

RESEARCH ARTICLE

Escherichia albertii, a novel human enteropathogen, colonizes rat enterocytes and translocates to extra-intestinal sites

Denise Yamamoto^{1*}, Rodrigo T. Hernandez^{1,2}, Ana Maria A. Liberatore³, Cecilia M. Abe⁴, Rodrigo B. de Souza³, Fabiano T. Romão^{1,5}, Vanessa Sperandio⁵, Ivan H. Koh³, Tânia A. T. Gomes^{1*}

1 Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, Escola Paulista de Medicina (UNIFESP-EPM), São Paulo, Brazil, **2** Departamento de Microbiologia e Imunologia, Instituto de Biociência, Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Botucatu, São Paulo, Brazil, **3** Departamento de Cirurgia, Universidade Federal de São Paulo, Escola Paulista de Medicina (UNIFESP-EPM), São Paulo, Brazil, **4** Laboratório de Biologia Celular, Instituto Butantan, São Paulo, Brazil, **5** Department of Microbiology and Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas, United States of America

* tatg.amaral@unifesp.br (TATG); dyamamoto@unifesp.br (DY)



OPEN ACCESS

Citation: Yamamoto D, Hernandez RT, Liberatore AMA, Abe CM, Souza RBd, Romão FT, et al. (2017) *Escherichia albertii*, a novel human enteropathogen, colonizes rat enterocytes and translocates to extra-intestinal sites. PLoS ONE 12 (2): e0171385. doi:10.1371/journal.pone.0171385

Editor: Michael Hensel, Universitat Osnabruck, GERMANY

Received: September 16, 2016

Accepted: January 18, 2017

Published: February 8, 2017

Copyright: © 2017 Yamamoto et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data availability statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by grants from FUNDAÇÃO DE AMPARO À PESQUISA DO ESTADO DE SÃO PAULO (FAPESP 2011/12664-5) to TATG and COORDENAÇÃO DE APERFEIÇOAMENTO DE PESSOAL DE NÍVEL SUPERIOR (CAPES AUX-PE-PNPD 2350/2011) to TATG and DY. The funders had no role in study

Abstract

Diarrhea is the second leading cause of death of children up to five years old in the developing countries. Among the etiological diarrheal agents are atypical enteropathogenic *Escherichia coli* (aEPEC), one of the diarrheagenic *E. coli* pathotypes that affects children and adults, even in developed countries. Currently, genotypic and biochemical approaches have helped to demonstrate that some strains classified as aEPEC are actually *E. albertii*, a recently recognized human enteropathogen. Studies on particular strains are necessary to explore their virulence potential in order to further understand the underlying mechanisms of *E. albertii* infections. Here we demonstrated for the first time that infection of fragments of rat intestinal mucosa is a useful tool to study the initial steps of *E. albertii* colonization. We also observed that an *E. albertii* strain can translocate from the intestinal lumen to Mesenteric Lymph Nodes and liver in a rat model. Based on our finding of bacterial translocation, we investigated how *E. albertii* might cross the intestinal epithelium by performing infections of M-like cells *in vitro* to identify the potential *in vivo* translocation route. Altogether, our approaches allowed us to draft a general *E. albertii* infection route from the colonization till the bacterial spreading *in vivo*.

Introduction

Escherichia albertii is a recently recognized close relative of *Escherichia coli*, which is an emerging human enteropathogen and avian pathogen [1,2]. Since it is difficult to discriminate from other species of *Enterobacteriaceae* by common biochemical identification assays [3], *E. albertii* isolates can be erroneously identified as *Hafnia alvei*, *Shigella boydii*, *Yersinia ruckeri* or *E. coli* [4–6].

design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Diarrheagenic *E. coli* (DEC) are classified into different categories according to their set of virulence genes. Enteropathogenic *E. coli* (EPEC), one of the DEC categories, are sub-grouped into typical (tEPEC) and atypical (aEPEC) based on the presence of the *bfp* operon (encoding the bundle forming pilus—BFP) in tEPEC [7,8] and its absence in aEPEC ([9] and reviewed in [10]).

Both EPEC groups use the type 3 secretion system (T3SS) to inject a number of effector proteins directly into the host cell, including Tir (translocated intimin receptor), which is inserted into the host cell membrane and acts as a receptor for the EPEC adhesin called intimin [11–13]. Altogether, these events promote microvilli effacement and intimate bacterial adherence to the enterocyte membrane leading to the so-called attaching and effacing (AE) lesion, in which pedestal-like structures that are rich in actin and other cytoskeleton components are formed [14–16]. The T3SS, Tir and Intimin-encoding genes are located in a pathogenicity island named the locus of enterocyte-effacement (LEE). Besides the Tir-intimin interaction, the presence of other potential adhesins such as *E. coli* common pilus (ECP) and type 1 pilus (T1P) have been reported in aEPEC strains [17–19].

We have previously shown that certain aEPEC strains may invade cultured cells *in vitro* [20–24] and that the aEPEC 1551–2 strain invades enterocytes through the basolateral surface more effectively than through the apical surface [22]. Invasive enteropathogens can reach the basolateral receptors and promote host cell invasion *in vivo* by transcytosis through M cells [25]. M cells are recognized as part of the innate immune response (reviewed by [26,27]), and are found on Peyer's patches (gut associated lymphoid tissue—GALT) in association with phagocytic cells, forming the follicle-associated epithelium (FAE) [26,27]. Hase et al., [28] demonstrated that deficiency of bacterial FimH (the T1P adhesin) or its receptor, host glycoprotein 2 (GP2), led to defects in bacterial transcytosis through M cells, resulting in an attenuation of antigen-specific immune responses in Peyer's patches.

There is little clinical evidence, but experimental data have indicated that, at least in the initial infection steps, M cells transport pathogens from the intestinal lumen to macrophages in the lamina propria of the mucosa [29]. In fact, some enteropathogens such as *Shigella*, *Salmonella* and *Yersinia* use M cells as the main entrance site in enterocytes [29,30]. After the translocation through M cells, *Shigella* is phagocytized, leading to the apoptosis of macrophages, and is released to access the enterocyte basolateral surface [31].

During a recent evaluation of a collection of aEPEC strains in our laboratory, using a polymerase chain reaction system [32] and a preliminary analysis of the genomic sequence of the LEE region of one strain (1551–2 strain), we have found out that six aEPEC strains, including the 1551–2 strain, are actually *E. albertii* ([6] and unpublished data).

It is known that *E. albertii* strains share with EPEC and EHEC (Enterohemorrhagic *E. coli*) the ability to promote AE lesions due to the presence of the LEE [4,6]. However, there is scarce information regarding the virulence mechanisms that favor the interaction of *E. albertii* with the intestinal mucosa [1].

Host colonization of the small and/or large intestines comprises the first step in the establishment of diarrheal diseases by bacterial pathogens, such as *Vibrio cholera*, EPEC and *Salmonella* spp. [30]. However, studies on the interaction of *E. albertii* strains of human origin with enterocytes are scarce and appropriate animal models to explore the interaction of *E. albertii* strains with the intestinal mucosa have not been described [33,34]. To contribute to this issue, we evaluated the rat intestinal mucosa as a model to study *E. albertii* adhesion, colonization and translocation from the intestinal lumen to extra-intestinal sites. In addition, we investigated the participation of the intimin-Tir interaction, the T3SS-translocon and T1P in this infection model, as well as *E. albertii* translocation into M-like cells.

Materials and methods

Ethics statement

The protocols involving animal handling were approved by the Research Ethics Committee of UNIFESP, project license number 0342/09. “Comitê de Ética em Pesquisa da UNIFESP/ Hospital São Paulo” (CEP UNIFESP/HU-HSP) is in accordance with Good Clinical Practice (GCP) of the International Council for Harmonisation (ICH), formerly the International Conference on Harmonisation (ICH). Animals are handled under “Brazilian Guidelines For The Care And Use Of Animals In Educational Activities Or Scientific Research” standards that are in accordance with Brazilian Law 11.794/2008, which defined procedures to be employed in the scientific use of animals.

Bacterial strains

The invasive *E. albertii* 1551–2 strain (intimin subtype omicron) and its isogenic mutants obtained in previous studies by our group (Table 1) were statically cultured in Luria Bertani broth (LB) for 18 h at 37°C. Antibiotics were added to select resistant strains as indicated in Table 1. The mutant strain 1551–2Δ*tir* was constructed employing the one-step allelic exchange recombination method [35]. Primers containing a 40-bp region homologous to the 5’ and 3’ ends of the *tir* gene and a specific sequence for the zeocin (*zeo*) resistance-encoding gene (*tir-zeo-F* ATG CCT 1 ATT GGT AAT CTT GGT CAT AAT CCC AAT GTG AGT GGT CAT CGC TTG CAT TAG AAA GG and *tir-zeo-R* TTA AAC GAA ACG ATT GGA TCC CGG CAC TGG TGG GTT ATT CGA ATG ATG CAG AGA TGT AAG) were used to amplify the *Zeo* cassette [36]. Amplicons obtained in the PCR reaction were electroporated into competent wild type bacteria harboring the pKOBEG-Apra plasmid. The selection of recombinant bacteria were done on *Zeo*-containing LB agar plates (60 µg/mL), and the *tir* deletion in the isogenic mutant was confirmed by PCR. In addition, the loss of pKOBEG-Apra plasmid was confirmed by testing the mutant strain for apramycin susceptibility.

Table 1. Bacterial strains.

Strain	Characteristics	Origin
<i>E. albertii</i> 1551–2 Nal ^R	Wild type strain (wt)	[21]
<i>E. albertii</i> 1551–2 <i>eae</i> ::Kn ^R	<i>eae</i> mutant (<i>eae</i>)	[21]
<i>E. albertii</i> 1551–2Δ <i>tir</i> (Zeo ^R)	<i>tir</i> mutant (Δ <i>tir</i>)	This study
<i>E. albertii</i> 1551–2 <i>escN</i> ::Kn ^R	<i>escN</i> (T3SS) mutant (<i>escN</i>)	[36]
<i>E. albertii</i> 1551–2 <i>escN</i> ::Kn ^R (p <i>EscN</i>)	<i>escN</i> mutant strain complemented with pACY184 carrying <i>escN</i> gene of tEPEC E2348/69 (p <i>EscN</i>)	[36,37]
<i>E. albertii</i> 1551–2Δ <i>fimA</i> (Zeo ^R)	<i>fimA</i> mutant (Δ <i>fimA</i>)	[36]
<i>E. albertii</i> 1551–2Δ <i>fimA</i> (p <i>FimA</i>) (Zeo ^R Amp ^R)	<i>fimA</i> mutant strain complemented with plasmid pBAD Myc carrying <i>fimA</i> gene of aEPEC 1551–2 (p <i>FimA</i>)	[36]
tEPEC E2348/69	tEPEC prototype strain (E2348/69)	[38]
R6	Rat <i>E. coli</i> strain that is able to translocate from lumen to extra-intestinal organs (R6)	[39]
<i>E. coli</i> K12 HB101	Non-pathogenic laboratory strain (HB101)	[40]

Nal^R: nalidixic acid resistant (20 µg/mL); Kn^R: kanamycin resistant (50 µg/mL); Zeo^R: Zeocin resistant (60 µg/mL); Amp^R: ampicillin resistant (100 µg/mL).

doi:10.1371/journal.pone.0171385.t001

Cell culture conditions

Caco-2 cells (ATCC HTB-37, purchased from Banco de Células do Rio de Janeiro, Rio de Janeiro, Brazil) and NIH 3T3 cells (ATCC CRL 2795, kindly provided by Dr. Beatriz Castilho, UNIFESP) were grown in DMEM (Gibco Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibco Invitrogen, USA), 1% non-essential amino acids (Gibco Invitrogen, USA) and 1% antibiotics (Pen Strep—Gibco Invitrogen, USA). Raji-B cells (ATCC CCL-86, kindly provided by Dr. Roger Chammas, Universidade de São Paulo, São Paulo, Brazil) were cultured in RPMI-1640 (Gibco Invitrogen, USA) supplemented with 10% fetal bovine serum, 200 mM glutamine (Gibco Invitrogen, USA) and 1% antibiotics. The cell lines were cultured at 37°C in an atmosphere of 5% CO₂.

Adhesion and Invasion assays

Quantitative assessment of bacterial association and invasion was performed as described previously [24,41]. Briefly, differentiated Caco-2 cells were infected with 10⁷ colony-forming units (CFU) of *E. albertii* strain 1551–2 and its isogenic mutants for 6 h. Thereafter, cell monolayers were washed three times with phosphate buffered saline (PBS). While one set of monolayer-containing wells was lysed in 1% Triton X-100 for 30 min at 37°C, another set was treated with 100 µg/mL of gentamicin (Sigma, USA) for one hour at 37°C, and then washed 5 times prior to lysis. Following cell lysis, bacteria were resuspended in PBS and quantified by plating serial dilutions onto MacConkey agar plates to obtain the total number of cell-associated bacteria and of intracellular bacteria. The invasion indexes were calculated as the percentage of the total number of cell-associated bacteria that were located in the intracellular compartment. Assays were carried out in triplicate, and the results from at least three independent experiments were expressed as the percentage of invasion (mean ± standard error).

Animals

Female Wistar-EPM rats, ~3 months-old and weighting 200–250 g, were obtained from the Central Animal Facility of Universidade Federal de São Paulo (UNIFESP). After 14 days of environment adaptation, stool samples were collected for coproculture and *E. coli* recovered from each animal were screened for the presence of the *eae* gene, which encodes the adhesin intimin, by PCR (AE11 5′-CCCGGCACAAGCATAAGCTAA-3′ and AE12 5′-ATGACTCATGCCAGCCGCTCA-3′, generating a fragment of 917 bp [42]). This procedure was performed to avoid the use of experimental animals that were colonized by either *E. coli* or *Citrobacter rodentium*, a murine pathogen that also promotes AE lesion formation [43]. Prior to the assays, animals were fasted for 24 h with access to water.

In vivo organ culture (IVOC) bacterial colonization assay

For removal of ileum fragments, rats were held under anesthesia (pre-atropinization, induction of inhalation anesthesia with isoflurane and maintenance with intramuscular injection of 0.1 mL/100 g body weight ketamine + xylazine (4:1 1). After antisepsis, rats were subjected to median laparotomy for the collection of intestinal fragments of ~0.5 cm². Briefly, ileal segments were removed, sectioned longitudinally at its antimesenteric border and placed onto a sterile filter paper with its serous portion facing the filter. This procedure allowed the exposure of the entire apical surface of the mucosa to the bacterial inoculum. Fragments were kept in Dulbecco's Modified Eagle Medium (DMEM—Gibco Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibco Invitrogen, USA) [44]. Fragments were infected with 10¹⁰ CFU for 6 h of incubation (37°C, 5% CO₂); fragments were then washed, macerated and suspended

in PBS and plated in serial dilution onto MacConkey agar plates containing 20 µg/mL nalidixic acid [21] for quantification (calculation of the total number of mucosa-associated bacteria). Infected IVOC preparations were also fixed for electron microscopy analysis.

In vivo bacterial translocation assay

Animals were maintained under anesthesia (intramuscular injection of 0.1 mL/100 g body weight of ketamine and xylazine (4:1) during the *entire* procedure. Additional half dose of anesthetic was administered when necessary. Bacterial translocation (BT) was induced by a midline incision, oroduodenal cannulation, injection of 10^{10} CFU/mL resuspended in 10 mL of saline through the catheter, and bacterial retention for a period of 2 h, within a portion between the duodenum and ileum, by means of ligatures [39]. The *E. coli* rat strain R6, which is devoid of the DEC virulence genes, such as the *eae* gene, was used as a BT-positive control strain [39], while the non-pathogenic *E. coli* strain HB101 was used as BT-negative control. Bacterial inoculation causes a transient dilation of the small bowel, which disappeared within a short period. Blood (1 mL), mesenteric lymph nodes (MLN), spleen and liver were then collected, weighed, macerated and suspended in PBS. Subsequently, bacterial colonies were enumerated after plating serial dilutions onto MacConkey agar plates containing 20 µg/mL nalidixic acid, to estimate the number of translocated bacteria. The results were expressed by mean \log_{10} values of CFU/g tissue.

M-like cell differentiation

M-like cells were obtained as previously described [45–47] with modifications. Briefly, Caco-2 cells (10^5 cells/filter) were seeded on the upper chamber of a Millicell filter (3.0-µm pore diameter, Millipore, USA) and kept in DMEM as described above for 10 days at 37°C in an atmosphere of 5% CO₂. The lower chamber was also filled with DMEM. During this incubation period, transmembrane electric resistance (TEER) was measured every two days using the Millicell[®] ERS (Electrical Resistance System, Millipore), until it reached ~420 mΩ. Afterwards, Raji-B cells (10^6 cells/mL) were seeded at the Millicell lower chamber and cultured in RPMI-1640, as described above, for 6 days. In parallel, in some filters, Caco-2 cells were kept in monoculture for an additional 6 days (non-differentiated cells). Since galectin-9 is expressed on M cells but not on Caco-2 cell surface [47], NIH 3T3 (positive control), Caco-2 (negative control) and M-like cells were fixed and incubated with galectin-9 (M-20):sc-19294 (Santa Cruz Biotechnology, INC), followed by Alexa Fluor 488 (donkey anti-goat IgG, Invitrogen) incubation. Green fluorescent cells indicated the presence of galectin-9. Alternatively, donkey anti-goat IgG-TR (Santa Cruz Biotechnology, INC), combined with phalloidin-FITC (Sigma, USA) and DAPI (Molecular Probes, USA) were used.

In vitro bacterial translocation assay

Bacterial suspensions (10^7 CFU) in DMEM (as described above, except for 1% antibiotics) were inoculated in the upper chamber of filters bearing either M-like/Caco-2 or Caco-2 cells only for 6 h. Filters were transferred to a well containing fresh medium (DMEM without antibiotics) every hour and the medium from the lower chamber was collected for bacterial quantification at 6 h [46]. In parallel, the transmembrane electric resistance (TEER) was measured. At the end of the infection period, monolayers were washed with PBS and fixed for microscopy.

Scanning electron microscopy (SEM)

For SEM, preparations were fixed in 2% glutaraldehyde, post-fixed in 1% osmium tetroxide, and dehydrated through a graded ethanol series (50, 70, 90, and 100%). After dehydration,

preparations were dried by the critical point method, mounted onto SEM stubs, coated with gold and examined under SEM (QUANTA 250—FEI Company, Netherlands) at 12.5 kV [48].

Transmission electron microscopy (TEM)

Infected monolayers and ileum fragments were first fixed in 2% glutaraldehyde (EMS, USA) for at least 24 h at 4°C. After primary fixation, cells and fragments were washed 3 times with PBS (10 min) and subjected to secondary fixation with 1% osmium tetroxide (EMS, USA) in 0.1 M sodium cacodylate buffer for 30 min. After being washed three times with distilled water, preparations were dehydrated through a graded ethanol series (50%, 75%, 85%, 95% and 100%), and propylene oxide (100%). Preparations were then gradually embedded in Araldite, which was allowed to polymerize for 24–48 h at 60°C. Ultrathin sections were placed on Formvar (EMS, USA) coated 200 mesh copper grids and stained with 4% aqueous uranyl acetate (Merck, Germany) and Reynold's lead citrate (Merck, Germany). Grids were examined under TEM (LEO 906E—Zeiss, Germany) at 80 kV [48].

Statistical analysis

Differences in bacterial adherence, invasion percentages and translocation or differences in TEER of infected M-like cells were assessed for significance by using an unpaired, two-tailed *t* test (GraphPad Prism 4.0).

Results

Intimin, Tir and T3SS are essential for invasion of human intestinal cells cultured *in vitro*

Strain 1551–2 had been previously evaluated regarding its ability to invade differentiated Caco-2 cells [24]. In this study, Caco-2 cells were infected with bacterial suspensions of the wild type or its isogenic mutant strains (Table 1). Compared to the wild type strain the adherence index of mutant strains was not altered (Fig 1A) while the invasion index decreased significantly (Fig 1B), except for *fimA* mutation that did not affect the adherence or invasion indexes (Fig 1A–1B). These results confirm that *E. albertii* 1551–2 invasion depends on intimin and/or proteins injected by the T3SS, such as Tir, but not on T1P. Besides that, as the T3SS mutant did not inject Tir into Caco-2 cells, it is possible that, in the absence of its receptor, the 1551–2 intimin might recognize another host cell membrane structure as site for adhesion, but not for invasion, as confirmed by results obtained in invasion assays with 1551–2 Δ *tir* strain. Complementation of T3SS mutant restored the invasion index to the wild type values (Fig 1B).

E. albertii 1551–2 colonizes rat enterocytes in *in vitro* organ culture (IVOC)

To evaluate whether *E. albertii* 1551–2 could colonize the rat intestinal mucosa, ileal fragments (approx. 0.5 cm²) were individually infected with bacterial suspensions of the wild type or its isogenic mutant strains (Table 1). Methylene blue staining of the intestinal fragments was performed to confirm that all tissue layers were well preserved (S1 Fig).

SEM images confirmed that the wild type strain strongly adhered to the intestinal mucosa (Fig 2A), whereas the T3SS-mutant comparatively showed a weaker adherence (Fig 2B). Non-infected fragments showed well-preserved bacterial-free brush borders (Fig 2C). Similarly to the wild type strain, the intimin, Tir and T1P mutants remained adherent to the intestinal mucosa (S2 Fig). Besides bacterial adherence, TEM images showed that the wild type strain

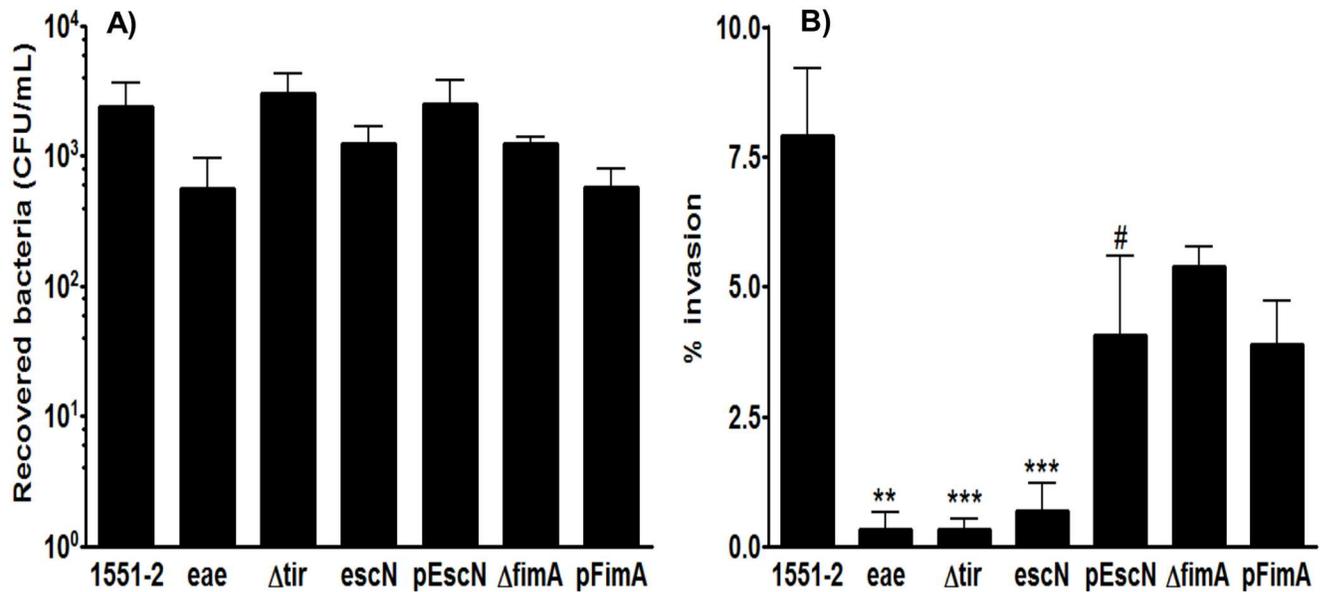


Fig 1. Quantitative assessment of association and invasion of *E. albertii* 1551-2 and its isogenic mutants to differentiated Caco-2 cells. Cells were infected with 10⁷ CFU for 6 h, monolayers were washed and one set of monolayer was lysed while another set was submitted to the gentamicin protection assay. The invasion indexes were calculated as the percentage of the total number of cell-associated bacteria that were located in the intracellular compartment. Assays were carried out in triplicate, and the results from at least three independent experiments were expressed as the percentage of invasion (mean ± standard error). A) Association. No statistical differences in the association of the wild type, mutant and complemented mutant strains were found. B) Invasion: (**) indicates statistical differences between 1551-2 and the intimin mutant (P = 0.0030). (***) indicates statistical differences between 1551-2 and Tir (P = 0.0002) or T3SS (P = 0.0004) mutants. (#) indicates statistical differences between T3SS mutant and complemented T3SS (P = 0.0238) strains.

doi:10.1371/journal.pone.0171385.g001

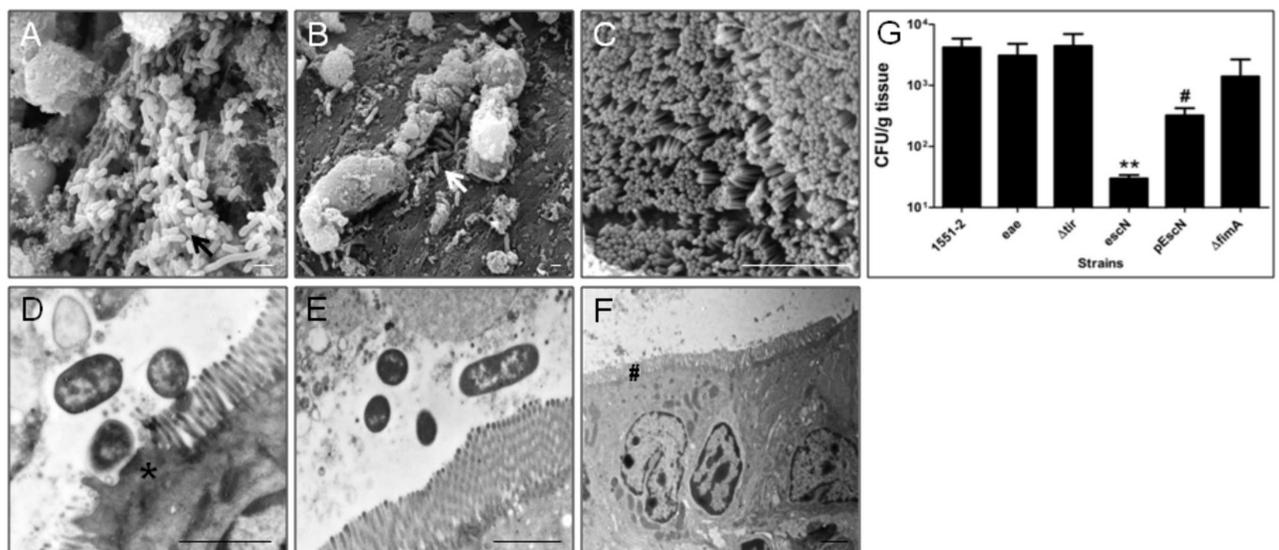


Fig 2. IVOC and *E. albertii* colonization assays. Ileal fragments collected from 3 Wistar-EPM rats were inoculated with 10¹⁰ CFU of each bacterial strain and incubated for 2 h at 37°C. SEM: (A) wild type *E. albertii* strain 1551-2 (black arrow), (B) T3SS mutant (white arrow), (C) non-infected control. TEM: (D) AE lesion underneath the wild type strain adherence site (#), (E) absence of AE lesion under the T3SS mutant interaction and (F) Non-infected control. (#) indicates preserved brush borders. Bars, 2 μm. (G) Quantification assay. After the incubation period, preparations were washed, macerated, and bacterial suspensions were diluted for seeding and CFU counting. (**) Significantly less adherent than wild type (p = 0.013), (#) significantly more adherent than T3SS mutant (P = 0.02). *eae*, intimin mutant; *Δtir*, Tir mutant; *escN*, T3SS mutant; *pEscN*, complemented T3SS mutant; *ΔfimA*, T1P mutant.

doi:10.1371/journal.pone.0171385.g002

caused AE lesions with characteristic pedestals underneath adhered bacteria on the rat mucosal surface (Fig 2D). In contrast, the T3SS-translocon mutant failed to cause AE lesions (Fig 2E), and non-infected fragments showed well-preserved bacterial-free brush borders (Fig 2F).

The number of CFU recovered from rat intestinal mucosa *in vitro* decreased significantly in the absence of the T3SS-translocon, while mutant strains deficient in intimin, Tir or T1P production, as well the T3SS mutant complemented strain, showed similar adherence levels in comparison with the wild type strain (Fig 2G).

E. albertii strain translocates across rat intestinal barrier *in vivo*

To reduce the number of animals utilized in the next approach, we selected the T3SS-translocon mutant for *in vivo* comparison with wild type strain based on results obtained with the IVOC infection assay. Our results demonstrated that *E. albertii* 1551–2 reached the liver, while the T3SS-translocon mutant was not recovered from this organ. These findings suggest that, as a consequence of the reduced adhesion of this mutant to the intestinal mucosa, as observed *ex vivo*, fewer bacteria were available to cross the intestinal barrier, reach and survive in the MLN (Fig 3).

E. albertii 1551–2 translocates across M-like cells

Considering our results in the BT assay described in Materials and Methods and that pathogens such as *Shigella* species use M cells to cross the intestinal barrier, we performed *E. albertii* infection of M-like cells *in vitro* to identify the potential BT route employed *in vivo*. Prior to infection, we confirmed the conversion of part of the Caco-2 cells to M-like cells as described elsewhere [47], by demonstrating the expression of galectin-9 on M-like cell surface but not on Caco-2 cells (S3 Fig). Moreover, cellular morphology alterations [45] were observed on M-like cells, such as a reduced number of microvilli, flattened apical surface and disorganized

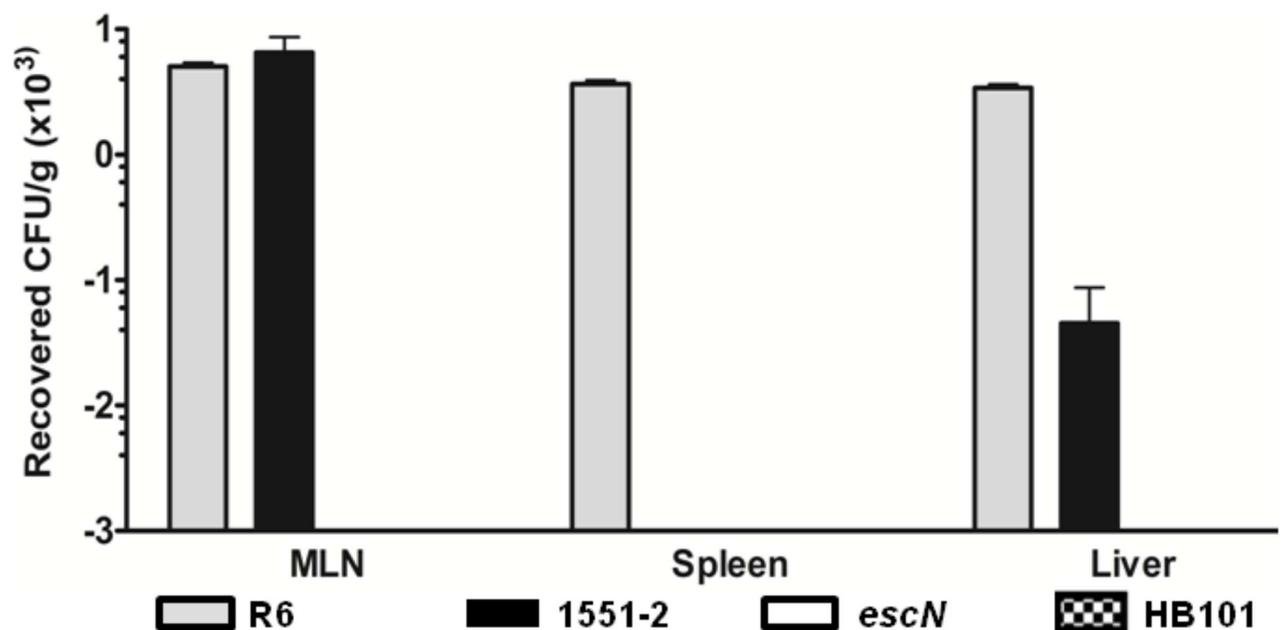


Fig 3. Bacterial translocation (BT) *in vivo*. *E. albertii* 1551–2 was recovered from the mesenteric lymph nodes (MLN) and liver. The T3SS mutant strain was not recovered from any tested organs. *E. coli* R6 (BT-positive control) was recovered from all examined organs while *E. coli* HB101 (BT-negative control) was not. *escN*, T3SS mutant.

doi:10.1371/journal.pone.0171385.g003

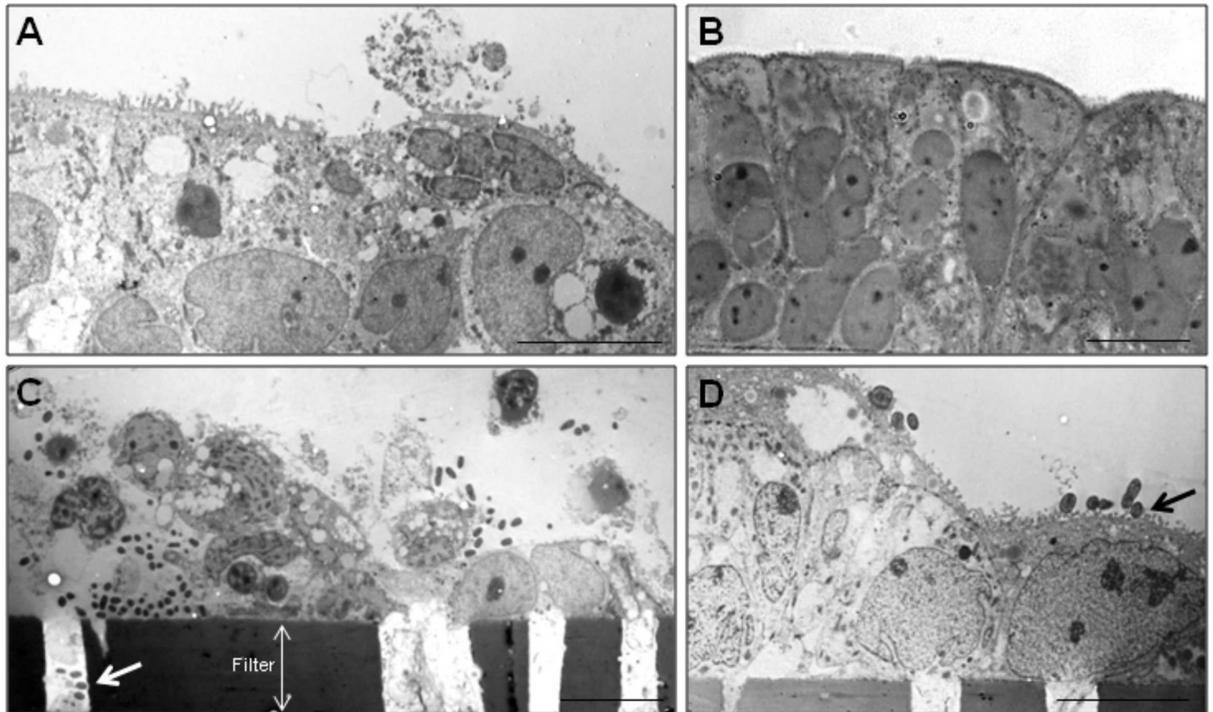


Fig 4. TEM of M-like and Caco-2 cells infected with *E. albertii*. M-like cells (A) showed few microvilli and a disorganized morphology, while on apical surface of differentiated Caco-2 cells (B) microvilli were still present. After *E. albertii* infection, M-like cells (C) allowed bacterial transcytosis (note bacteria crossing the filter membrane pore—white arrow), while in Caco-2 cells (D), *E. albertii* remained on the cells surface (black arrow). Bars, 10 μ m.

doi:10.1371/journal.pone.0171385.g004

cytoplasm (Fig 4A), while fully differentiated Caco-2 cells displayed preserved brush borders (Fig 4B). The presence of M-like cells significantly increased bacterial translocation (Fig 4C) as compared to differentiated Caco-2 cells (Fig 4D).

For quantitative *E. albertii* 1551–2 translocation assessment, tEPEC prototype strain E2348/69 was used as control [46]. We demonstrated that *E. albertii* translocation through M-like cells was significantly more effective than through differentiated Caco-2 cells ($2,962.0 \pm 546.0$ and 184.2 ± 91.6 , $p = 0.0024$, respectively) (Fig 5A), and as previously demonstrated [46], the presence of M-like cells did not increase the transcytosis of tEPEC E2348/69 in a significant manner as compared to differentiated Caco-2 cells (1.203 ± 0.528 and 0.417 ± 0.247 , $p = 0.1480$, respectively). Additionally, *E. albertii* 1551–2 translocated through M-like cells more effectively than tEPEC E2348/69 ($p = 0.033$, Fig 5A). In order to exclude bacterial paracellular migration due to increased permeability as an invasion route, transepithelial electrical resistance was measured hourly during the infection period (S3 Fig). Comparison between M-like cells infected with the wild type or the T3SS mutant strains demonstrated a significant decrease in bacterial recovery ($p = 0.0029$, Fig 5B) with the latter strain, while complementation of the mutant strain restored its translocation capacity ($p = 0.0418$, Fig 5B and S4 Fig). Contrarily, non-significant differences between CFU recovered from T1P mutant and its complemented strains were observed with M-like cells (Fig 5B).

Discussion

Previous data from our laboratory showed that the 1551–2 strain invaded HeLa cells [21] with invasion being dependent on the intimin-Tir interaction, since the intimin mutant (1551-

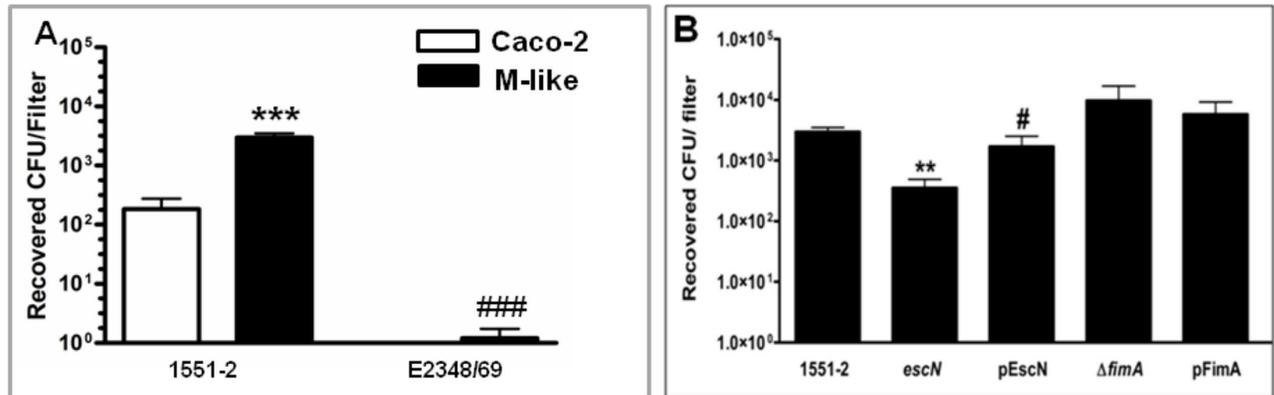


Fig 5. Bacterial Translocation through the M-like cells. Caco-2 cells were cultured for 10 days on Millicell filter. One group was co-cultured with Raji-B cell and control group were maintained in monoculture for 6 days. Then, upper chambers containing Caco-2 cells only or M-like cells were infected with *E. albertii* 1551–2, isogenic mutants or complemented strains for 6 h, or with tEPEC E2348/69 for 3 h. Media from the lower chamber were collected in three different periods during the incubation and seeded onto MacConkey agar for CFU counting. (A) Translocation of *E. albertii* and tEPEC through the Caco-2 and M-like cells: (***) indicates statistical differences between the two lineages infected with *E. albertii* strain ($P < 0.05$). (##) indicates differences between M-like cells infected with 1551–2 and E2348/69 strains ($P < 0.05$). (B) Translocation of *E. albertii*, isogenic mutants and complemented strains: (**) indicates differences between M-like cells infected with wt and T3SS mutant ($P < 0.05$). (#) indicates difference between M-like cells infected with the T3SS mutant and its complemented strain ($P < 0.05$). No statistical differences were found between in the transcytosis of wt and T1P mutant or between T1P mutant and its complement strains. *eeae*, intimin mutant; *tir*, Tir mutant; *escN*, T3SS mutant; pEscN, complemented T3SS mutant; Δ *fimA*, T1P mutant. pFimA, complemented T1P mutant.

doi:10.1371/journal.pone.0171385.g005

2eae::Kn) was non-invasive [21]. Later on, we demonstrated that, in contrast with the wild type 1551–2 strain that displayed a localized pattern of adherence (formation of compact bacterial clusters) in HeLa cells, its T3SS-mutant adhered weakly, while the intimin mutant adhered, showing a T3SS-dependent diffuse pattern of adherence [36]. In addition, Pacheco et al., 2014 [24] showed that the 1551–2 strain invades, persists and multiplies inside differentiated Caco-2 cells up to 48 h.

In this work, we demonstrated for the first time that intimin, Tir and T3SS are essential for invasion of enterocytes *in vitro*, since mutations in the corresponding genes abolished bacterial uptake. Bacterial adherence was preserved in mutants, including the T3SS mutant, which did not adhere on HeLa cells in a previous study [36]. This fact might be due to the interaction between either intimin or T1P and Caco-2 cell surface receptors. It has been previously demonstrated that Tir and Map, and EspF can induce tEPEC invasion of HeLa and Caco-2 cells, respectively [49,50].

We have previously shown that an aEPEC strain, 1711–4, is able to translocate across the rat gastrointestinal barrier and be isolated from the MLN, spleen and liver [51]. The mechanisms promoting this bacterial translocation, however, are unknown. Generally, studies on colonization and infection by enteropathogens are conducted with Caco-2 cells, but although this cell line mimics enterocytes from the human small intestine, it does not represent the complex intestinal mucosa, since it is devoid of the mucosal layer and other intestinal cell types. It was demonstrated that EHEC [52] as well as tEPEC E2348/69 [44] colonize human IVOC. More recently, Etienne-Mesmin et al., [53] demonstrated that EHEC colonize and translocate into ileum fragments from mice, where Peyer’s patches are available, but quantification was not performed. In the present study, we evaluated *E. albertii* capacity to colonize the rat intestinal mucosa in the IVOC model, to mimicry the first steps that lead to bacterial translocation from the intestinal lumen to the extra-intestinal sites demonstrated *in vivo*. We showed for the first time the interaction of *E. albertii* with rat intestinal mucosa *ex vivo*, which

could be an alternative model to study AE-producing pathogens' interaction with more complex intestinal tissues. In this model, colonization was detected after 30 min of infection, and invasiveness was revealed after 2 h, when *E. albertii* 1551–2 could be found inside the enterocytes. Additionally, we demonstrated that *E. albertii* adherence to the rat IVOC depends on T3SS, as previously demonstrated in human IVOC for tEPEC E2348/69 [44], but not on intimin, Tir or T1P, since in the absence of these genes, bacterial adherence was qualitatively and quantitatively preserved. Thus, the use of this model may optimize the selection of potentially invasive strains to be tested *in vivo*, thus reducing the number of animals used to assess the fate of invasive *E. albertii* from the intestinal lumen to extra-intestinal sites.

We selected the T3SS mutant to compare to the wild type strain, since this mutant strain had previously shown a significantly reduced capacity to interact with the host epithelium in an *ex vivo* model, losing the capacity to invade cultured intestinal cells *in vitro*.

It has been reported that some *E. albertii* strains isolated from birds are able to adhere and to invade HEp-2 cells [54] and to reach the liver and spleen of one day-old chicks *in vivo*, possibly by disrupting the intestinal barrier, despite the minor intestinal mucosa alterations [54]. In this study, using an *in vivo* bacterial translocation assay in rats, we recovered the *E. albertii* 1551–2 strain in the MLN and liver but not spleen, while the T3SS mutant completely lost translocation capacity. It has been reported that T3SS-dependent effectors such as EspF, Map and NleA disrupt tight junctions that contribute to the integrity of the intestinal barrier [55–57]. In addition, some infectious processes can disturb the intestinal epithelium, for example, neutrophil migration during inflammation; this event promotes a transitory epithelial barrier destabilization, which exposes the basolateral side, either allowing enterocyte invasion [58] or offering an alternative route for bacterial translocation from the intestinal lumen to extra-intestinal niches.

Based on our finding that *E. albertii* 1551–2 can reach the MLN and liver *in vivo* and that the invasion level through the basolateral surface is higher than at the apical surface of T84 cell monolayers [22], we investigated how *E. albertii* might cross the intestinal epithelium. It is well known that enteropathogens can reach basolateral receptors and promote enterocyte invasion *in vivo* by transcytosis through M cells [25,59]. According to Hase and coworkers [28], bacterial translocation depends on T1P-GP2 interaction, since isogenic mutant or non-T1P producer strains were unable to translocate through M-like cells. On the other hand, Inman and Cantey [60] described that a rabbit EPEC strain (RDEC-1) produced AE lesion on the M cell membrane, suggesting that AE lesions could prevent bacterial internalization, thus preventing transcytosis and antigen presentation, thereby delaying the immune response.

In this study, *E. albertii* 1551–2 translocation was significantly more effective through M-like cells than Caco-2 cells only. This could not be observed with tEPEC as previously demonstrated by [46]. We also demonstrated that translocation depended on functional T3SS, and that T1P mutation did not compromise bacterial translocation, contrary to what was found by Hase et al., [28]. These differences could be due to allelic FimH alterations in T1P in different strains. Therefore, these data suggest that *E. albertii* 1551–2 may reach the enterocyte basolateral surface *in vivo* after M cell translocation. Etienne-Mesmin et al., [53] also found that EHEC O157:H7 and O113:H2 and their respective intimin and Shiga toxin mutants translocated more effectively through M-like cells in comparison with Caco-2 cells. Cieza et al., [61] reported that the translocation of adherent-invasive *E. coli* (AIEC) through M-like cells depends on IbeA (an invasin); however, *E. albertii* strain 1551–2 is devoid of the *ibeA* gene (not shown) and other invasion-related genes [62], reinforcing that the bacterial translocation ability of this *E. albertii* strain is due to intimin-Tir interaction.

Altogether, our results demonstrated for the first time that both *ex vivo* and *in vivo* bacterial infection of rat intestinal mucosa are useful models to study *E. albertii* interaction with the

host. We also showed that *E. albertii* 1551–2 may also cross the intestinal mucosa *in vivo* possibly using M cells as a route to reach extra-intestinal organs.

Supporting Information

S1 Fig. Light microscopy of rat intestinal mucosa prior to *ex vivo* infection. Ileal fragments (approx. 0.5 cm²) were stained with Methylene blue to confirm, by the presence of neurons from the myenteric plexus (Black arrows), that all tissue layers (as indicated) are well preserved. Magnification 400x.

(TIF)

S2 Fig. IVOC and *E. albertii* isogenic mutants colonization assays. Ileal fragments collected from 3 Wistar-EPM rats were inoculated with 10¹⁰ CFU of each bacterial strain and incubated for 2 h at 37°C. SEM: (A) intimin mutant, (B) Tir mutant (white arrow), (C) Type 1 pilus mutant. Bars: A and B = 5 μm; C = 200 nm.

(TIF)

S3 Fig. Caco-2 cells conversion into M-like cells and measurement of Transepithelial Electrical Resistance during *E. albertii* infection. To verify the M-like cell conversion, immunofluorescence staining was performed to detect Galectin-9 (green): A) NIH 3T3 cells (positive control), B) Differentiated Caco-2 cells, C) M-like cells. D-E, a monolayer containing M-like cells infected with *E. albertii* 1551–2 strain immunostained for Galectin-9 (red), combined with phalloidin-FITC and DAPI, for actin (green) and nucleic acid (blue), respectively. D) Merge image of actin and nucleic acid: insert (red square) indicates zoom of the white square area, showing actin accumulation underneath bacterial adhesion sites, E) Merge image of Galectin-9 and nucleic acid: insert (red square) indicates zoom of the white square area, showing bacterial adhesion site (Original Magnification: A-C 1,000x and D-E 400x). F) Transepithelial Electrical Resistance of monolayer containing M-like cells during infection with the 1551–2 strain. Non-significant differences were observed ($p > 0.05$).

(TIF)

S4 Fig. TEM of M-like and Caco-2 cells infected with T3SS mutant and complemented *E. albertii* strain 1551–2. After infection of M-like (A) and Caco-2 (B) cells with mutant strain, none or rare bacteria were observed adhered to the host cell surface. Complementated strain infecting M-like cells (C) presented intimate adherence and pedestal formation (AE lesion—arrow), while the same strain infecting Caco-2 cells (D) loosely adhered to the tips of cells microvilli. Bars, 1 μm.

(TIF)

Author contributions

Conceptualization: DY.

Formal analysis: DY.

Funding acquisition: DY TATG.

Investigation: DY AMAL CMA RBS FTR VS IHK.

Methodology: DY IHK.

Project administration: DY.

Resources: TATG IHK.

Supervision: TATG.

Validation: DY.

Writing – original draft: DY RTH TATG.

Writing – review & editing: DY RTH CMA IHK TATG.

References

- Oaks JL, Besser TE, Walk ST, Gordon DM, Beckmen KB, Burek KA, et al. *Escherichia albertii* in wild and domestic birds. *Emerg Infect Dis*. 2010;
- Ooka T. Human Gastroenteritis Outbreak Associated with *Escherichia albertii*, Japan. *Emerg Infect Dis*. 2013; 19.
- Huys G, Cnockaert M, Janda JM, Swings J. *Escherichia albertii* sp. nov., a diarrhoeagenic species isolated from stool specimens of Bangladeshi children. *Int J Syst Evol Microbiol*. 2003; 53: 807–810. doi: [10.1099/ijs.0.02475-0](https://doi.org/10.1099/ijs.0.02475-0) PMID: [12807204](https://pubmed.ncbi.nlm.nih.gov/12807204/)
- Albert MJ, Faruque SM, Ansaruzzaman M, Islam MM, Haider K, Alam K, et al. Sharing of virulence-associated properties at the phenotypic and genetic levels between enteropathogenic *Escherichia coli* and *Hafnia alvei*. *J Med Microbiol*. 1992;
- Abbott SL, O'Connor J, Robin T, Zimmer BL, Janda JM. Biochemical properties of a newly described *Escherichia* species, *Escherichia albertii*. *J Clin Microbiol*. 2003;
- Ooka T, Seto K, Kawano K, Kobayashi H, Etoh Y, Ichihara S, et al. Clinical significance of *Escherichia albertii*. *Emerg Infect Dis*. 2012; 18: 488–492. doi: [10.3201/eid1803.111401](https://doi.org/10.3201/eid1803.111401) PMID: [22377117](https://pubmed.ncbi.nlm.nih.gov/22377117/)
- Girón JA, Ho AS, Schoolnik GK. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. *Science*. 1991; 254: 710–713. PMID: [1683004](https://pubmed.ncbi.nlm.nih.gov/1683004/)
- Donnenberg MS, Kaper JB. Enteropathogenic *Escherichia coli*. *Infect Immun*. 1992; 60: 3953–3961. PMID: [1398907](https://pubmed.ncbi.nlm.nih.gov/1398907/)
- Trabulsi LR, Keller R, Gomes TAT. Typical and Atypical Enteropathogenic *Escherichia coli*. *Emerg Infect Dis*. 2002; 8: 508–513. doi: [10.3201/eid0805.010385](https://doi.org/10.3201/eid0805.010385) PMID: [11996687](https://pubmed.ncbi.nlm.nih.gov/11996687/)
- Hernandes RT, Elias WP, Vieira MAM, Gomes TAT. An overview of atypical enteropathogenic *Escherichia coli*. *FEMS Microbiol Lett*. 2009; 297: 137–149. doi: [10.1111/j.1574-6968.2009.01664.x](https://doi.org/10.1111/j.1574-6968.2009.01664.x) PMID: [19527295](https://pubmed.ncbi.nlm.nih.gov/19527295/)
- Jerse A, Yu J, Tall BD, Kaper JB. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proc Natl Acad Sci U S A*. 1990; 87: 7839–7843. PMID: [2172966](https://pubmed.ncbi.nlm.nih.gov/2172966/)
- Garmendia J, Frankel G C V. Enteropathogenic and Enterohemorrhagic *Escherichia coli* Infections: *Infect Immun*. 2005; 73: 2573–2585. doi: [10.1128/IAI.73.5.2573-2585.2005](https://doi.org/10.1128/IAI.73.5.2573-2585.2005) PMID: [15845459](https://pubmed.ncbi.nlm.nih.gov/15845459/)
- Croxen M a, Finlay BB. Molecular mechanisms of *Escherichia coli* pathogenicity. *Nat Rev Microbiol*. 2010; 8: 26–38. doi: [10.1038/nrmicro2265](https://doi.org/10.1038/nrmicro2265) PMID: [19966814](https://pubmed.ncbi.nlm.nih.gov/19966814/)
- Moon HW, Whipp SC, Argenzio RA, Giannella RA. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. 1983; 41: 1340–1351. PMID: [6350186](https://pubmed.ncbi.nlm.nih.gov/6350186/)
- Knutton S, Baldwin T, Willians PH M A. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect Immun*. 1989; 57: 1290–1298. PMID: [2647635](https://pubmed.ncbi.nlm.nih.gov/2647635/)
- Navarro-Garcia F, Serapio-Palacios A, Ugalde-Silva P, Tapia-Pastrana G, Chavez-Dueñas L. Actin cytoskeleton manipulation by effector proteins secreted by diarrheagenic *Escherichia coli* pathotypes. *Biomed Res Int*. 2013; 2013: 374395. doi: [10.1155/2013/374395](https://doi.org/10.1155/2013/374395) PMID: [23509714](https://pubmed.ncbi.nlm.nih.gov/23509714/)
- Elliott SJ, Kaper JB. Role of type 1 fimbriae in EPEC infections. *Microb Pathog*. 1997; 23: 113–8. doi: [10.1006/mpat.1997.0135](https://doi.org/10.1006/mpat.1997.0135) PMID: [9245623](https://pubmed.ncbi.nlm.nih.gov/9245623/)
- Hernandes RT, Velsko I, Sampaio SCF, Elias WP, Robins-Browne RM, Gomes TAT, et al. Fimbrial adhesins produced by atypical enteropathogenic *Escherichia coli* strains. *Appl Environ Microbiol*. 2011; 77: 8391–8399. doi: [10.1128/AEM.05376-11](https://doi.org/10.1128/AEM.05376-11) PMID: [21926222](https://pubmed.ncbi.nlm.nih.gov/21926222/)
- Romão FT, Hernandez RT, Yamamoto D, Osgui L, Popi A, Gomes TAT. Influence of environmental factors in the adherence of an atypical enteropathogenic *Escherichia coli* strain to epithelial cells. *BMC Microbiol*. 2014; 14: 299. doi: [10.1186/s12866-014-0299-y](https://doi.org/10.1186/s12866-014-0299-y) PMID: [25527183](https://pubmed.ncbi.nlm.nih.gov/25527183/)
- Rosa ACP, Vieira MAM, Tibana A, Gomes TAT, Andrade JRC. Interactions of *Escherichia coli* strains of non-EPEC serogroups that carry eae and lack the EAF and stx gene sequences with undifferentiated

- and differentiated intestinal human Caco-2 cells. FEMS Microbiol Lett. 2001; 200: 117–122. PMID: [11410359](#)
21. Hernandez RT, Silva RM, Carneiro SM, Salvador FA, Fernandes MCDC, Padovan ACB, et al. The localized adherence pattern of an atypical enteropathogenic *Escherichia coli* is mediated by intimin omicron and unexpectedly promotes HeLa cell invasion. Cell Microbiol. 2008; 10: 415–425. doi: [10.1111/j.1462-5822.2007.01054.x](#) PMID: [17910741](#)
 22. Yamamoto D, Hernandez RT, Blanco M, Greune L, Schmidt MA, Carneiro SM, et al. Invasiveness as a putative additional virulence mechanism of some atypical Enteropathogenic *Escherichia coli* strains with different uncommon intimin types. BMC Microbiol. 2009; 9: 146. doi: [10.1186/1471-2180-9-146](#) PMID: [19622141](#)
 23. Sampaio SCF, Andrade JRC, Sampaio JLM, Carneiro CRW, Freymüller E, Gomes TAT. Distinct Interaction of Two Atypical Enteropathogenic *Escherichia coli* Strains with Enterocytes In Vitro. Open Microbiol J. 2011; 5: 65–71. doi: [10.2174/1874285801105010065](#) PMID: [21792379](#)
 24. Pacheco VCR, Yamamoto D, Abe CM, Hernandez RT, Mora A, Blanco J, et al. Invasion of differentiated intestinal Caco-2 cells is a sporadic property among atypical enteropathogenic *Escherichia coli* strains carrying common intimin subtypes. Pathog Dis. 2014; 70: 167–175. doi: [10.1111/2049-632X.12112](#) PMID: [24339197](#)
 25. Grutzkau A, Hanski C, Hahn H, Riecken EO. Involvement of M cells in the bacterial invasion of Peyer's patches: a common mechanism shared by *Yersinia enterocolitica* and other enteroinvasive bacteria. Gut. 1990; 31: 1011–1015. PMID: [2210445](#)
 26. Corr SC, Gahan CCGM, Hill C. M-cells: Origin, morphology and role in mucosal immunity and microbial pathogenesis. FEMS Immunol Med Microbiol. 2008; 52: 2–12. doi: [10.1111/j.1574-695X.2007.00359.x](#) PMID: [18081850](#)
 27. Gill N, Wlodarska M, Finlay BB. Roadblocks in the gut: Barriers to enteric infection. Cell Microbiol. 2011; 13: 660–669. doi: [10.1111/j.1462-5822.2011.01578.x](#) PMID: [21392202](#)
 28. Hase K, Kawano K, Nochi T, Pontes GS, Fukuda S, Ebisawa M, et al. Uptake through glycoprotein 2 of FimH(+) bacteria by M cells initiates mucosal immune response. Nature. Nature Publishing Group; 2009; 462: 226–30.
 29. Sansonetti PJ, Phalipon A. M cells as ports of entry for enteroinvasive pathogens: mechanisms of interaction, consequences for the disease process. Semin Immunol. 1999; 11: 193–203. doi: [10.1006/smim.1999.0175](#) PMID: [10381865](#)
 30. Sansonetti PJ. War and peace at mucosal surfaces. Nat Rev Immunol. 2004; 4: 953–964. doi: [10.1038/nri1499](#) PMID: [15573130](#)
 31. Sansonetti PJ. Rupture, invasion and inflammatory destruction of the intestinal barrier by *Shigella*: The yin and yang of innate immunity. Can J Infect Dis Med Microbiol. 2006; 17: 117–119. PMID: [18418486](#)
 32. Ooka T, Ogura Y, Katsura K, Seto K, Kobayashi H, Kawano K, et al. Defining the genome features of *Escherichia albertii*, an emerging enteropathogen closely related to *Escherichia coli*. Genome Biol Evol. 2015; 7: evv211.
 33. Law RJ, Gur-arie L, Rosenshine I, Finlay BB, Behnsen J, Deriu E, et al. In Vitro and In Vivo Model Systems for Studying Enteropathogenic *Escherichia coli* Infections. 2013; doi: [10.1101/cshperspect.a009977](#) PMID: [23457294](#)
 34. Dupont A, Sommer F, Zhang K, Repnik U, Basic M, Bleich A, et al. Age-Dependent Susceptibility to Enteropathogenic *Escherichia coli* (EPEC) Infection in Mice. PLOS Pathog. 2016; 12: e1005616. doi: [10.1371/journal.ppat.1005616](#) PMID: [27159323](#)
 35. Chaveronche MK, Ghigo JM, d'Enfert C. A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*. Nucleic Acids Res. 2000; 28: E97. PMID: [11071951](#)
 36. Hernandez RT, De la Cruz MA, Yamamoto D, Girón JA, Gomes TAT. Dissection of the role of pili and type 2 and 3 secretion systems in adherence and biofilm formation of an atypical enteropathogenic *Escherichia coli* strain. Infect Immun. 2013; 81: 3793–3802. doi: [10.1128/IAI.00620-13](#) PMID: [23897608](#)
 37. Gauthier A, Finlay BB. Translocated Intimin Receptor and Its Chaperone Interact with ATPase of the Type III Secretion Apparatus of Enteropathogenic *Escherichia coli*. J Bacteriol. 2003; 185: 6747–6755. doi: [10.1128/JB.185.23.6747-6755.2003](#) PMID: [14617638](#)
 38. Levine MM, Nataro JP, Karch H, Baldini MM, Kaper JB, Black RE, et al. The diarrheal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. J Infect Dis. 1985; 152: 550–559. PMID: [2863318](#)
 39. Koh IH, Silva R. Novel in vitro small intestinal graft model for study of bacterial translocation in the rat. Transpl Proc. 1996; 28: 2667–2668.

40. Boyer HW, Roulland-Dussoix D. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J Mol Biol.* 1969; 41: 459–472. PMID: [4896022](#)
41. Luck SN, Bennett-Wood V, Poon R, Robins-Browne RM, Hartland EL. Invasion of epithelial cells by locus of enterocyte effacement-negative enterohemorrhagic *Escherichia coli*. *Infect Immun.* 2005; 73: 3063–3071. doi: [10.1128/IAI.73.5.3063-3071.2005](#) PMID: [15845514](#)
42. Gannon VP, Rashed M, King RK, Thomas EJ. Detection and characterization of the *eae* gene of Shiga-like toxin-producing *Escherichia coli* using polymerase chain reaction. *J Clin Microbiol.* 1993; 31: 1268–1274. PMID: [8501228](#)
43. Deng W, Li Y, Vallance BA, Finlay BB. Locus of enterocyte effacement from *Citrobacter rodentium*: Sequence analysis and evidence for horizontal transfer among attaching and effacing pathogens. *Infect Immun.* 2001; 69: 6323–6335. doi: [10.1128/IAI.69.10.6323-6335.2001](#) PMID: [11553577](#)
44. Shaw RK, Cleary J, Murphy MS, Frankel G, Knutton S. Interaction of Enteropathogenic *Escherichia coli* with Human Intestinal Mucosa: Role of Effector Proteins in Brush Border Remodeling and Formation of Attaching and Effacing Lesions. *Infect Immun.* 2005; 73: 1243–1251. doi: [10.1128/IAI.73.2.1243-1251.2005](#) PMID: [15664974](#)
45. Kernéis S, Bogdanova A, Kraehenbuhl JP, Pringault E. Conversion by Peyer's patch lymphocytes of human enterocytes into M cells that transport bacteria. *Science.* 1997; 277: 949–952. PMID: [9252325](#)
46. Martinez-Argudo I, Sands C, Jepson M. Translocation of enteropathogenic *Escherichia coli* across an in vitro M cell model is regulated by its type III secretion system. *Cell Microbiol.* 2007; 9: 1538–1546. doi: [10.1111/j.1462-5822.2007.00891.x](#) PMID: [17298392](#)
47. Pielage JF, Cichon C, Greune L, Hirashima M, Kucharzik T, Schmidt MA. Reversible differentiation of Caco-2 cells reveals galectin-9 as a surface marker molecule for human follicle-associated epithelia and M cell-like cells. *Int J Biochem Cell Biol.* 2007; 39: 1886–1901. doi: [10.1016/j.biocel.2007.05.009](#) PMID: [17596995](#)
48. Knutton S. Electron microscopical methods in adhesion. *Methods Enzymol.* 1995; 253: 145–158. PMID: [7476383](#)
49. Jepson MA, Pellegrin S, Peto L, Banbury DN, Leard AD, Mellor H, et al. Synergistic roles for the Map and Tir effector molecules in mediating uptake of enteropathogenic *Escherichia coli* (EPEC) into non-phagocytic cells. *Cellular Microbiology.* 2003.
50. Weflen AW, Alto NM, Viswanathan VK, Hecht G. *E. coli* secreted protein F promotes EPEC invasion of intestinal epithelial cells via an SNX9-dependent mechanism. *Cell Microbiol.* 2010; 12: 919–929. doi: [10.1111/j.1462-5822.2010.01440.x](#) PMID: [20088948](#)
51. Liberatore AMA, Moreira FC, Gomes TAT, Menchaca-Diaz JL, Koh IHJ. Typical and atypical enteropathogenic *Escherichia coli* bacterial translocation associated with tissue hypoperfusion in rats. *Brazilian J Med Biol Res.* 2011; 44: 1018–1024.
52. Fitzhenry RJ, Reece S, Trabulsi LR, Heuschkel R, Murch S, Thomson M, et al. Tissue tropism of enteropathogenic *Escherichia coli* strains belonging to the O55 serogroup. *Infect Immun.* 2002; 70: 4362–4368. doi: [10.1128/IAI.70.8.4362-4368.2002](#) PMID: [12117946](#)
53. Etienne-Mesmin L, Chassaing B, Sauvanet P, Denizot J, Blanquet-Diot S, Darfeuille-Michaud A, et al. Interactions with M cells and macrophages as key steps in the pathogenesis of enterohemorrhagic *Escherichia coli* infections. *PLoS One.* 2011; 6.
54. La Ragione RM, McLaren IM, Foster G, Cooley WA, Woodward MJ. Phenotypic and genotypic characterization of avian *Escherichia coli* O86:K61 isolates possessing a gamma-like intimin. *Appl Environ Microbiol.* 2002;
55. McNamara BP, Koutsouris A, O'Connell CB, Nougayréde J, Sonnenberg MS, Hecht G. Translocated EspF protein from enteropathogenic *Escherichia coli* disrupts host intestinal barrier function. *J Clin Invest.* 2001; 107: 621–629. doi: [10.1172/JCI11138](#) PMID: [11238563](#)
56. Peralta-Ramírez J, Hernandez M, Manning-Cela R, Luna-Muñoz J, Garcia-Tovar C, Nougayréde JP, et al. EspF interacts with nucleation-promoting factors to recruit junctional proteins into pedestals for pedestal maturation and disruption of paracellular permeability. *Infect Immun.* 2008; 76: 3854–3868. doi: [10.1128/IAI.00072-08](#) PMID: [18559425](#)
57. Wong ARC, Pearson JS, Bright MD, Munera D, Robinson KS, Lee SF, et al. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: even more subversive elements. *Mol Microbiol.* 2011; 80: 1420–38. doi: [10.1111/j.1365-2958.2011.07661.x](#) PMID: [21488979](#)
58. McCormick THA, Siber AM, Maurelli AT. Requirement of the *Shigella flexneri* virulence plasmid in the ability to induce trafficking of neutrophils across polarized monolayers of the intestinal epithelium. *Infect Immun.* 1998; 66: 4237–4243. PMID: [9712773](#)
59. Kraehenbuhl J-P, Neutra MR. Epithelial M Cells: Differentiation and Function. *Annu Rev Cell Dev Biol.* 2000; 16: 301–332. doi: [10.1146/annurev.cellbio.16.1.301](#) PMID: [11031239](#)

60. Inman LR, Cantey JR. Specific adherence of *Escherichia coli* (strain RDEC-1) to membranous (M) cells of the Peyer's patch in *Escherichia coli* diarrhea in the rabbit. *J Clin Invest*. 1983; 71: 1–8. doi: [10.1172/JCI110737](https://doi.org/10.1172/JCI110737) PMID: [6129261](https://pubmed.ncbi.nlm.nih.gov/6129261/)
61. Cieza RJ, Hu J, Ross BN, Sbrana E, Torres AG. IbeA, the invasin of Adherent-Invasive *Escherichia coli* (AIEC) mediates interaction with intestinal epithelia and macrophages. *Infect Immun*. 2015; 83: 1904–1918. doi: [10.1128/IAI.03003-14](https://doi.org/10.1128/IAI.03003-14) PMID: [25712929](https://pubmed.ncbi.nlm.nih.gov/25712929/)
62. Vieira M, Andrade J, Trabulsi L, Rosa A, Dias A, Ramos S, et al. Phenotypic and genotypic characteristics of *Escherichia coli* strains of non-enteropathogenic *E. coli* (EPEC) serogroups that carry EAE and lack the EPEC adherence factor and Shiga toxin DNA probe sequences. *J Infect Dis*. 2001; 183: 762–772. doi: [10.1086/318821](https://doi.org/10.1086/318821) PMID: [11181153](https://pubmed.ncbi.nlm.nih.gov/11181153/)