that the rapid drop in OD was due to lysis of the bacteria but typical signs of bacterial lysis (increased viscosity, reduced pellet size) was not observed in the bacterial suspensions. Another possibility is that *B. theta* might not have been "working properly" under the aerobic experimental conditions and in PBS buffer. If that was the case, however, the heat treated/inactivated/dead *B. theta*, would most likely have demonstrated a faster decline in OD than the alive, but such faster sedimentation was not observed here.

TEM pictures from Iversen et al, unpublished results (Fig. 4) and light microscopy from this work indicated that a tight cell-cell adhesion does occur between EHEC and *B. theta.* The co-aggregation results did, however, not show a co-aggregation between the two species. A higher co-aggregation in the mixed species suspensions than observed in the mono-cultures is a sure sign that co-aggregation occurs. However, the results from the co-aggregation analyses were difficult to interpret because, in most of the cases, the average of the two species' auto aggregation was higher than the co-aggregation. The seemingly very high auto-aggregation of *B. theta*, ruins the credibility of the experiment due to the fact that the equation isn't designed for species with high divergence auto-aggregation behavior. The high auto-aggregation of *B. theta* will make the average of the auto-aggregation of *B. theta* and EHEC combined unnaturally high, and make the co-aggregation seem non-existent in comparison.

The TEM analyses were performed on bacterial cultures where both species were in an exponential growth phase. In the aggregation assays performed here, both bacteria are in an inactive state in respects to proliferation, and due to lack of access to nutrients (PBS) they can be assumed to be metabolically inactive. Since, the environmental factors were very different in the aggregation assay and the co-culturing conditions, one could expect that the bacteria would behave differently during the aggregation assay versus in co-cultures.

Another problem with interpreting these results is that the significance of difference in motility between the co-incubated species is difficult to determine. While both pathogenic

strains were motile, both *E. faecalis* and *B. theta* are non-motile[36, 76]. If one hypothetically assumed that no auto-aggregation is happening in any of the cuvettes, one could assume that the motile species would be able to stay in the liquid phase longer, and hence have a slower decrease in optical density. It is a possibility that this is the case in this experiment. In respect to this, and because of high variation between each biological sample, more replicates should have been done to be able to draw any certain conclusions.

Another argument against the possibility of no occurrence of aggregation, is firstly that both co-aggregation and auto-aggregation was observed by microscopic examination of cultures in exponential growth phase. There is also a noticeable difference in co-aggregation between the heat-treated cells and the live cells. This shows that there is an interaction that is most likely aggregation between EHEC and *B. theta*, and this interplay is weakened in dead cells. The weak co-aggregation results can give an impression that when *B. theta* adheres to EHEC, EHEC's strong motility prevents *B. theta* from falling down to the bottom by holding the *B. theta* suspended in the liquid phase, thereby showing a false "negative" of weakened or inexistent co-aggregation.

Heat inactivated co-cultures have weaker co-aggregation than live *B. theta* with EHEC

As discussed earlier, there is a possibility of damage to *B. theta* during heat treatment and centrifugation. However, any kind of cell that is inactivated/killed, regardless of which method is used and how gentle it is, will have its properties somewhat changed.

In our experiment, the co-aggregation was reduced when *B. theta* cells were inactivated. The reason for this can be many, but since aggregation abilities often are connected with surface structure it is most likely that it is because the treatment of the cells (heat- treatment and washing in PBS with centrifugation) has damaged the cell surface, and thereby the aggregation abilities of the bacteria.

B. theta is covered by short fimbriae (see Fig. 3). Fimbriae (also called pili) are important for adherence[77], and damaged fimbriae will hence affect aggregation abilities. Since fimbriae are "extracellular", we can assume that they are more vulnerable for destruction by heat,

but also by cell compaction caused by centrifugation (in comparison with organelles that are protected inside the bacterial cell).

There has not been a study that shows fimbria to be important for co-aggregation with *Bacteroidetes,* but it has been shown to be crucial in co-aggregation between *Lactobacilli* and *Escherichia coli* [78]. In Mizuno et al [78] there was shown that both fimbriae and LPS in *E. coli* were necessary for co-aggregation with various *Lactobacilli* (that also had fimbriae), it can therefore be assumed that a weakened/damaged fimbrial coat on *B. theta* would reduce aggregation/adhesion with EHEC, and this is most likely the main factor explaining why the co-aggregation was lower with dead *B. theta*. It can be assumed that damage to LPS is less likely to be the cause of lessened aggregation in the heat inactivated cell, because of LPS' robustness and high heat tolerance [79].

In comparison with the part of the assay conducted with *B. theta*, the results from EHEC's aggregation with *E. feacalis* showed a clear co-aggregation, because of a higher co-aggregation than the average from the auto-aggregation. While *E. faecalis* is not known for having "furry" fimbriae, it does have pili on the surface that are of importance for *Enterococcal* adhesion to epithelial cells and aggregation (biofilm formation) [80].

FAS assay

The results from the actin FAS assay in this work showed no heightened adhesion or pedestal formation of EHEC when it was inoculated on HeLa cells with *B. theta* present, versus when EHEC was inoculated on HeLa cells alone.

Iversen et al [47] successfully showed an increase in adhesion of EHEC to HeLa cells when co-cultured with *B. theta*, and Curtis et al [48] demonstrated an increase in pedestal formation in their FAS assay with *B. theta* present with EHEC. Based on these studies, the expected result in this assay was an elevated colonization by EHEC with *B. theta* present. This was however not the case, and the reason for not being able to replicate the published results probably lies within the methodology, and aspects of this will be discussed below.

Incubation time

The first time the FAS assay was run, the bacteria was stained with propidium iodide (PI). PI has the ability to stain nucleic acids by inserting itself between successive bases in DNA and form fluorescent complexes [81], and this makes it suitable to stain bacteria and the nucleus of eukaryotic cells. PI is a strong dye, and in this experiment the staining of the eukaryotic cells was too strong (staining the whole eukaryotic cell), and made counting of adherent bacteria impossible. This made us discard the use of this dye further (in favor of staining with antibodies). If it had been possible to count adherent bacteria, we would probably have noticed that at 3 h, there would be many of the adhering EHEC that hadn't had time to induce pedestals in the HeLa cells. This is also logical, since bacteria will adhere during the whole incubation time, and pedestal formation does not happen instantaneously. This made the counting of pedestals an inaccurate measurement of adherence.

This was discovered when the counts were made after EHEC was stained with immunofluorescence. Since the number of pedestals did not equate to the number of adhering EHEC, the decision to count total amount of EHEC present and amount of cells with EHEC attached was made. If there would have been time, the assay would have been done once more a washing step at 3h and a total of 6 h incubation time. The washing would have removed non-adhering bacteria, and the prolonged incubation would allow pedestals to form, and the number of adhering bacteria would probably correspond better with the amount of pedestals. Also, we would have examined a larger area of cells to get more counts to achieve a higher statistical relevance.

Bacterial inoculums

In the planning stages of the FAS assay, two types of the bacterial inoculums were considered; either an inoculum of a co-culture of EHEC and *B. theta*, or inoculums of separately grown overnight cultures of EHEC and *B. theta*.

In Iversen et al [47], there was recorded increased adherence of EHEC to HeLa cells. The bacterial suspension of EHEC and *B. theta* was, in contrast to this work, co-cultured for 3.5 h before inoculation on HeLa cells. In Curtis et al [48] however, the first encounter of the two bacterial species was when both overnight cultures was inoculated in the wells with the HeLa cells. The decision to do the inoculation according to Curtis et al[48] was based on the

observation that when *B. theta* and EHEC are co-cultured for approximately 3.5 h, a formation of clusters in various sizes occur (Fig. 4). In an *in vivo* situation, EHEC in clusters with other bacteria would have a very poor chance of reaching the epithelial cells. The epithelium is protected by two layers of mucus, the outer layer is loose and inhabited by commensal microbes. The inner layer however, is dense and anchored to the epithelial cells [82]. If EHEC forms aggregates with *B. theta*, it will lose a great deal of motility, which is needed for passage through the dense mucus layer. If the decreased motility would not be enough for a failed infection, the larger size of the aggregates would be enough to stop them from passing through.

Since HeLa cells have no mucus layer for defense, neither of inoculations considered would perfectly imitate how it would happen *in vivo.* Still, it seemed to be most true to reality that the bacterial cells should be inoculated on HeLa cells from separate cultures, imitating how they would encounter each other in the outer layer of the mucus. If they were to be incubated on the HeLa cells after a 3.5 h co-incubation, the inoculum would contain "ready-made" clusters of *B. theta* and EHEC.

If EHEC's up-regulation of LEE genes in co-culture is in fact due to a surface contact that occurs in clustering, the EHEC in clusters will have a very high LEE gene expression. In an *in vivo* situation however, these clusters that pre-co-culturing causes would probably not be able to pass through the dense inner mucus layer. HeLa cells that lack this mucus layer, would get infected with cluster of co-cultured EHEC. These clusters of *B. theta* and EHEC, would have higher adhesion gene expression. It is however impossible to say if EHEC has mechanisms for preventing these types of situations without examining it specifically.

It can be discussed how well aggregation assays and adhesions in co-cultures in vitro can enlighten on how aggregation/adhesion happens between the pathogen and indigenous commensal microflora *in vivo.* Not only will the composition/immense diversity in the commensal flora compared to the in vitro di-cultures with only two bacterial species in large quantities and uneven ratios, make it difficult to predict how adhesion/aggregation would potentially occur, but also the fact that the indigenous bacteria the pathogens encounter are enveloped in a layer of mucus [82, 83]. This mucus layer consist mostly of mucin polymers, that has many properties, one of them: the ability to prevent bacterial aggregation by

maintaining bacteria in a free swimming state [83]. This was however only shown for non-planktonic bacteria, which EHEC is not.

It would be interesting to see if aggregation between the strains used in this work would be possible with mucin present.

Could higher Menadione sodium bisulfite concentrations have influenced adherence?

Since MSB content seems to influence LEE gene expression (as discussed earlier), there is a chance that it could influence adherence in this type of assay as well, especially when Curtis et al [48] followed a very similar method (without MSB) and got an increased adherence. It would have been interesting to re-do the FAS assay with the exact same conditions as in this work, with the exception of adding the originally intended amount of MSB [50], instead of ten times as much.

Colonization pattern

As stated earlier, the colonization pattern of EHEC was peculiar. Instead of infecting all HeLa cells, some cells were especially targeted, and microcolonies where several EHEC infected the cells in close proximity to each other formed. The microcolony formation can be explained as a step in biofilm formation, a survival strategy where the bacteria ensure continued infection of the cell by a "strength in numbers" strategy [84]. How EHEC decides which cells to target however is more difficult to explain. Tissue tropism of EHEC is very dependent on intimin binding to nucleolin [28]. Nucleolin synthesis is correlated with cell proliferation, and is expressed in newly divided cells in the body in mid and late G1 in the cell cycle [85]. In the intestines, this means that EHEC will attach to the newest epithelial cells, as older cells has no expression of nucleolin [85]. This will ensure them to stay in the body for as long as possible, since renewal of epithelial cells happen continuously.

In Sinclair et O'brien [19], it is demonstrated that EHEC colonizes HEp-2 cells in accordance to nucleolins expression pattern. In HeLa cells (and many other cell cultures) nucleolin is expressed at all times due to their cancerous origin [85, 86]. Cancer cells never stop proliferation, and nucleolin is therefore always present on the cell surface. It is however likely, that HeLa cells like non-tumor cells, has the highest amount of nucleolin expressed

when they are newly divided. Maybe EHEC colonize certain cells based on the quantity of nucleolin present on the surface, and that is the explanation for the pattern of colonization?

B. theta influence on HeLa cells

The seeming negative influence *B. theta* has on HeLa cells, is unexpected and a surprise, seeing as the cell experiment was done under aerobic conditions, and *B. theta* (as an obligate anaerobe) have a severely retarded metabolism when it is exposed to air [66]. There is however one metabolic product, Lactate, which synthesis increases greatly under aerobic conditions in *B. theta* [66]. This might have an effect on the health of the cells, but without analyzing the broth for lactate content, it is difficult to say if the levels of lactate produced in 3h would be sufficient to make a difference.

Since *B. theta* is supposed to be close to metabolically dormant, if one disregards lactate production[66] there might be virulence mechanisms that can work under oxygenated conditions that are unknown to us. In the closely related obligate anaerobe *B. fragilis,* exposure to O_2 can elevate the virulence potential of the bacterium in anaerobic infections [87]. There may be something similar that happens in *B. theta* or something else completely that is unaffected by access to oxygen.

As mentioned in the introduction, *B. theta* has been shown to have the ability to induce severe ulcerative disease in mice predisposed for IBD [66], which entails destruction of epithelial cells. While the mechanism for how it causes disease isn't shown in the study done by Bloom et al [66], it might be possible that *B. theta* can affect HeLa cells in a similar destructive manner as it does the epithelial cells in the mice models.

Part 5- Conclusion

The main conclusion based on the results from this thesis is that cell-cell contact is necessary for elevated LEE gene expression when EHEC O103:H25 is co-cultured with *B. theta* under the conditions of this thesis. One could also conclude that there is a difference on how serotype O103:H23 and O157:H7 reacts to co-culturing. While O103:H25 got a strong up-regulation of adherence related genes, O157:H7 did not. This suggested that O157:H7 might have a different interaction pattern with commensal bacteria.

The results from this thesis, that oppose findings from Curtis et al [48] and Iversen et al [47] in some experiments, emphasizes the fact that a the conditions of an experiment can have tremendous effects on the outcome. This shed light on how difficult it is to imitate the environment in the bowels *in vitro.* One small component can throw a whole system out of balance.

The results from this thesis leave many questions unanswered, and open for further research.

Future prospects include looking into MSB's effect on LEE expression in the two EHEC serotypes in co- and monoculture, with various MSB concentrations. Other adhesion related genes could also be included (e.g. *ompA*).

EHEC NIPH-11060424 had an especially high occurrence of HUS and hence high virulence in the outbreak in 2006 [49]. A FAS assay, with and without high amounts of MSB, that compare the two EHEC strains' adherence efficiency might give indications to if the high virulence from the 2006 outbreak could be related to adherence abilities.

As mentioned earlier it is extremely difficult to imitate *in vivo* conditions in a laboratory, and since EHEC predominantly is a human pathogen, no good animal models are available. A step in the right direction of emulating conditions of the bowel, would be inoculating EHEC and *B. theta* on *ex vivo* tissue cultured colonic biopsies. In tissue cultures there are some functions that occur *in vivo*, but not on cell cultures (e.g. mucin production). Examining how EHEC with the presence of *B. theta* adheres and damages tissue etc. in comparison to EHEC alone, can give answers that might eventually be important for developing strategies to treat or prevent disease.

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Appendix

Appendix 1

Media, buffers and solutions

TAE- Buffer (1 L, 50x concentration) : 242g Tris base, 57.1ml 1M acetic acid , O.5M EDTA pH8 in H_2O.

SET Buffer: 25mM EDTA, pH8.0, 20mM Tris HCL pH7.5 and 75mM NaCl

TE- Buffer (1L): 10 ml 1M TrisHCl (pH 8.0), 200ml 0,5M EDTA, 790ml milliQ-H_2O

0,5M EDTA pH8.0 (1L): 232.6g disodium ethylenediamintetraacetate, 1L dH20, adjustment to pH8 with approximately 25g NaOH.

1M TrisHCL pH8.0(1L): 121g Tris base, 800ml dH_2O, 42ml HCl, pH adjusted to 8 by addition of HCL, dH_2O up to 1L

PBS pH 7,2 (1L): 130mM NaCl, 10mM Na_2HPO_4, H_2O, pH adjusted with concentrated HCL

Hepes buffer pH7,4 (1L): 115mM NaCl, 1,2mM $CaCl_2$, 1,2mM $MgCl_2$, 2,4mM K_2HPO_4, 4,77 g HEPES + 1L of H_2O. pH was adjusted to 7,4 by addition of 5M NaOH and sterilized by filtration using a 0,22µm filter (Minisart, Sartorius Stedim Biotech, Goettingen, Germany)

Appendix 2

Pre- coating of coverslips for heightened adherence

When culturing cells on glass surfaces, there can be a problem with cell-glass adherence. To prevent shedding/release of cells from the glass during wash steps a coating treatment of the glass to improve adherence properties can be desirable. There was performed prior to incubation of the cell into the wells

Both poly-D-lysine and Poly-L-lysine MW30 000-150 000 (lower molecular weight is toxic to the cells) can be used as a coating agent that will give the surface a positive charge and hence improve attachment to the glass. Some cell lines will release proteases, and only Poly-D-lysine is unaffected by protease activity.[88]

For this reason, Poly-D-lysine was used in this experiment.

Protocol:

1. A stock solution of 1mg/mL poly-D-lysine-HBr (MW 30 000-70 000) in milliQ water was prepared and sterilized with a Millipore filter membrane of 0,22µm pore size.[89]
2. The sterilized samples was distributed into aliquots and either stored at -20°C or applied immediately.
3. A working solution of 0.1mg/mL poly-D-lysine was prepared with a 1:10 dilution in milliQ water.
4. 1 mL of work solution was added to each coverslip and incubated in room-temperature for 5 min in a flow hood.
5. Remove the poly-D-lysine solution from the coverslips with a syringe or pasteur pipette and rinse thoroughly with dH20.
6. Let the coverslips dry in a fume hood for 2 h, to ensure that there is no free poly-lysine introduced to the cell medium(This can inhibit cell division). [90]

The coating procedure is either done aseptically or sterilized later with UV radiation. [89]

Appendix 3

Gel Electrophoresis

Protocol:

1. 0,600g of SeaKem®LE agarose was weighed and added to a clean Erlenmeyer flask.
2. 60ml of TAE buffer was added to the flask and mixed.
3. The solution was heated in the microwave over on the highest power, until bubbles appeared.
4. The Beaker/flask was removed from the microwave and gently swirled to re-suspend potentially settled powder or gel pieces. (The importance of handling the microwaved solution with gentleness is caused by the possibility of superheating and hence the danger of foaming over when it is agitated. This can cause severe burn accidents.)

5. The solution was then boiled in the microwave for 1 minute, or until all residues of particles was dissolved.
6. The solution was chilled to approximately 50-60°C, and 10mg/ml (ca. a drop) of Ethidium bromide was added before casting of the gel.
7. 10 µl of Coomassie Blue loading dye was added to 50 µl of PCR product and vortexed.
8. 10 µl of the dyed PCR product was loaded onto the gel as well as 10 µl of a 1Kb ladder.
9. The gel was run for 40 min at 100V while soaked in TAE buffer.
10. The gels were then photographed with Gel Logic 200 imaging system (Kodak), to display possible bands that indicate successful primer binding capacity.

Appendix 4

Accordinng to the DSMZ strain passport *Enterococcus faecalis* DSM 20478 the optimal growth medium was Tryptic Soy Yeast Extract broth (TSYE). A growth study was conducted comparing TSYE and mBHI, with measureremnts of optical densitu A_{600} over a period of 6 h (until the growth stagnated).

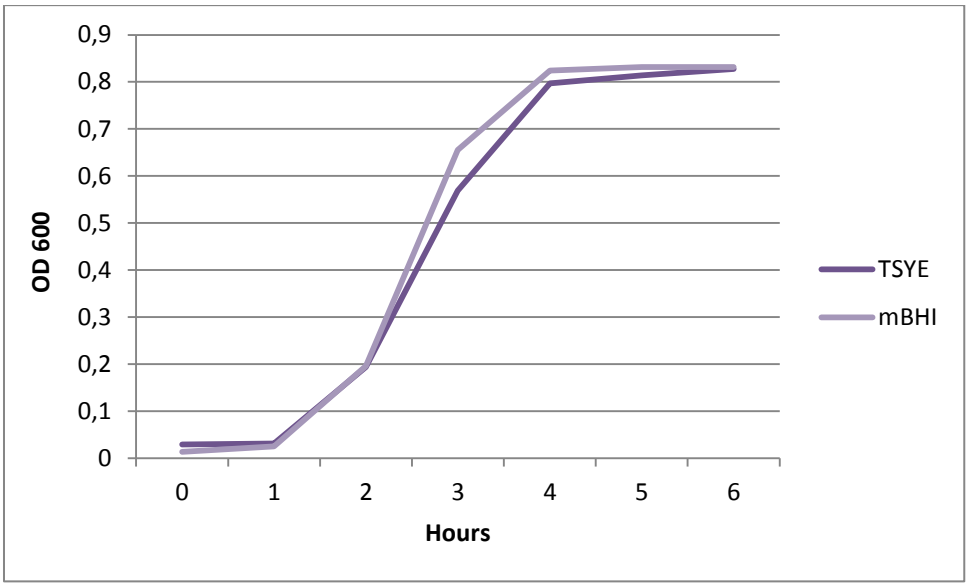


Figure 3. A graph that illustrates growth of *E. faecalis* in two different broths: Tryptic Soy Yeast Extract broth (TSYE) and modified BHI. pH In TSYE was 5,26 at the last OD measurement and 5,90 I mBHI

Since *E. faecalis* had an equally high (or higher) growth in mBHI, mBHI was used throughout the thesis for cultures with *E. faecalis.*

Appendix 5

Growth of EHEC inhibited by *L. acidophilus*

Co- cultures with EHEC and *L. acidophilus* was attempted because of *L. acidopilus'* affiliation to the Firmicutes phylum and its properties as a probiotic[91, 92]

The growth of EHEC was significantly crippled in co-culture with compared with EHEC growing alone. While *L. acidophilus* is considered a probiotic that resides in the commensal colonic microbiota, it is probably most known as Lactic Acid Bacteria (LAB). Amongst the LAB *L. acidophilus* is one of, if not the strongest acid producer[92]. In addition the acid production inhibiting growth, it also produces bacteriocins[92], making co-cultures with *L. acidophilus* an especially hostile growth environment for EHEC. This makes it a difficult bacteria to use in co-culturing experiments, as the bacteria used in the experiments needs to be at least mildly acidophilic, which EHEC is not. *L. acidophilus* was therefore not further used.

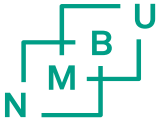
Appendix 6

Heat inactivation of *B. theta*

The aim of the heat treatment experiment was to find the lowest possible temperature that killed *B. theta*, for a gentle as possible inactivation of the cells that would be used in further experiments. (Co-cultures with dead cells and aggregation assay). One of the hypothesis to why EHEC up-regulates LEE expression in co-culture with *B. theta*, is that it is caused by cell to cell contact[74]. As surface structures can be very important for adherence[93], choosing a method that preserved surface proteins/structure was very important. All preparatory procedures of the cells in all the experiments in this thesis, was done with an intention of maintaining the surface structure of the cells. Because It has been showed that temperatures from 80°C and up destroys aggregation ability, and alters the cells appearance[75], much lower temperatures was tested. The results from the heat treatment are shown in the table below.

Temperature	Time	Growth
50°C	**15min**	**Growth**
	30min	**Growth**
55°C	**15min**	**Growth**
	30min	**No growth**
60°C	**15min**	**Growth**
	30min	**No growth**
Roomtemperature (untreated)	**30 min**	**Growth**
		Growth

The temperature chosen for further use throughout the thesis was 55°C for 30 min. It was the lowest and therefore considered the most delicate heat treatment for killing *B. theta*.



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