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Increased EHEC survival and virulence gene expression indicate an enhanced pathogenicity upon simulated pediatric gastrointestinal conditions

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BACKGROUND: Enterohemorrhagic *Escherichia coli* (EHEC) are major foodborne pathogens that constitute a serious public health threat, mainly in young children. Shiga toxins (Stx) are the main virulence determinants of EHEC pathogenesis but adhesins like intimin (eae) and Long polar fimbriae (Lpf) also contribute to infection. The TNO GastroIntestinal Model (TIM) was used for a comparative study of EHEC O157:H7 survival and virulence under adult and child digestive conditions.

METHODS: Survival kinetics in the *in vitro* digestive tract were determined by plating while bacterial viability was assessed by flow cytometry analysis. Expression of *stx*, *eae*, and *lpf* genes was followed by reverse transcriptase-quantitative PCR (RT-qPCR) and Stx production was measured by ELISA (enzyme-linked immunosorbent assay).

RESULTS: Upon gastrointestinal passage, a higher amount of viable cells was found in the simulated ileal effluents of children compared to that of adults (with 34 and 6% of viable cells, respectively). Expression levels of virulence genes were up to 125-fold higher in children. Stx was detected only in child ileal effluents.

CONCLUSION: Differences in digestive physicochemical parameters may partially explain why children are more susceptible to EHEC infection than adults. Such data are essential for a full understanding of EHEC pathogenesis and would help in designing novel therapeutic approaches.

Enterohemorrhagic *Escherichia coli* (EHEC), mainly from the serotype O157:H7, are food and waterborne pathogens that constitute a serious public health concern. EHEC are responsible for hemorrhagic colitis and bloody diarrhea that can evolve toward life-threatening age-dependent complications (1). The hemolytic uremic syndrome (HUS), defined by the triad of acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, mainly affects young children under 3 y of age (1–3) while thrombotic thrombocytopenic purpura, characterized by central nervous system damages, more commonly afflicts adults and elderly. In Europe and France, EHEC infections are the leading cause of renal failure in children. Even if Shiga toxins (Stx) are the main virulence determinants of EHEC, other factors such as intimin (eae) and adhesins (including Long polar Fimbriae (Lpf)) also contribute to human pathogenesis by mediating bacterial colonization (4,5).

Survival and virulence of EHEC strains in the human gastrointestinal (GI) tract are key factors in the infectious process (6) but they remain poorly described, mainly in children, due to a lack of relevant models. Studies in humans are obviously prohibited and results obtained in animal models are hampered by differences between animal and human digestive physiology. *In vitro* digestion methods emerge as an appropriate alternative to *in vivo* assays providing that such models can reproduce physiologically relevant conditions. Among the available systems, the dynamic multicompartmental and computer-controlled TNO gastrointestinal model (TIM) is currently the most complete simulator of the upper GI tract (7) and has been validated for microbial applications against *in vivo* data (8). Previous works in this model have assessed the survival of *E. coli* O157:H7 when ingested within a food matrix (9,10), but experiments were carried out only under adult conditions and no data are available on virulence gene expression during GI transit.

In this context, the aim of this study was to use the TIM model for a comparative analysis of *E. coli* O157:H7 survival and virulence under adults and young children (from 6 mo to 2 y)

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digestive conditions, upon simulated ingestion of a glass of contaminated water.

RESULTS

Survival and Physiological State of EHEC O157:H7 in the Simulated Stomach

Whatever the simulated age group (adult or child), no significant difference was observed between the survival kinetics obtained for O157:H7 and the transit marker, showing that the growth ability of the pathogen (as assessed by numeration of cultivable cells) was not affected by gastric conditions (**Figure 1a**). In contrast, flow cytometry analysis of gastric effluents showed marked differences in the physiological state of bacteria between adult and child conditions (**Figure 1b**). At 60 min digestion, a higher percentage of viable cells was observed under child conditions compared with adult ones (59.0 vs. 21.9%), together with a lower number of damaged cells (18.0 vs. 46.2%), whereas similar initial viability profiles were observed between adult and child conditions. Our results indicate that cells that are likely to reach the small intestine would be less damaged in infants compared with adults.

Survival and Physiological State of EHEC O157:H7 in the Simulated Small Intestine

In the duodenal simulated compartment, EHEC survival was unaffected in both age conditions. In contrast, bacterial mortality was noticed halfway the jejunum- and ileum-simulated digestion time, but only under adult conditions (**Figure 2a**). In adults, bacterial recovery percentages after 120 min were 2.8 ± 0.4% (n = 4) vs. 27.5% for the transit marker (P < 0.01) in the jejunum and 11.4 ± 5.5% (n = 4) vs. 37.9% in the ileum (P < 0.001). At the end of digestion (240–360 min), the trend reversed with the recoveries of bacteria exceeding those of the transit marker, thus indicating bacterial outgrowth. EHEC growth was much more pronounced under simulated child conditions with differences between bacteria and transit marker being significant both in the jejunal and ileal compartments (P < 0.05). At 360 min during the child assays, a survival percentage of 87.3 ± 30.7% (n = 4) was found for O157:H7 compared with 2.2% for the marker (P < 0.001) in the jejunum and 441.7 ± 341.2% (n = 4) compared with 13.5% in the ileum (P = 0.063). Results based on flow cytometry analysis of ileal effluents (**Figure 2b**) strengthened those obtained by cultivation. The percentages of damaged cells remained constant around 15% throughout child digestions while they regularly increased during adult experiments (from 5.4% at t0 to 37.9% at 300 min). Conversely, the percentages of viable cells dramatically decreased during adult digestion (from 77.1% at t0 to 6.3% at 300 min) whereas they decreased then increased in child assays (from 77.1% at t0 to 21.9% at 180 min; and 33.9% at 360 min). This last feature is in accordance with the cell mortality followed by growth observed by cultivation, mainly under child conditions. Our results also indicate that, when entering the colon, most of bacterial cells are viable under child digestive conditions while in adults most of them have a damaged membrane.

Expression of EHEC Virulence Genes in the Simulated GI Tract

Gastric effluents of the simulated child conditions in TIM (**Figure 3a**) displayed significant up-regulated *stx1* and *stx2* at 10, 20, 40, and 60 min (with a fold change up to 125 for *stx1* and up to 12 for *stx2* compared with t0, P < 0.001). However, *stx* upregulation was not observed under simulated adult conditions, resulting in significantly higher *stx1* and *stx2* expression levels upon child gastric conditions compared with adult ones (P < 0.01). In the ileal effluents (**Figure 3b**), *stx1*, and *stx2* were up-regulated from 60 to 240 min in the child digestions, yet significant upregulation levels were only obtained at 180 min with a fivefold to sevenfold increase (P < 0.05). In adult ileal effluents, *stx* genes were significantly up-regulated in the same order of magnitude, but at 120 and 180 min for *stx1* (P < 0.05); and at 60 and 240 min for *stx2* (P < 0.001). *stx* expression levels were significantly higher in children compared with adults only at 60 min for *stx1* (P < 0.01). Results provided by enzyme-linked immunosorbent assay (**Figure 4**) fully support the difference obtained between adults and children with RT-qPCR. No Stx was evidenced under adult conditions while toxins were detected in the ileal effluents of children from 60 min and the amount produced regularly increased to reach 0.89 ± 0.12 ng/ml at 300 min (n = 4). Stx concentrations were significantly higher during child digestions compared with the adult ones from 120 min (P < 0.05).

In the gastric effluents from children (**Figure 5a**), *eae* expression was significantly (P < 0.001) repressed compared with the initial conditions from 10 to 60 min. On the contrary, under adult conditions, *eae* was over-expressed at 10 and 20 min (P < 0.05) with a fold change up to 15, resulting in significant higher expression levels in adults compared with children from 10 to 40 min (P < 0.01). Notably, at the end of gastric digestion (60 min), the reverse was found with *eae* mRNA levels higher in children (P < 0.01). In the ileal effluents (**Figure 5b**), *eae* mRNA expression levels tended to increase under child conditions from 180 min (P > 0.05), while they were significantly higher than t0 in adults at 60 and 120 min (P < 0.001, with up to ninefold change). At the beginning of *in vitro* digestion (until 120 min), *eae* was significantly overexpressed in adults compared with children, while the opposite trend was observed at 180 and 240 min (P < 0.01).

Lastly, in the gastric effluents (**Figure 6a**), *lpf1*, and *lpf2* were overexpressed compared with t0 under both adult and child conditions, with a fold change up to 12 for *lpf1* (P < 0.001, in adults and children) and up to 40 for *lpf2* (P < 0.001, in children). *lpf1* and *lpf2* expression levels were significantly higher in adults at 10 min (P < 0.05), while the reverse was observed at 40 and 60 min with significant higher levels in children (P < 0.05). In the ileal effluents (**Figure 6b**), *lpf* genes were overexpressed in both age conditions, but higher mRNA levels were found in children with a maximum fold increase of 30 and 60 at 240 min for *lpf1* and *lpf2*, respectively. *lpf* expression levels were significantly higher under child conditions compared with adult ones, but only at 180 min for *lpf1* (P < 0.01).

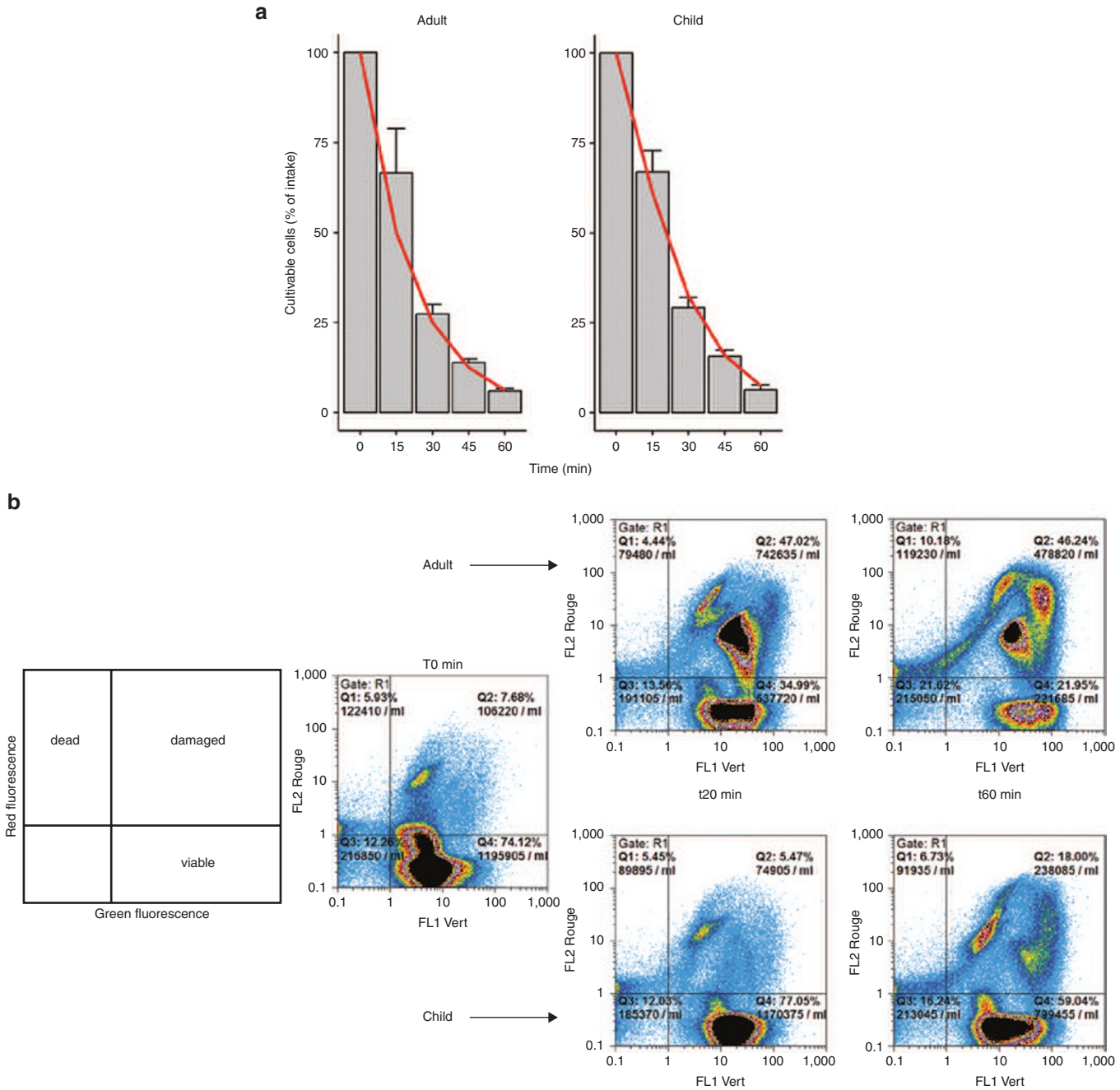

Figure 1. Survival and physiological state of EHEC O157:H7 in the TIM stomach. (**a**) The number of cultivable cells in the gastric compartment was determined by plating. Results are expressed as survival percentages ± SEM ($n = 4$) and compared with that obtained with the transit marker (red line). (**b**) Physiological state of O157:H7 was assessed by live/dead cytometry analysis. Bacteria recovered from gastric effluents were double-stained using green-fluorescent SYTO 9 (all cells) and red-fluorescent PI (bacteria with damaged membranes). TIM, TNO gastrointestinal model; EHEC, Enterohemorrhagic *Escherichia coli*.

DISCUSSION

EHEC survival and virulence in the human digestive tract are key features in bacterial pathogenesis (6). Overall, our data suggest that, in comparison with adults, a higher amount of cells, what is more less damaged, may reach the distal parts of the small intestine and the colon in children. Expression of major virulence genes, such as *stx*, is also higher under child digestive conditions.

Up to date, there was only scarce information on the behavior of EHEC strains under human simulated GI conditions (9–11). In addition, none of these studies took into account specific child conditions, despite young children forming a high-risk population for EHEC infection. The aim of this study was to assess and compare the survival and virulence of the reference strain O157:H7 EDL 933 under adult and child digestive conditions by using the well-validated dynamic and multicompartmental TIM model. An exhaustive survey of the

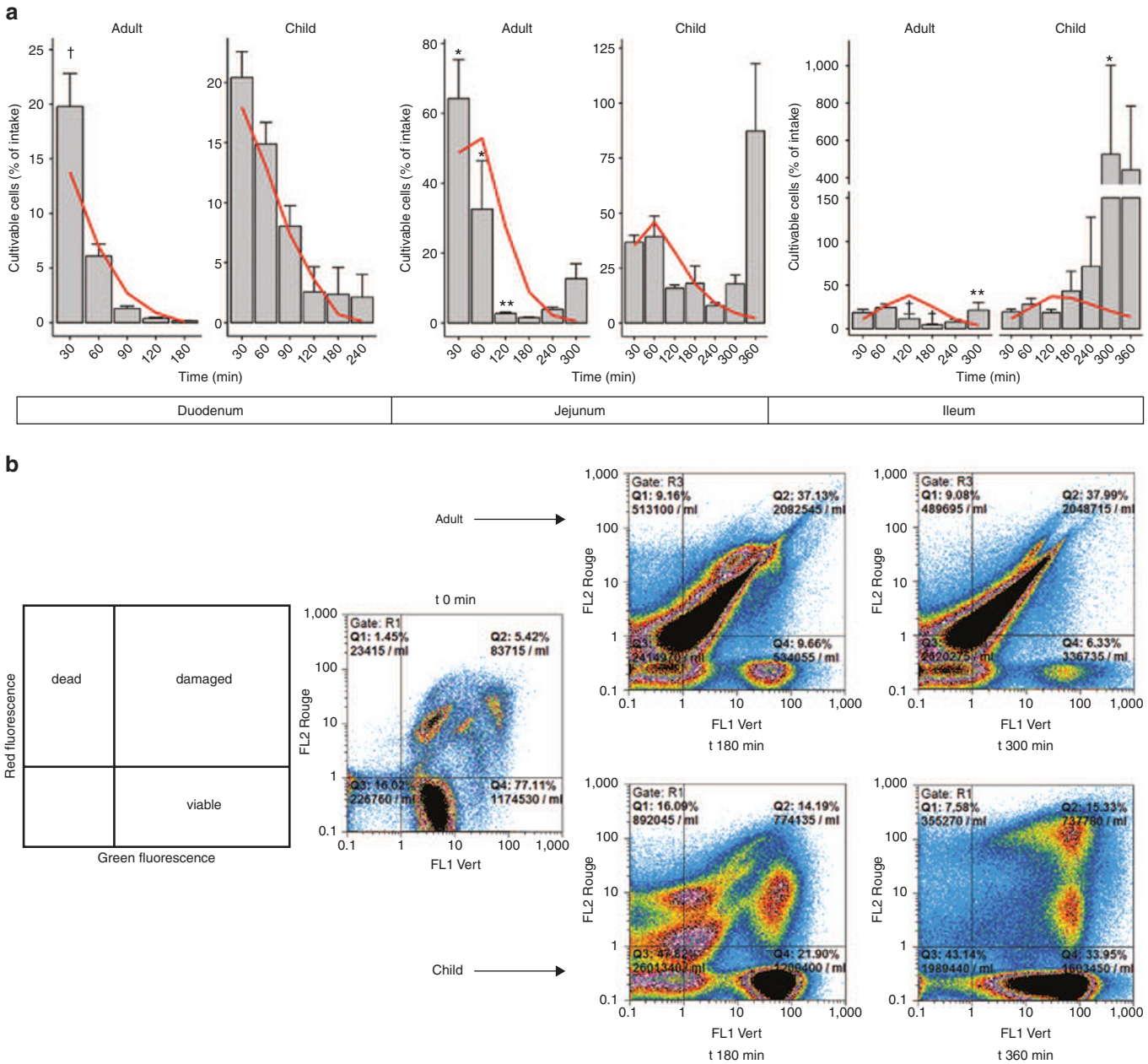

Figure 2. Survival and physiological state of EHEC O157:H7 in the TIM small intestine. (**a**) The number of cultivable cells in the small intestinal compartments was determined by plating. Results are expressed as survival percentages ± SEM ($n = 4$) and compared with that obtained with the transit marker (red line). O157:H7 significantly different from the transit marker at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (†). (**b**) Physiological state of O157:H7 was assessed by live/dead cytometry analysis. Bacteria recovered from ileal effluents were doubled-stained using green-fluorescent SYTO 9 (all cells) and red-fluorescent PI (bacteria with damaged membranes). TIM, TNO gastrointestinal model; EHEC, Enterohemorrhagic *Escherichia coli*.

literature was made to identify the physicochemical parameters unique to the child and adult GI tract in a healthy state and subsequently implement them into the TIM program. The survey was restricted to studies involving young children aged between 6 mo and 2 y because (i) children <3 y are a high-risk population for EHEC infection and HUS (3), (ii) the newborns (under 6 mo) mostly have an exclusive milk diet and subsequently show particular digestive physiology (12), and (iii) children >2 y show a mature GI tract, with no difference compared with the adult digestion process. Contaminated water which has been involved in major EHEC outbreaks (13) was chosen as a vehicle for bacteria. A supraphysiological dose of EHEC (10^7 Colony Forming Unit (CFU)/ml compared with the infectious dose of 10–100 CFU) was used to get enough RNAs in TIM digestive samples for transcriptomic analysis.

EHEC survival kinetics were first established in the TIM model. Our results suggest that O157:H7 is less sensitive to child than adult gastric conditions. This divergence may be explained by differences in stomach pH acidification less rapid and pronounced in children (14,15), associated with gastric

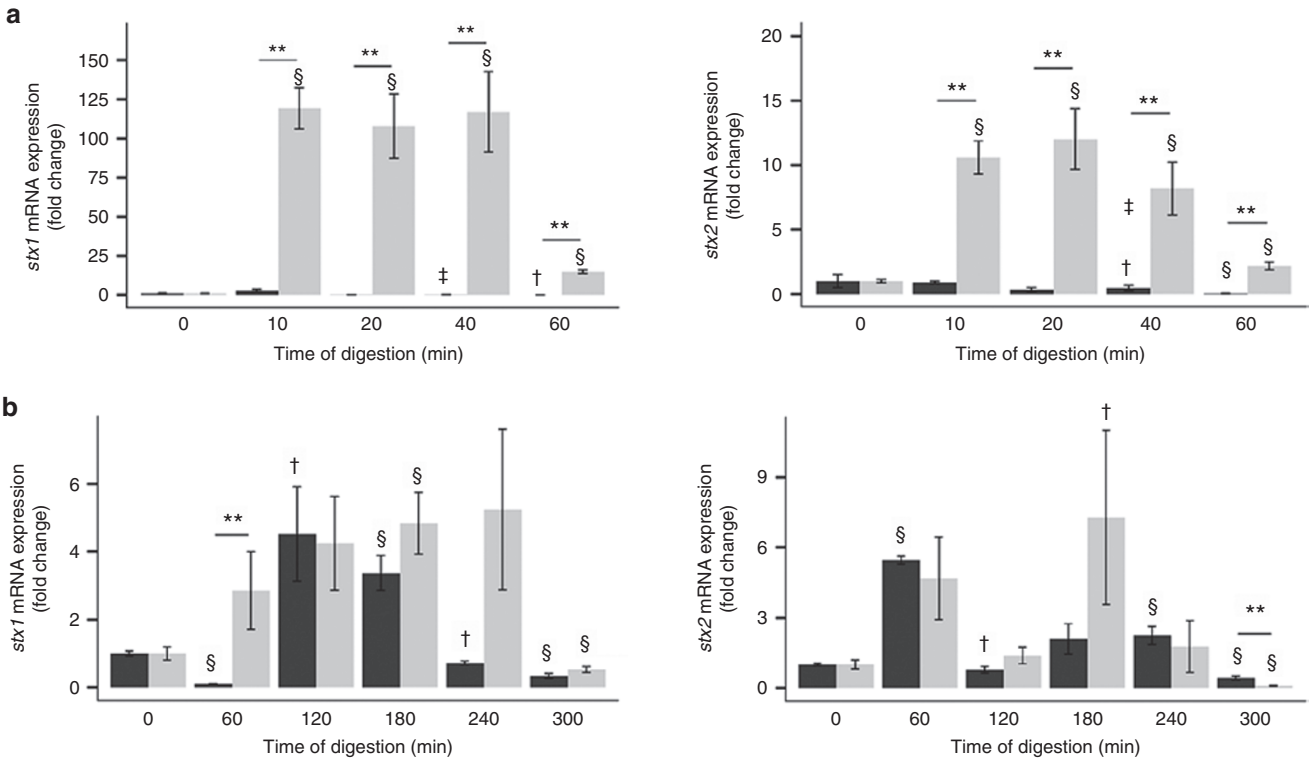

Figure 3. Expression of Shiga toxin-encoding genes in the TIM system. *stx 1/2* expression levels were measured by RT-qPCR in the gastric (**a**) and ileal (**b**) effluents of the TIM under child (in gray) and adult (in black) digestive conditions. Results are expressed as means of fold-induction ± SEM ($n = 4$). Time points statistically different from t0 at $P < 0.05$ (†), $P < 0.01$ (‡), and $P < 0.001$ (§). Child statistically different from adult at $P < 0.01$ (**).TIM, TNO gastrointestinal model; RT-qPCR, reverse transcriptase-quantitative PCR.

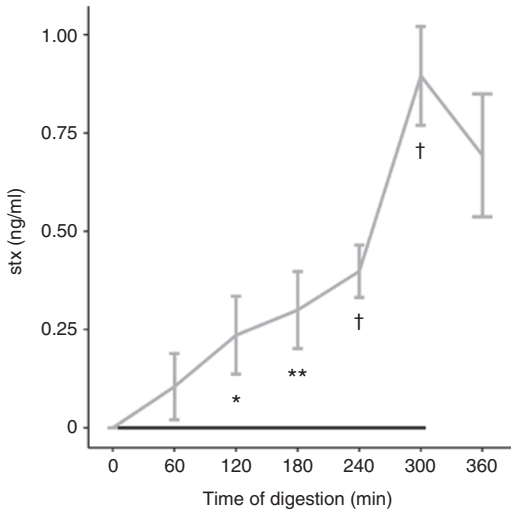

Figure 4. Shiga-toxin production in the ileal effluents of the TIM system. Stx 1/2 were measured in the ileal effluents by ELISA under child (in gray) and adult (in black) digestive conditions. Results are expressed as cumulative amounts in ng/ml ± SEM ($n = 4$). Child statistically different from adult at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (†). TIM, TNO gastrointestinal model; ELISA, enzyme-linked immunosorbent assay.

emptying rates quite similar under both age conditions (16,17). In the small intestinal compartments, a loss of viability was noticed at the middle of digestion in the jejunum and ileum, but only during adult assays. The higher mortality observed in adults might be linked to a twofold higher concentration in bile salts and pancreatic secretions compared with children (18–24). The bactericidal effect of bile salts toward *E. coli* strains is well known and attributed to their detergent properties that may lead to the disruption of cell membrane. This phenomenon was maybe enlarged by the fact that in adults, when entering into the small intestine, bacterial cells were more damaged than under child conditions. Conversely, at the end of *in vitro* digestions, bacterial growth was noticed in the jejunum and ileum under both age conditions, but mainly in children. This growth renewal may be linked to the occurrence of less stringent conditions, such as pH values close to neutrality and lower bile salts concentrations due to their passive reabsorption (as occurs in humans). The higher growth observed during child digestions may be explained by the occurrence of less stressful conditions together with a higher small bowel transit time (25,26), as well as the "better" physiological state of bacteria when entering the small intestine. As terminal ileum and colon are assumed to be the main sites of EHEC pathogenesis (27), this finding may partly explain why young children are a high-risk population for EHEC infection.

The next step was dedicated to the assessment of O157:H7 virulence gene expression in the TIM model. Up to now, regulation of EHEC virulence genes was only investigated in oversimplified *in vitro* approaches integrating a single digestive parameter, such as acidic pH or bile, and never under child conditions. Three genes mainly involved in the virulence of EHEC strains were studied: (i) *stx* encoding Shiga

toxin, known as the main virulence trait of the pathogen and responsible for systemic complications (4), eae encoding intimin, required from intimate attachment to the host intestinal mucosa (5) and, (iii) *lpf* loci encoding Long Polar Fimbriae, adhesive factors assumed to be involved in key steps of EHEC pathogenesis, such as adhesion, translocation, and inflammation (28).

We showed for the first time that *stx* genes were upregulated during gastric and small intestinal transit in the TIM system. These results suggest that EHEC O157:H7 would be able to produce its toxins from the stomach in the upper GI tract even if Stx-mediated cytotoxicity is generally associated with distal parts of the small intestine or large intestine (29). Interestingly, we found that expression levels of *stx* genes and related toxin production were larger under child compared with adult conditions, which is in accordance with the higher sensitivity of young children to EHEC infection and HUS. The large expression levels found in the gastric compartment in children may be related to less acidic conditions, as *stx* genes were not up-regulated under adult conditions where bacteria have to cope with a higher acidity. This hypothesis was supported by additional assays in the TIM system under "modified child" gastric conditions (child gastric pH curve combined with adult transit time) where *stx* was still over-expressed (data not shown). Our results are in accordance with those obtained by Yuk and Marshall (30) and Huang *et al.* (31) who showed a decrease in Stx production in O157:H7 after acid challenge *in vitro*, but not with those of Yin *et al.* (32) who observed the opposite trend in acid treated pig ligated intestine. In the small intestine, *stx* genes were up-regulated both under adult and child conditions. It may be related to the occurrence of neutral pH, but probably not linked to bile salts which do not increase *stx* expression in LB medium (33,34). Overexpression of *stx* genes did not result in Stx detection in the ileal effluents of adults, maybe due to concentrations under the detection limit and/or degradation under low gastric pH or by proteases. It is well established that Stx synthesis requires induction of Stx prophages, caused by any stress conditions provoking the SOS response (35). In our study, surprisingly, the high percentages of damaged cells observed under adult conditions in the TIM system were not associated with any detectable toxin, while significant amounts of Stx were measured in children where most of EHEC bacteria were unspoiled. We can then hypothesize that toxin production observed during child digestions may be mostly linked to *stx1* overexpression, as it does not necessarily require prophage induction and may also occur under conditions of low iron levels (29).

As for *stx*, *eae*, and *lpf* genes were up-regulated in the gastric and small intestinal compartments of the TIM system. This implies that the expression of EHEC adhesins, such as intimin and Lpf, can be induced even if the pathogen does not come into contact with the host cells. For both adhesins, higher expression levels were found under child conditions compared with adult ones, but only at the end of gastric or GI *in vitro* digestions. It is worth noting that *eae* and *lpf* overexpression occurs in children when most of bacterial cells have reached

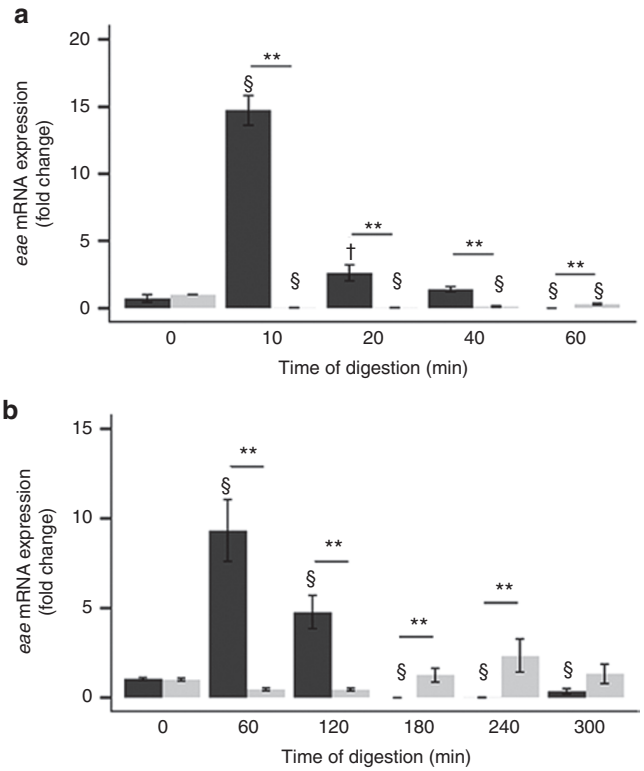

Figure 5. Expression of intimin-encoding gene in the TIM system. *eae* expression levels were measured by RT-qPCR in the gastric (**a**) and ileal (**b**) effluents of the TIM under child (in gray) and adult (in black) digestive conditions. Results are expressed as means of fold-induction ± SEM ($n = 4$). Time points statistically different from t0 at $P < 0.05$ (†) and $P < 0.001$ (§). Child statistically different from adult at $P < 0.01$ (**). TIM, TNO gastrointestinal model; RT-qPCR, reverse transcriptase-quantitative PCR.

the distal parts of the small intestine. As initial EHEC binding is assumed to occur at the follicle-associated epithelium of Peyer's Patches and villi of terminal ileum or colon (27), these results may suggest a higher ability to colonize in children. Very few studies have investigated the effect of digestive parameters on *eae* or *lpf* expression. A significant decrease in *eae* gene transcripts was observed *in vitro* when bile salts were added (33,36) and in pig intestinal loops acidified at pH 2.5 (32) while acid challenge (37) and bile salts (32,38) led to an up-regulation of *lpf* genes. Bile salts increase *lpf2* but not *lpf1* in O157:H7 (32), which may explain why *lpf2* mRNA levels were higher than *lpf1* ones in the small intestine. Current knowledge from literature does not provide any explanation from why *lpf* expression levels were higher under child conditions where a milder acidity and lower bile salt concentrations are found, nor why *eae* was mainly overexpressed in adults until 120 min digestion when the highest bile concentrations were found in the small intestinal compartments.

To conclude, our study shows that TIM model can provide meaningful insights into the comprehension of EHEC pathogenesis. Taken together, our results indicate that differences in digestive physicochemical parameters related to age conditions may partly explain the highest isolation rate of O157:H7 in the feces of children and their higher sensitivity to EHEC

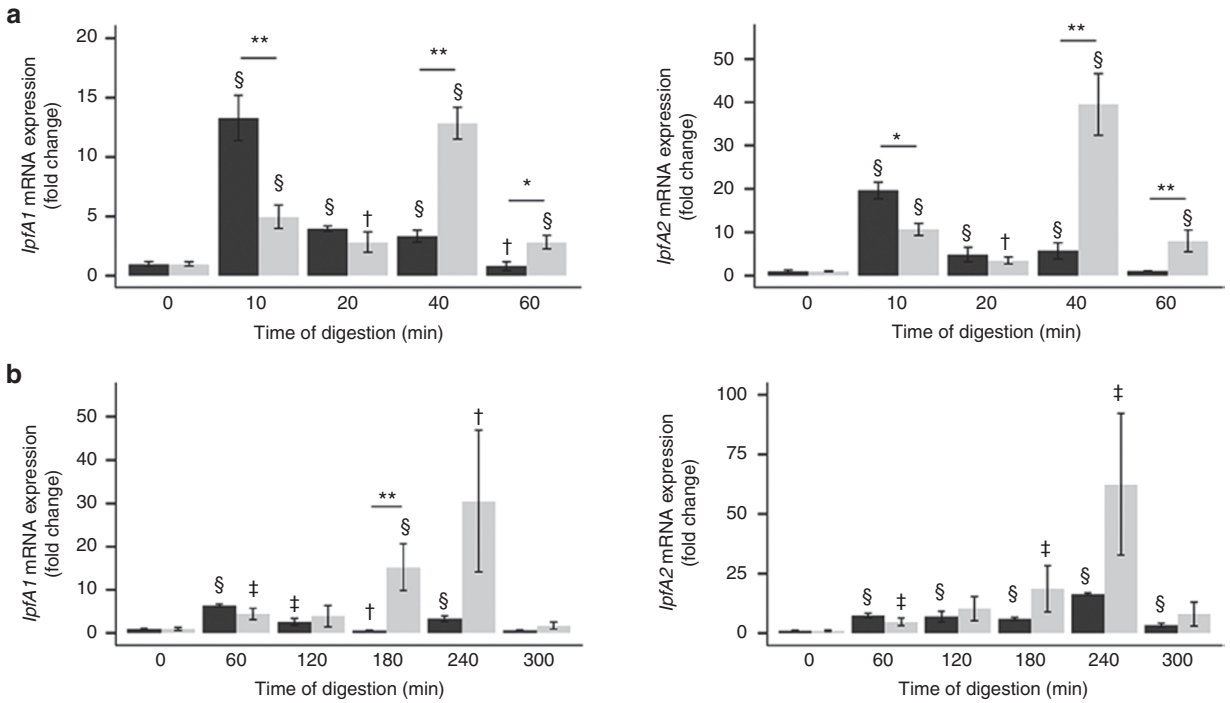

Figure 6. Expression of Long polar fimbriae-encoding genes in the TIM system. *lpf 1/2* expression levels were measured by RT-qPCR in the gastric (**a**) and ileal (**b**) effluents of the TIM under child (in gray) and adult (in black) digestive conditions. Results are expressed as means of fold-induction ± SEM ($n = 4$). Time points statistically different from t0 at $P < 0.05$ (†), $P < 0.01$ (‡) and $P < 0.001$ (§). Child statistically different from adult at $P < 0.05$ (*) and $P < 0.01$ (**). TIM, TNO gastrointestinal model; RT-qPCR, reverse transcriptase – quantitative PCR.

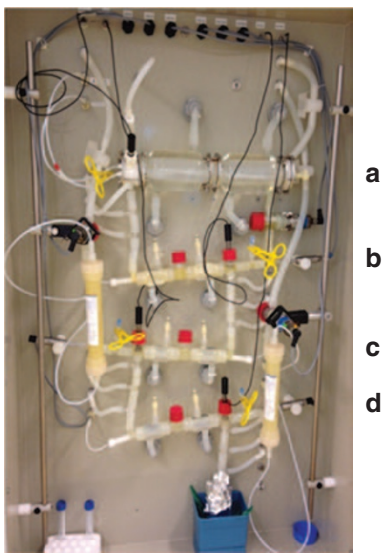

Figure 7. Gastric and small intestinal TNO gastrointestinal model (TIM). The TIM model is composed of four successive compartments simulating the human stomach (**a**) and the three parts of the small intestine, i.e., the duodenum (**b**), jejunum (**c**) and ileum (**d**).

infection and HUS (1–3). Nevertheless, other extra-digestive factors, such as immaturity of the immune system and higher expression of Stx receptor on the surface of renal cells (39) are also known to contribute to the higher susceptibility of infants to HUS. Besides, it should be noted that the results obtained in this study may be strain-specific (9). To get a more complete picture of EHEC pathogenesis, similar experiments should be carried out with other non-O157 strains that are also involved in EHEC disease. Such data are essential in the designing of novel therapeutic approaches, particularly in the young children high-risk population.

METHODS

TIM Gastrointestinal Model

The TIM model (TIM2013, TNO, Zeist, Netherlands) consists of four successive compartments simulating the human stomach, duodenum, jejunum, and ileum (**Figure 7**). The main parameters of digestion, such as body temperature, pH, peristaltic mixing and transport, gastric, biliary, and pancreatic secretions and passive absorption of small molecules and water, are reproduced as accurately as possible. Briefly, each compartment is composed of glass units with a flexible inner membrane. Peristaltic mixing and body temperature are achieved by pumping water at 37°C into the space between the glass jacket and the flexible wall at regular intervals. Mathematical modeling of gastric and ileal deliveries with the Elashoff power exponential equation ($f = 1–2^{-(t/t1/2)\beta}$ where t is the time of emptying and β a coefficient describing the shape of the curve) is used for the computer control of chyme transit. Chyme transport through the TIM is regulated by the peristaltic valves that connect the successive compartments. The volume in each compartment is monitored by a pressure sensor and pH is computer-monitored and continuously controlled by adding either HCl or $NaHCO_3$. Simulated gastric, biliary and pancreatic secretions are introduced into the corresponding compartments by computer-controlled pumps. Water and products of digestion are removed from the jejunal and ileal compartments by pumping dialysis liquid through hollow fibers (SF 09L, Nipro, Zaventem, Belgium; cut-off 10 KDa).

In vitro Digestions

The TIM system was programmed to reproduce, based on *in vivo* data (16–26), the physicochemical digestive conditions observed in a healthy adult or young children (from 6 mo to 2 y) when a glass

Table 1. Set-point parameters of gastrointestinal digestions in the TIM system

Parameters of *in vitro* digestion	Adult	Child from 6 mo to 2 y old
pH	Gastric compartment (min/pH)	Gastric compartment (min/pH)
	$t = 10 \rightarrow 3.2$	$t = 10 \rightarrow 5.7$
	$t = 20 \rightarrow 2.4$	$t = 20 \rightarrow 5.3$
	$t = 40 \rightarrow 1.8$	$t = 40 \rightarrow 4.5$
	$t = 60 \rightarrow 1.6$	$t = 60 \rightarrow 3.2$
	$t = 90 \rightarrow 1.5$	$t = 90 \rightarrow 2.0$
	Duodenal compartment: 6.4	Duodenal compartment: 6.4
	Jejunal compartment: 6.9	Jejunal compartment: 6.9
	Ileal compartment: 7.2	Ileal compartment: 7.2
Transit time	Based on *in vivo* pH (14)	Based on *in vivo* pH (15)
	Stomach: $t_{1/2} = 15$ min, $\beta = 1$	Stomach: $t_{1/2} = 20$ min, $\beta = 1.2$
	Ileum: $t_{1/2} = 150$ min, $\beta = 2.4$	Ileum: $t_{1/2} = 190$ min, $\beta = 1.7$
	Based on *in vivo* transit time (16,25)	Based on *in vivo* transit time (17,26)
Digestive secretions	Gastric compartment[a]	
	130 IU/min of pepsin (18) (P7012 Sigma-Aldrich, St Quentin, France)	130 IU/min of pepsin (19) (P7012 Sigma-Aldrich)
	5 IU/min of lipase (18) (DS Amano Pharmaceutical, Aichi, Japan)	5 IU/min of lipase (19,20) (DS Amano Pharmaceutical)
	0.25 ml/min of HCl 0.3 mol/l (according to pH)	0.25 ml/min of HCl 0.1 mol/l (according to pH)
	Duodenal compartment	
	Bile salts[b] 4% during the first 30 min and then 2% (21) (B8631 and F48305 Sigma-Aldrich)	Bile salts[b] 1% (22) (B8631 and F48305 Sigma-Aldrich)
	Pancreatic juice 7% (18) (P1750 Sigma-Aldrich)	Pancreatic juice 3.5% (23) (P1750 Sigma-Aldrich)
	Trypsin 3.4 mg (24) (T4665 Sigma-Aldrich)	Trypsin 3 mg (19) (T4665 Sigma-Aldrich)
	0.25 ml/min of intestinal electrolytes solution	0.25 ml/min of intestinal electrolytes solution
	0.25 ml/min of $NaHCO_3$ 0.5 mol/l (according to pH)	0.25 ml/min of $NaHCO_3$ 0.5 mol/l (according to pH)
	Jejunal compartment	
	0.25 ml/min of $NaHCO_3$ 0.5 mol/l (according to pH)	0.25 ml/min of $NaHCO_3$ 0.5 mol/l (according to pH)
	Ileal compartment[a]	
	0.25 ml/min of $NaHCO_3$ 0.5 mol/l (according to pH)	0.25 ml/min of $NaHCO_3$ 0.5 mol/l (according to pH)
Jejunal and ileal dialysis	10 ml/min	10 ml/min
Ileal absorption	0.4 ml/min	0.4 ml/min

The table gives the main parameters of the TNO gastrointestinal model (TIM) when simulating digestive conditions of a healthy adult or child after intake of a glass of water. [a]A power exponential equation ($f = 1–2^{-(t/t_{1/2})^{\beta}}$ where f represents the fraction of meal delivered, t the time of delivery, $t_{1/2}$ the half-time of delivery, and β a coefficient describing the shape of the curve) was used for the computer control of gastric and ileal deliveries. [b]Bile is composed of porcine bile extract 1/3 and bile salts 2/3 (deoxycholate and cholate).

of water is ingested (Table 1). The bacterial suspension (200 ml) that was introduced into the TIM system consisted of mineral water experimentally contaminated with the reference strain EHEC O157:H7 EDL 933 (ATCC 43895) at a final concentration of 10^7 CFU/ml. This strain was isolated from Michigan ground beef that was linked to a multistate US outbreak in 1982 involving contaminated hamburgers, in which *E. coli* O157:H7 was first associated with human disease (40). Two types of experiments were performed: gastric digestions where the gastric compartment was solely used (total duration of 60 min) and GI digestions using the entire TIM model (total duration of 300 and 360 min for the adult and child protocol, respectively). During digestion, gastric, and ileal effluents were kept on ice and pooled on 0–10, 10–20, 20–40, and 40–60 min for gastric digestions and hour-by-hour for GI digestions. Digestions were run in quadruplicate. Samples were taken in the initial bacterial suspension (t0) and regularly collected during digestion in each digestive compartment (stomach, duodenum, jejunum, and ileum) and/or in the gastric and ileal effluents.

Bacterial Counting

Survival kinetics in each digestive compartment of the TIM model was determined by direct plating onto Luria Bertani (LB) agar (overnight incubation at 37°C). Results were expressed as percentages of initial intake and cross-compared with those obtained with a theoretical nonabsorbable transit marker provided by the TIM system and indicating a 100% survival rate for bacteria. Bacterial curves below that of the transit marker will reflect cell mortality, while curves above the transit marker will be indicative of bacterial growth.

Physiological State of Bacteria

Physiological state of bacteria was determined by a live/dead analysis with flow cytometry. Bacteria from gastric and ileal effluents were double stained (LIVE/DEAD BacLight, Molecular probes, Whaltham, MA) with the green-fluorescent DNA stain SYTO 9 labelling all bacteria and the red-fluorescent Propidium Iodide (PI) only penetrating and staining cells with damaged membranes. Adequate volumes of gastric or ileal effluents were centrifuged

Table 2. Primers used in RT-qPCR assay

Name	Sequence 5′-3′	Target	Annealing final temperature (°C)	References
VT1c	ACCCTGTAACGAAGTTTGCG	*stx1* EHEC Shiga-toxin 1	60	(41)
VT1d	ATCTCATGCGACTACTTGAC			
Stx2-F	TTGCTGTGGATATACGAGGGC	*stx2* EHEC Shiga-toxin 2	60	(42)
Stx2-R	TCCGTTGTCATGGAAACCG			
Eae-F	CCCGAATTCGGCACAAGCATAAGC	*eae* EHEC intimin	63	(32)
Eae-R	CCCGAATCCGTCTCGCCAGTATTCG			
LPF154-F	TATGGCAGGTCACCTACAGG	*lpfA2* major fimbrial subunit LpfA2	60	(32)
LPF154-R	AGGTTTCCGGGCATTGAGTC			
LPFA141-F	CATCACTATCACCACTAAAGC	*lpfA1* major fimbrial subunit LpfA1	60	(32)
LPFA141-R	ATTTACAAGGGCATTCGCTGT			
Eco1457-F	CATTGACGTTACCCGCAGAAGAAGC	*16S Enterobacteriaceae*	63	(43)
Eco1652-R	CTCTACGAGACTCAAGCTTGC			

EHEC, enterohemorrhagic *Escherichia coli*; eae, intimin; LPF, long polar fimbriae; RT-qPCR, reverse transcriptase-quantitative PCR; Stx, Shiga toxins.

(9,000×*g*, 20°C, 5 min) and bacterial pellets were suspended in phosphate buffer at pH 7.3 to get a final concentration of ~10^6 CFU/ml. Bacterial suspensions were incubated for 15 min at room temperature in the dark with SYTO 9 (5 µmol/l) and PI (30 µmol/l), according to the manufacturer's instructions. Flow cytometry analysis was performed on a CyFlow SL cytometer and data were collected with FlowMax software version 2.3 (Partec, Sainte-Geneviève-des-Bois, France). Gating on forward-angle light scatter/side-angle light scatter was used in order to differentiate bacteria from the background, then the combined red and green fluorescence dot-plots were used to distinguish among the various subpopulations. Statistical tables that show percentages of marked cells determined by each detector were used to analyze data.

Expression of Virulence Genes

Reverse transcription (RT)-PCR was used to follow the expression of *stx 1*, *stx 2*, *eae*, *lpf 1*, and *lpf 2* virulence genes in the gastric and ileal effluents of the TIM. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). RNAs were reversely transcribed using the PrimeScript RT Reagent Kit (TAKARA Bio, Shiga, Japan). q-PCR was performed using SYBR Green qPCR Master Mix (Agilent Technologies, Waldbronn, Germany) on a CFX96 Touch Real-Time PCR Detection System (Biorad, Hercules, CA) with the specific primers indicated in **Table 2**. *Enterobacteriaceae* rRNA 16S was used as housekeeping gene for quantification of mRNA expression. Fold-induction was calculated using the Ct method as follows:

$$\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}})_{\text{at time t}} - (Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}})_{\text{at t0}}$$

and the final data were derived from $2^{-\Delta\Delta Ct}$.

Toxins Production

Stx1 and Stx2 produced by O157:H7 in the ileal effluents of the TIM system were dosed by enzyme-linked immunosorbent assay using the Ridascreen Verotoxin kit (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions. Stx concentrations were determined by measuring a change in absorbance of the digestive samples using the Multiskan spectrum reader (Thermo Scientific, Waltham, MA) set at 450 nm. Supernatant from an overnight culture of EDL 933 was used to establish standard calibration curves.

Statistical Analysis

Values are given as means and SEM (n = 4). Significant differences in survival between treatments and time points were testing using a nonparametric analysis of repeated measures with the "f1.ld.f1" function of the R package "nparLD" in R 3.1.2 R (Development Core Team 2015). In case of a significant treatment effect, Tukey contrast effects of survival between the two treatments for each time point were calculated using the function "nparcomp" of the R package "nparcomp". In case of a significant interaction effect, a linear mixed effect model with a random intercept on experiments taking into account the repeated measures was performed and followed by function "diffsmeans" of the package "lmerTest". The kinetics of virulence genes expression was tested with the "ld.f1" function of the R package nparLD. In case of a significant time effect, pairwise comparisons with Bonferroni adjustment were performed.

Ethics Statement

Statement of study approvement by the Institutional Review Board: As this study was only performed in an *in vitro* model, no subject was enrolled and no human sample was used. Therefore, study approvement by our Institutional Review Board was not required.

Statement of subject and/or parental informed consent: As this study was only performed in an *in vitro* model, no subject was enrolled and therefore subject or parental informed consent was not required.

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