Chapter 1

Enterotoxigenic and Enterohemorrhagic *Escherichia coli*: Survival and Modulation of Virulence in the Human Gastrointestinal Tract

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) and Enterohemorrhagic *Escherichia coli* (EHEC) are major food- and water-borne pathogens that constitute a serious public health threat in low-income and developed countries, respectively. Survival and expression of virulence genes in the human digestive tract are key features in bacterial pathogenesis, but the mechanisms behind these processes remain largely unknown due to obvious prohibition of human studies. Use of well-controlled and multi-parametric *in vitro* models can aid in addressing knowledge gaps in ETEC and EHEC pathogenesis. After a general description of the physiopathology of ETEC and EHEC infections, this chapter will give an overview of all the *in vitro* studies that have investigated the effect of the main physicochemical and biotic parameters of the human gut on pathogen survival and expression of virulence factors. We bring a picture of how ETEC and EHEC are able to adapt to each of the successive environments of the human gastrointestinal tract by reading many cues provided by both the host and the gut microbiota.

Keywords: enterotoxigenic *Escherichia coli* (ETEC), enterohemorrhagic *Escherichia coli* (EHEC), survival, virulence genes expression, human gastrointestinal tract, *in vitro* models

1. Introduction

Since its identification in 1885, *Escherichia coli* (*E. coli*) has become one of the most comprehensively studied bacterial species. While *E. coli* is widely found in the environment and foods and is an important member of the commensal microbiota of mammals, some strains have evolved to include pathogenic mechanisms that cause significant diseases in humans and animals. In humans, *E. coli* strains can cause diverse enteric/diarrheagenic or extra-intestinal infections by means of virulence factors that affect a wide range of cellular processes. Pathogenic *E. coli* associated with gastrointestinal illness have been divided into eight pathotypes based on their virulence profiles: (i) enteropathogenic *E. coli* (EPEC), (ii) enterohaemorrhagic *E. coli* (EHEC), (iii) enterotoxigenic *E. coli* (ETEC), (iv) enteroinvasive *E. coli* (EIEC), (v) enteroaggregative *E. coli* (EAEC), (vi) diffusely adherent *E. coli* (DAEC), (vii) adherent invasive *E. coli* (AIEC) and (viii) Shiga toxin-producing enteroaggregative *E. coli* (STEAEC) [1]. This chapter will cover only two of them: ETEC and EHEC, which show opposite trends during their pathogenic processes. Even if in both cases human infections are primarily acquired through consumption of contaminated food products or drinking water, ETEC is a major cause of infantile diarrhea in developing countries, while EHEC is one of the main *E. coli* pathotypes associated with food poisoning outbreaks in the developed world.

To cause human illness, pathogenic enteric *E. coli* must not only survive the passage through the human gastrointestinal (GI) tract but also accomplish their pathogenic process by a complex and coordinated multistage strategy, including adherence to the host intestine and toxin/virulence protein production. The current chapter will provide a state of the art of ETEC and EHEC physiopathology, then focus on pathogen survival in the human digestive tract and regulation of virulence determinants by GI cues. As studies on humans are ethically inconceivable and small animal models do not recapitulate human pathogenesis, we will introduce the potential of dynamic *in vitro* digestion systems for increasing our understanding of ETEC and EHEC pathogenesis in a physiologically relevant GI environment.

2. Physiopathology of ETEC and EHEC infections

2.1. Epidemiological data

ETEC are a significant cause of watery diarrhea in developing countries where sanitation and clean water remain scarce and a main cause of traveler's diarrhea [2]. In contrast, EHEC are a major public health concern of developed countries [3] (**Figure 1**). Hence, ETEC are among the top four pathogens causing moderate to severe diarrhea among children in Africa and South Asia, while EHEC are the third most common zoonotic pathogen in Europe associated with large food poisoning outbreaks in EU, the USA, Canada, and Japan. The most common serogroups implicated in outbreaks and sporadic cases are O6, O78, O8, O128, and O153 for ETEC and O157:H7, O26:H11, O45:H2, O103:H2, O111:H8, O121:H19, and O145:H28 for EHEC.

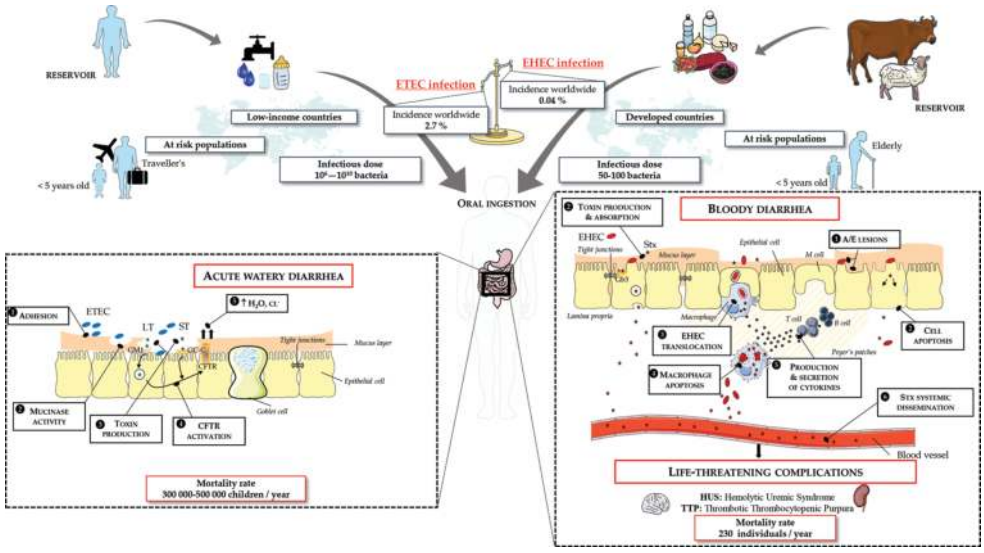

Figure 1. ETEC and EHEC pathogenesis including epidemiological data on the infections and at-risk populations, reservoir, mode of transmission and virulence factors of the pathogen, and clinical signs are described. A/E: Attaching and effacing; CFTR: cystic fibrosis transmembrane regulator; GC-C: guanylyl cyclase C; GM1: monosialoganglioside receptor; LT: heat-labile enterotoxins; ST: heat-stable enterotoxins; Stx: Shiga toxin.

ETEC cause approximately 280 million episodes of diarrhea worldwide, leading to hundreds of thousands of deaths per year [4]. With regard to EHEC, it is estimated that the pathogen is responsible for 2,801,000 acute illnesses, 3890 cases of haemolytic and uremic syndrome (HUS), 270 cases of permanent end-stage renal disease, and 230 deaths worldwide [3]. For both pathogens, infants less than 5 years old are a high-risk population. ETEC are responsible for 20–25% of diarrhea in young children, mostly in low-income countries, and up to 40% of traveler's diarrhea [5]. In developing countries, children suffer from diarrhea attacks 7–8 times a year, with a peak incidence occurring between 6 and 18 months, and ETEC strains are responsible for one of each three attacks [6, 7]. In such countries, ETEC infections have then shown to play a significant part in the complex association between malnutrition and repeated bouts of diarrheal illness among young children. The impact of EHEC is also greater in infants and children, compared to other ages with 42% of cases of HUS and 29% of deaths occurring in children between the ages of 0 and 4 years [3].

While the lack of ongoing monitoring systems makes it difficult to understand ETEC pathogenesis worldwide, dedicated surveillance systems of human EHEC infections have been developed in most of the industrialized areas of the world [8]. In Europe, the surveillance of EHEC infections is embedded in the Food and Waterborne Diseases and Zoonoses (FWD) surveillance system coordinated by the European Center for Disease Prevention and Control (ECDC). FWD is a passive surveillance system, collecting data on EHEC infections including laboratory-confirmed cases, probable cases, and possible cases. Cases of HUS are specifically recorded through a network of pediatric nephrologists and infection-control practitioners on the basis of clinical diagnosis.

2.2. Reservoir and route of transmission

Both ETEC and EHEC infections are typically acquired through the ingestion of contaminated food or water (**Figure 1**). However, a major difference between ETEC and EHEC is that ETEC only have a human reservoir of infection while EHEC are zoonotic pathogens [2, 9]. The main source of ETEC infection is contaminated water, such as surface water and drinking water (especially for weaning food) suffering from a lack of adequate sanitation and sewage facilities [2]. Nevertheless, a variety of food items including vegetables and herbs imported from endemic countries have also been recently implicated in uncommon sporadic cases or outbreaks in industrialized countries. Ruminants, especially cattle, are a natural reservoir of EHEC, and hence entry into the food chain through fecal contamination. Food (mainly undercooked beef products, unpasteurized milk, and vegetable) and water are the principal sources of human contamination with EHEC. Person-to-person transmission of EHEC may significantly contribute to outbreaks from a primary source, whereas this mode of transmission is not likely under most circumstances for ETEC infection.

The infective dose widely differs between ETEC and EHEC. It fluctuates between 10^8 and 10^{10} cells for ETEC in adults, but vulnerable populations such as infants may be susceptible to infection at lower doses [7, 10]. The infective dose for EHEC is recognized to be much lower: less than 50 to a few hundred organisms are usually sufficient to lead to the clinical signs [11].

2.3. Clinics and treatments

ETEC or EHEC show similar clinical pictures at the beginning of infections: watery diarrhea leading to rapid dehydration, usually associated with nausea, vomiting, and abdominal cramps [2, 11]. With regard to ETEC, following an incubation period of 10–72 hours, the duration of illness is typically 3–5 days, and resolved usually without antimicrobial treatment, even though symptoms can persist for 2–3 weeks. ETEC infections are generally self-limited and cannot be distinguished from Cholera on clinical grounds. Symptoms are much more severe in children from developing countries where diarrhea and malnutrition combine to form a vicious cycle leading to declining health status and death. Unlike ETEC, EHEC infections may evolve toward extra-digestive complications. EHEC infections typically progress from watery to bloody diarrhea and resolve within a week or 10 days in the majority of infected individuals. Nevertheless, in 5–7% of cases, the infection may lead to life-threatening complications, namely HUS and thrombotic thrombocytopenic purpura (TTP), and death [11, 12]. HUS is characterized as a triad of acute kidney failure, microangiopathic hemolytic anemia, and thrombocytopenia, and remains the most common cause of acute renal failure in children in the EU and US. The elderly mostly develop TTP, which differs from HUS because of neurological symptoms including lethargy, severe headache, convulsions, and encephalopathy.

Currently, treatment for ETEC and EHEC infections consists primarily of supportive therapy, with oral rehydration to prevent dehydration and loss of electrolytes. For EHEC, general supportive measures also include peritoneal dialysis or hemodialysis and management of anemia with transfusion of whole blood or packed red cells [13]. Conventional antibiotic treatment is generally not recommended for EHEC-infected patients as it increases HUS or

neurological complications [14]. The use of antimicrobials is also problematic during ETEC infection since an etiologic diagnosis cannot be made rapidly, mainly in childhood diarrhea [2]. Fluoroquinolones are shown to be effective during ETEC traveler's diarrhea [15] but should be used with caution due to the rise of antimicrobial resistance worldwide and the risk of side effects. For both pathogens, antimotility agents can be prescribed but need to be carefully administered as they can prolong the residence time of bacteria or their toxins in the intestine.

In this context, alternative prophylactic or therapeutic strategies are currently under development for ETEC and EHEC. Vaccines against the pathogens are still not commercially available, although vaccine strategies have been developed and used with variable success in animal models and/or humans [16, 17]. However, Dukoral®, a vaccine commercialized for *Vibrio cholerae*, can be prescribed to prevent traveler's diarrhea due to ETEC. Global alternative approaches involving dietary supplementation or probiotics have also been considered for both ETEC [18, 19] and EHEC [20, 21], with various levels of evidence from *in vitro* and *in vivo* studies. Other therapeutic options targeting a specific step in bacterial pathogenesis have been developed, mainly for EHEC, such as the use of agents that link toxins or block their binding at the cell surface [13] or antibodies that inhibit the terminal complement complex formation [22].

2.4. Virulence factors

After ingestion by humans, ETEC and EHEC pursue a strategy of infection involving colonization of the intestinal mucosal surface and production of toxins. The main sites of colonization differ between the two pathogens: from the upper jejunum to the ileum for ETEC [23, 24] and terminal ileum and colon for EHEC [25–27]. Notably, EHEC show a preferential tropism to the follicle-associated epithelium (FAE) of small intestinal Peyer's patches [25, 28], which has not been described for ETEC. Even if for both pathogens toxins are clearly identified as their main virulence factor, bacterial pathogenesis is not limited to toxin-mediated effects, and a combination of virulence traits is required to make ETEC and EHEC strains fully pathogenic to humans. This part describes the main virulence factors that have been identified for ETEC and EHEC.

2.4.1. Acid resistance

After being ingested, the pathogens must first breach the acidic barrier of the human stomach to reach their intestinal niche. It is well described that *E. coli* strains have intricate acid resistance (AR) systems that enable their survival in the harsh gastric environment, the glutamate-dependent AR system providing the highest level of acid protection [29]. Such acid resistance is a critical virulence trait of the infection, especially for EHEC for which the infectious dose is typically very low.

2.4.2. Colonization factors

ETEC adhere to the intestinal epithelium by means of several colonization factors (CFs). More than 25 CFs that are antigenically and structurally diverse, have been identified in ETEC and

include fimbrial and fimbrillar structures. Among them, seven are generally more prevalent than others: CFA/I (colonization factor antigen) and CS1 to CS6 (*coli* surface antigen) [30]. Most CF receptors have not been yet identified, but CFs are thought to bind to glycoprotein conjugates in mucus fraction from the small intestine and on the surface of host cells. Non-fimbrial adhesins such as TibA, a glycosylated autotransporter; Tia, an outer membrane protein; and EtpA, which acts as a molecular bridge binding host cell receptors to the tips of ETEC flagella, have also been implicated in the pathogenesis [31].

EHEC colonization involves attaching and effacing (A/E) lesions on the enterocytes, which are characterized by ultra-structural changes, including loss of microvilli and intimate attachment of the bacterium to the cell surface [32]. Genes encoding A/E lesion formation are localized on a pathogenicity island, the locus for enterocyte effacement (LEE), which encodes a bacterial type III secretion system (T3SS). Colonization is mainly mediated by the primary adhesin, namely intimin (encoded by *eae* gene), but other putative adherence factors have been described, such as long polar fimbriae—Lpf—or curli [33]. A number of other non-fimbrial EHEC adhesins have been implicated in adhesion including the plasmid-encoded *toxB*, the chromosomally encoded adhesins Iha, Cah, and OmpA [32, 33].

Mucin-degrading enzymes, which allow temporary access to intestinal cell membrane and promote bacterial adhesion have been recently identified in both ETEC and EHEC. In ETEC, YghJ, a mucin-binding metalloprotease [34] and EatA, a member of serine protease autotransporters of the Enterobacteriaceae (SPATE) family [35] have been described. In EHEC, one protein has been shown to have mucinase activity: StcE, an extracellular zinc metalloprotease which specifically recognizes α-O-glycan-containing substrates [36].

2.4.3. Secretion of toxins

Toxins are considered as the main virulence factor for both ETEC and EHEC as they are responsible for the main clinical symptoms and/or systemic complications. In ETEC, secretory diarrheas are mediated through the action of heat-stable (ST) and/or heat-labile (LT) enterotoxins.

ETEC strains are able to secrete either one or two toxins (LT and/or ST), but it has been shown that LT toxin is less likely to cause disease than ST or LT/ST ETEC toxins [7]. LT toxins encoded by the *eltAB* gene are similar in structure and function to Cholera toxin by sharing 80% homology. LT shows an AB_5 configuration with a catalytically active LT_A subunit and a pentameric ring of LT_B subunits responsible for binding and internalization [37]. LT are mainly secreted associated with outer membrane vesicles (OMVs) and bind irreversibly to monoganglioside (GM1) on the host cell. LT leads to an increase in cAMP that induces cystic fibrosis transmembrane regulator (CFTR) phosphorylation, eliciting massive fluid loss and watery diarrhea. In addition to causing diarrhea, LT plays multiple roles in modulating host cell function and providing a competitive advantage for ETEC adherence to cultured intestinal epithelial cells. ST toxins encoded by the *estAB* gene are small cysteine-rich peptides which mimic the human hormone guanylin. They are divided into two structural and antigenically distinct groups: STa and STb which reversibly bind to guanylyl cyclase C (GC-C) and sulphatide, respectively [37], leading to CFTR activation and diarrhea.

Shiga toxins (Stx) are produced by EHEC in the lumen of the intestine, and then cross the epithelial barrier by poorly described mechanisms to eventually reach their target organs [38]. Two toxin families encoded in the genomes of lysogenic lambdoid phages are produced by the bacteria, namely Stx1 and Stx2, the latter being associated with the most severe complications [39]. Stx contain two major structural subunits, A and B [40]. The B subunit binds to the toxin cellular receptor, globotriaosylceramide-3 (Gb3), expressed on host microvascular endothelial cell surfaces (kidney, intestine, and brain). This explains the life-threatening complications associated with EHEC infections. The A subunit exhibits an RNA N-glycosidase activity against the 28S rRNA, resulting in inhibition of protein synthesis and cell death.

3. Bacterial survival in the human digestive tract

Bacterial survival in the human GI tract is a key parameter in ETEC and EHEC physiopathology. Nevertheless, how pathogens can survive in the human digestive environment remains largely unknown as studies in humans are impossible. For regulatory, ethical, technical, and cost reasons, artificial digestive systems are increasingly used as an alternative to *in vivo* studies in humans. Until now, almost no data are available for ETEC under human digestive simulated conditions while a number of studies have assessed the survival of EHEC during human *in vitro* digestion.

Masters et al. [41] have shown that after exposure to pH 2, ETEC became undetectable by plate counting after 2 hours. A recent study using flow cytometry analysis indicated that there was no significant difference in the percentage of live bacteria when ETEC were subjected to pH 5 or pH 7 [42]. Only one study has investigated the impact of 30 g/L bile on the survival of ETEC *in vitro*. Despite the known bactericidal effect of bile in the intestine, growth curves for ETEC in Luria Bertani (LB) media and LB-bile showed similar slopes during the exponential growth phase [43].

With regard to EHEC, most of the studies have been carried out, like for ETEC, using simplified *in vitro* approaches integrating a limited number of digestive parameters, such as acid pH or bile salts [44, 45]. Even if the pathogen is considered as acid resistant, large variations in survival rates have been obtained for *E. coli* O157:H7 in acidified culture media or in simulated gastric fluid [45, 46]. This wide range of response may be explained by differences in culture conditions, bacterial strains, and pH values used to simulate the gastric phase. Other more recent studies have evaluated the survival of EHEC strains by using dynamic multicompartmental *in vitro* models that closely mimic the gastric, small intestinal, and colonic human digestive conditions. In the TNO GastroIntestinal model (TIM), which simulates the stomach and the three segments of the human small intestine, it has been shown that EHEC survival was affected in the stomach and duodenum (when ingested within a food matrix but not with a glass of water), while bacterial growth was observed at the end of digestion in the jejunum and ileum [47–49]. This growth renewal in the distal parts of the small intestine was probably linked to the occurrence of less stringent conditions, such as neutral pH and lower concentrations of bile salts due to their reabsorption (as occurred *in vivo*). EHEC survival in the TIM model was found to be strain/serotype dependent [48] and influenced

by food matrices [47–49] and age conditions [48]. In particular, thanks to the potential of the TIM model, Roussel et al. [49] have shown that differences in digestive physicochemical parameters related to age conditions may partly explain the higher susceptibility of children (compared to adults) to EHEC infections and HUS. Additional studies performed under human-simulated colonic conditions (including colonic microbiota) have shown that EHEC strains were not able to colonize [50], probably due to the barrier effect of gut microbiota or to the high short-chain fatty acid (SCFAs) concentrations found in the colon and known to inhibit EHEC growth [51, 52]. Taken together, these data suggest that the ability of EHEC to colonize the human gut would be rather linked to growth renewal of the pathogen in the distal parts of the small intestine than the ability to maintain in the colon.

4. Regulation of virulence genes by gastrointestinal cues

To be fully pathogenic, bacteria must not only survive in the human GI tract but also coordinate expression of virulence determinants in response to localized gut microenvironments. An increased number of *in vitro* or *in vivo* studies have shown that both ETEC and EHEC are able to respond to various GI cues and employ these cues to modulate the expression of their virulence factors [33, 53], as described below (**Figure 2**). Compared to ETEC, where all

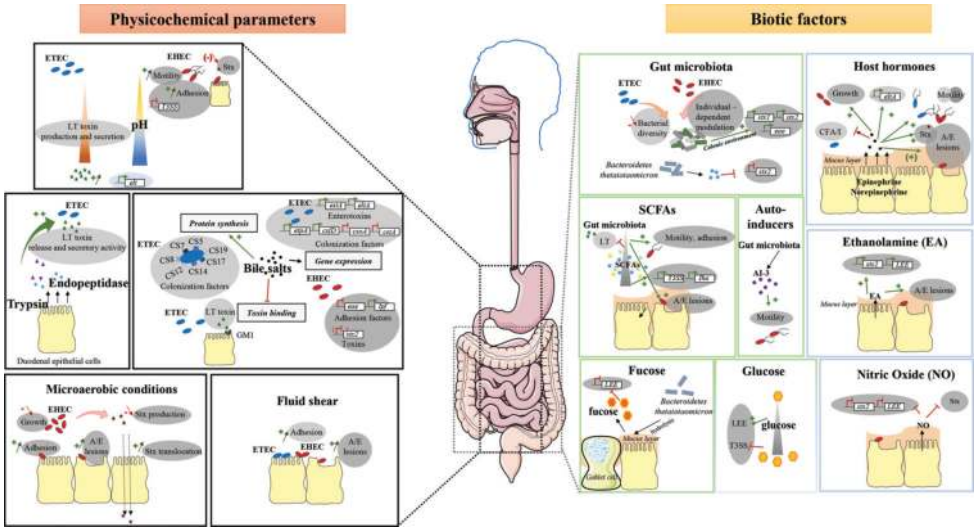

Figure 2. The figure provides a state of the art on the effects of biotic and abiotic parameters of the human gut on ETEC and EHEC virulence, as assessed by *in vitro* studies. Data related to ETEC and EHEC are surrounded by light grey and dark grey, respectively. A/E: Attaching and effacing; AI: autoinducer; CFA: colonization factor antigen; CS: *coli* surface; EA: ethanolamine; *elt*: heat-labile enterotoxin encoding gene; *est*: heat-stable enterotoxin encoding gene; *etpA*: ETEC two-partner protein A encoding gene; GM1: monosialoganglioside receptor; *IhA*: IrgA homologe adhesion encoding gene; LEE: locus for enterocyte effacement; *lpf*: long polar fimbriae encoding gene; LT: heat-labile enterotoxins; NO: nitric oxide; SCFA: short-chain fatty acids; Stx: Shiga toxin; T3SS: type 3 secretion system.

the available studies have been performed in simple *in vitro* digestive conditions, recent data have been obtained for EHEC in more physiological conditions simulated by dynamic multi-compartmental models.

4.1. Regulation by physicochemical parameters of the human gut

4.1.1. pH

Once ingested, pathogens are exposed to the host digestive tract characterized by acid conditions in the stomach where pH gradually decreases during digestion from around 6 to 2, followed by pH close to neutrality in the small intestine.

For ETEC, the release of ST seems to be not pH-dependent [54], while it is acknowledged that extracellular pH has an influence on the release of LT toxin which increases with alkalinity [55, 56]. ETEC seems to use the pH gradient in the GI tract to modulate LT toxin production and secretion: when bacteria reach the small intestine, alkaline pH induces both transcription and maximal release of LT [42].

For EHEC, House et al. [57] have examined, using DNA microarrays, the gene expression profiles of EHEC O157 that had been acid stressed and then neutralized relative to the same unstressed strain. Virulence factors associated with adhesion, motility, and type III secretion were significantly modulated leading to enhancement of motility and host cell adhesion. The T3SS genes encoding proteins that mediate colonization and infection in the large intestine were downregulated following acid stress [33, 57]. Impact of low pH on Stx gene expression and production is not yet fully understood: House et al. [57] have shown no change whereas other studies have revealed that acid pH decreases Stx production [58, 59]. In the gastric and small intestinal TIM model, Roussel et al. [49] have shown that *stx1* and *stx2* genes were upregulated in the gastric compartment even if Stx-mediated cytotoxicity is generally associated with distal parts of the small intestine or large intestine. Higher expression levels were observed under child digestive conditions compared to adult ones where less acidic conditions are found, which is in accordance with the results of Yuk et al. [58] and Huang et al. [59].

4.1.2. Bile

Once the small intestine is reached, bile salts form a major challenge to pathogens, with bile concentrations sequentially decreasing from duodenum to colon due to reabsorption.

Chatterjee and Chowdhury [60] have shown *in vitro* that 2 g/L crude bile can prevent the binding of LT toxin to GM1 and that this effect was associated to arachidonic, linoleic, and oleic unsaturated fatty acids detected in crude bile. The same authors demonstrated *in vivo* in rabbit ileal loops that linoleic acid prevented LT-mediated fluid accumulation in a dose-dependent manner [60]. In another study by Nicklasson et al. [61], 1.5 g/L crude bile and 2 g/L bile salts sodium deoxycholate and sodium glycocholate-induced *in vitro* the expression of CS5-encoding gene *csfD*. A global transcriptional analysis of two ETEC strains showed that bile salts at a concentration of 30 g/L in LB medium upregulated *estA*, *eltA*, or *etpA* (encoding for STa, LTa enterotoxins, and EtpA, respectively) while *csoA* and *cstA* (encoding for CS1 and

CS3 colonization factors) were downregulated [43]. In this study, the transcriptional response to bile salts was strain-dependent, suggesting that the results should not be extrapolated to the entire pathovar without further investigation. Finally, at the protein level, 1.5 g/L bile salts were required for surface expression of at least CS5, CS7, CS8, CS12, CS14, CS17, and CS19 [62–64]. Haines et al. [62] have shown that bile salts seem not to be required for the expression of CS1, CS2, and CS3, while the opposite was demonstrated by Sjoling et al. [63]. These results suggest that both interaction of LT toxin with its receptor and expression of ETEC colonization factors may be differentially induced along the human intestine where bile acid concentrations range from 2 to 20 g/L.

Studies have also shown that bile may serve as an environmental cue for EHEC by modulating the expression of specific virulence factors [33]. DNA microarray analysis of EHEC O157:H7 treated with 1.5 g/L bile salts showed upregulation of *acrA* and *acrB* genes encoding a bile salts efflux pump [65]. Expression of several other well-known virulence factors including those encoded on the LEE pathogenicity island, was not altered by bile salt treatment. On the contrary, a significant decrease in *eae* gene transcripts was observed *in vitro* by other authors when 5–8 g/L bile salts were added [66, 67]. Bile salts also modulate the expression of other adhesins, such as Lpf: Arenas-Hernández et al. [68] and Yin et al. [66] found that concentrations of 1.5–5 g/L led to an upregulation of *lpf* genes. In the TIM system, *eae* and *lpf* overexpression occurred under child digestive conditions at the end of *in vitro* digestion, when most of the bacterial cells have reached the distal parts of the small intestine [49]. This might suggest a higher ability of EHEC to colonize the terminal ileum or colon in children compared to adults. Lastly, there is no consensus for the effect of bile salts on *stx* gene expression. Kus et al. [65] reported that 1.5 g/L bile salt downregulated *stx2* genes, whereas no influence was observed by Hamner et al. [67] with concentrations of 8 g/L.

4.1.3. Digestive enzymes

Very few studies have investigated how human digestive enzymes may influence the expression of virulence genes in pathogens, none in EHEC and only two in ETEC. In the latter, *in vitro* studies have shown that trypsin, an endopeptidase secreted by duodenal epithelial cells, is able to increase LT release [55] and its secretory activity [69].

4.1.4. Oxygen levels

Various oxygen levels can be found in the human GI tract with concentrations decreasing from the upper to the lower digestive tract and from mucosal surfaces to gut lumen. Up to date, the effect of various oxygen concentrations on pathogen virulence has been studied only in EHEC.

In an *in vitro* cell culture model, James and Keevil [70] have shown that the presence of oxygen enhanced EHEC ability to adhere to epithelial cells. In other recent studies, polarized human colon carcinoma cells in a vertical diffusion chamber system were used to investigate the influence of reduced apical oxygen levels on EHEC colonization [38] and Stx production [71]. The authors demonstrated that both EHEC-host adhesion and expression and translocation of T3SS

effector proteins were increased under microaerobic conditions (1–2% oxygen). Microaerobiosis also significantly reduced bacterial growth as well as Stx production and release into the medium, while Stx translocation across the epithelial monolayer was enhanced. The role of oxygen levels on modulation of EHEC virulence was further confirmed by Lewis et al. [27] who showed in *in vitro* organ culture (IVOC) of human colonic biopsy samples that A/E lesion formation was dependent on oxygen levels. These lesions were suppressed under oxygen-rich culture conditions routinely used for IVOC. Taken together, these results suggest that the microaerobic environment adjacent to the intestinal microvilli may upregulate the expression of EHEC virulence factors that promote successful colonization of the large intestine.

4.1.5. Fluid shear

Fluid shear can be defined as distribution of frictional forces due to the hydrodynamic flow generated by GI peristaltic activity against the surface of intestinal epithelial cells. In the human gut, there is a decreasing gradient of fluid shear stress from mucosa to gut lumen. It has been generally assumed that shear stress inhibits pathogen adhesion, thereby serving as a non-specific host defense against bacterial colonization [72]. For both ETEC and EHEC, this concept has been very poorly described in the literature.

Tchesnokova et al. [72] have shown, using *in vitro* erythrocytes and Caco-2 cell models, a shear-enhanced binding of intestinal CfaE, the tip-localized minor subunit of CFA/I, in both prototypical and clinical ETEC strains. EHEC attachment to host cells is also enhanced by levels of shear force similar to peristaltic forces in the intestinal tract, which are required to fully activate LEE-encoded virulence mechanisms [73]. These preliminary data suggest that, in addition to a range of chemical environmental signals, ETEC and EHEC are capable of sensing and responding to mechanical cues in the human GI tract.

4.2. Regulation by biotic factors of the human gut

4.2.1. Gut microbiota and their metabolites

4.2.1.1. Gut microbiota

During passage through the human gut, enteric pathogenic bacteria such as ETEC and EHEC also have to face a high number of commensal bacteria that compete with them for nutrients and space. There is scarce data on the interactions of EHEC, but even more so for ETEC, with human gut microbiota.

For ETEC, only two studies have investigated gut microbiota changes during ETEC challenge [74, 75]. The authors conclude that ETEC infections are associated with a rapid and reversible change in gut microbial community structure as well as a significant decrease in overall bacteria diversity. However, there is no available data on how gut microbiota may influence ETEC virulence.

With regard to EHEC, Thévenot et al. [50] have recently shown in an *in vitro* model of the human colon, that *E. coli* O157:H7 has an individual dependent effect on the colonic micro-

biota, as assessed by qPCR analysis on major phyla and genus. The same authors also showed that EHEC infection led in the *in vitro* colonic environment to a significant increase in *stx1*, *stx2*, and *eae* expression 9–12 h post-administration. Besides, it has been also proposed that EHEC was sensing autoinducers produced by the GI microbiota, such as the quorum signaling molecule AI-3. EHEC respond to AI-3 by increasing flagellar synthesis and motility that allow the pathogen to more closely approach the mucosal epithelium at the site of colonization [76]. On the contrary, other soluble factors secreted by the normal gut microbiota may protect the host against EHEC infection. De Sablet et al. [77] have shown, in cecal contents of gnotobiotic rats colonized with human microbiota, that small molecules produced in part by *Bacteroides thetaiotaomicron*, a predominant species of the normal human intestinal microbiota, repressed *stx2* mRNA expression. Mutants of *B. thetaiotaomicron* with impaired production of a specific transporter of vitamin B12 were no longer able to inhibit the production of Stx2 [78]. This work suggests that concentration of vitamin B12 in the gut and by extension, activities of commensal bacterial species producing and/or consuming vitamin B12, may modulate the production of the main virulence factor of EHEC. Other studies have also demonstrated that the interplay between the nutrient requirements of normal flora and EHEC is important in determining pathogen virulence [76]. Njoroge et al. [79] uncovered the importance of glucose availability in regulating T3SS by EHEC: high-glucose growth media suppressed type III secretion while low-glucose conditions induced LEE expression. EHEC also use fucose that is made available from mucus by the microbiota (especially by *Bacteroides thetaiotaomicron*) to modulate their own metabolism and virulence. Pacheco et al. [80] described a novel two-component system that enables regulation of virulence gene expression and carbon-source choice by EHEC upon sensing fucose, resulting in a decrease in LEE transcript levels. All these results tend to indicate that differential microbiota composition may contribute to host resistance or susceptibility to EHEC infections. Then, differences in diet and antibiotic regimens, which cause shifts in the composition of the GI microbiota may also influence the outcome of the disease.

4.2.1.2. Short-chain fatty acids

Several studies have investigated how ETEC and EHEC may respond to gut microbiota metabolites such as SCFAs. The three main SCFAs present in the intestine are acetate, propionate, and butyrate and their concentrations vary from the small intestine to the colon.

A single study with ETEC has shown that addition of SCFAs from C-2 to C-7 at a concentration of 2 mg/mL in the culture medium significantly reduced or even abolished LT production [81]. A higher number of studies have evaluated how EHEC may sense SCFAs. Acetate (10–40 mM) and propionate (2–10 mM) had no effect on Stx2 production levels *in vitro* [78] while acetate production by *Bifidobacterium* strains was associated with an anti-infectious activity through the inhibition of Stx production and translocation [82]. Low SCFA concentrations (particularly of butyrate—from 6.25 to 25 mM), more typical of the distal ileum, enhanced the expression of EHEC virulence genes involved in motility, adhesion, and induction of A/E lesion formation [51, 52]. Other studies reported that high concentrations of SCFAs (above 50 mM), typically found in the distal colon, were associated with increased expression of T3SS [83] and Iha adhesin [84]. Very recently, Lackraj et al. [85] have investigated how EHEC modulate flagella expression and motility in response to SCFA mixes typical in compositions and concentrations of the small and large intestines.

They showed that when EHEC were exposed to SCFA mixes representative of the small intestine, there was a significant upregulation of flagellar genes, flagellar protein FliC, and motility, while the opposite was observed with SCFA mixes representative of the large intestine. Lastly, a high-fiber diet, via enhanced butyrate levels, increased host's expression of Gb3 and susceptibility of mice to disease [86]. Conversely, increased levels of microbiota-derived acetate protected animals from disease that is caused by the toxin. Collectively, these data suggest that molecular cues secreted by commensal microbiota such as SCFAs may modulate EHEC motility, adhesion, and toxin production, differently in the small and large intestines.

4.2.2. Host hormones

Microbial endocrinology is a newly recognized microbiology research area investigating the interactions of bacteria with stress-associated hormones, such as catecholamine. Among these hormones, only epinephrine and norepinephrine have been investigated as environmental cues for ETEC and EHEC.

Lyte et al. [87] demonstrated that physiological concentrations of norepinephrine increased the *in vitro* growth of an ETEC strain isolated from calf, as well as the expression of the virulence factor F5 fimbrial adhesin. On the contrary, Sturbelle et al. [88] did not observe any effect of norepinephrine or epinephrine on the *in vitro* growth of a piglet ETEC strain, and Haines et al. [62] found a significant inhibition of porcine ETEC growth by norepinephrine. However, a significant increase in motility and expression of F4 fimbriae and LT toxin-encoding genes was shown in the ETEC culture supplemented with conditioned medium (containing autoinducers) and epinephrine [88]. Lastly, Haines et al. [62] found that norepinephrine inhibited CFA/I expression in an ETEC strain isolated from humans.

As described for ETEC, Lyte et al. [89] found that norepinephrine increased *in vitro* EHEC growth. EHEC also use norepinephrine as a signal for differential regulation of virulence factors mediating invasion, motility, and A/E lesion formation [90]. Regulation of EHEC virulence by epinephrine and norepinephrine is still not fully understood but it has been shown that the pathogen uses the histidine sensor kinases QseC and QseE as sensors of the two hormones [33, 76]. So, host-derived hormones epinephrine and/or norepinephrine seem to assist ETEC and EHEC in cueing their site of colonization and enhance approach to the epithelial layer through increased motility and adhesion.

4.2.3. Other factors

The influence of other GI factors, such as ethanolamine (EA) and nitric oxide (NO), has been studied on EHEC virulence, but not on ETEC. However, the nature of the associated regulations is still not fully understood.

EA comes from the turnover of intestinal epithelial cells and commensal microbiota and is generated from the breakdown of phosphatidylethanolamine. EHEC cultured in minimal media containing EA showed increased expression of both s*tx2* and genes encoded on the LEE pathogenicity island, as well as a higher number of attaching and effacing (A/E) lesions on host epithelial cells [91].

NO is an essential mediator of the innate immune response of infected colonic mucosa. Chemical or cellular sources of NO have been shown to inhibit *stx*- and LEE-encoded genes mRNA expression and Stx synthesis, without altering EHEC viability [92, 93].

5. Conclusion

This chapter shows that we get clearer evidence that the food- and water-borne pathogens ETEC and EHEC are able to adapt to each of the successive environments of the human GI tract by reading many cues provided by both the host and the gut microbiota. Exposure to different environmental cues may impact pathogen survival but also alter the expression of virulence genes. Nevertheless, the data obtained until now show many gaps and inconsistencies. In particular, most of the current studies have been carried out using oversimplified *in vitro* approaches, and what is still missing is the integration of signals delivered in a sequential but not in an isolated fashion. Relevant alternatives to better understand how ETEC and EHEC respond to these various cues in a temporal-spatial fashion may imply relevant animal models (e.g., human microbiota-associated animals) [94] or digestion models closely mimicking the human digestive tract, such as the TIM or the SHIME (Simulator of the Human Intestinal Microbial Ecosystem) [95]. In particular, TIM and SHIME would be of high interest to (i) assess how the modalities of ingestion (e.g., infectious dose, growth phase, and food vehicle) and age conditions (adult, infant, and elderly) may influence pathogen survival and virulence in the human GI tract, (ii) investigate how ETEC and EHEC interact with luminal and mucosal gut microbiota under physiological fluid shear stresses and microaerobic conditions, and (iii) study host-microbiota-pathogen interactions by using intestinal cells in culture coupled with TIM or SHIME models, like in the HMI (host microbe interactions) module [96]. For an in-depth understanding of pathogen behavior in the human GI tract, these models should be used in combination with new technologies such as -omics or quantitative imaging technologies.

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