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Bacteroides fragilis prevents *Salmonella* Heidelberg translocation in co-culture model mimicking intestinal epithelium

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RESEARCH ARTICLE

Abstract

Salmonella Heidelberg is one of the most common serovar causing foodborne illnesses. To limit the development of digestive bacterial infection, food supplements containing probiotic bacteria can be proposed. Commensal non-toxigenic *Bacteroides fragilis* has recently been suggested as a next-generation probiotic candidate. By using an original triple co-culture model including Caco-2 cells (representing human enterocytes), HT29-MTX (representing mucus-secreting goblet cells), and M cells differentiated from Caco-2 by addition of Raji B lymphocytes, bacterial translocation was evaluated. The data showed that *S.* Heidelberg could translocate in the triple co-culture model with high efficiency, whereas for *B. fragilis* a weak translocation was obtained. When cells were exposed to both bacteria, *S.* Heidelberg translocation was inhibited. The cell-free supernatant of *B. fragilis* also inhibited *S.* Heidelberg translocation without impacting epithelial barrier integrity. This supernatant did not affect the growth of *S.* Heidelberg. The non-toxigenic *B. fragilis* confers health benefits to the host by reducting bacterial translocation. These results suggested that the multicellular model provides an efficient *in vitro* model to evaluate the translocation of pathogens and to screen for probiotics that have a potential inhibitory effect on this translocation.

Keywords: *Bacteroides*, M cells, enterocytes, goblet cells, gut permeability

1. Introduction

The intestinal epithelium is composed of multiple cell types including enterocytes, goblet cells and specialised epithelial cells called M cells. Enterocytes are responsible for nutrient absorption and are characterised by the presence of brush borders associated to sucrase-isomaltase (SI), a glycoprotein hydrolase (Kenny and Maroux, 1982). Goblet cells secrete mucins, predominantly mucin-2 (Muc2) forming a barrier between the epithelial cells monolayer and the luminal content that does not consist only of nutrients, but is also loaded with potentially pathogenic microorganisms (McGuckin *et al.,* 2011). M cells are implicated in the uptake and eventual presentation of luminal antigens to the immune system (Kanaya *et al.,* 2020). These intestinal cells are interconnected by a set of apical proteins called tight junctions, essential for the integrity of barrier function (Groschwitz and Hogan, 2009). Occludin and the intracellular adapter proteins zonula occludens (ZO-1) are the main proteins of tight junctions (Bauer *et al.,* 2010).

In vitro intestinal models are developed to facilitate the study of intestinal function and disease (Costa and Ahluwalia, 2019; Mattei *et al.,* 2014). Caco-2 cells are the most widely used cell model. Caco-2 cell differentiation starts when the cells achieve confluence, around 7 days after seeding and is completed within 21 days. That is when the cells are polarised and connected to each other through tight junctions, exhibiting an apical brush border structure with the expression of several enzymes, transporters and receptors (Antunes *et al.,* 2013). Nevertheless, the Caco-2 monoculture does not contain other important factors that influence the functionality of enterocytes. Models composed of a combination of the different intestinal

epithelial cells are needed. Models consisting of a co-culture of Caco-2 and mucus-producing HT29 cell lines are developed (Béduneau *et al.,* 2014). Schimpel *et al.* (2014) have also reported that Raji B cells can promote M cell phenotype in some Caco-2 cells which strongly impacted nanoparticle uptake.

The surface of the intestinal epithelium can be exposed to a large variety of pathogens which can gain access to the lamina propria. Thus, bacteria have been observed within the basal paracellular space of polarised enterocyte monolayers (Nazli *et al.,* 2004, 2006). Translocation of bacteria across the intestinal epithelium may occur via a transcellular route, involving an endocytic uptake followed by intracellular trafficking. In addition, the translocation of some members of the intestinal microbiota can occur through M cells which possess a high phagocytic and transcytotic capacity (Rios *et al.,* 2016). Several pathogens, such as *Salmonella*, *Shigella*, and *Yersinia* exploit M cells to invade mucosal tissues and cross the digestive epithelial barrier before reaching the bloodstream (Westphal *et al.,* 2008).

Among *Salmonella enterica,* the *Salmonella* Heidelberg (*S.* Heidelberg) is one of the most common serovar causing severe extra-intestinal infections (Wilmshurst and Sutcliffe, 1995). Within the natural population of *S.* Heidelberg, some strains display a hypermutator phenotype related to the frequent occurrence of mutations in the genes involved in methyl mismatch repair system (Le Gall *et al.,* 2009; Le Gall-David *et al.,* 2015). The hypermutator phenotype allows bacteria to adapt to adverse and stringent environmental conditions including the pressure of antibiotic exposure (Blázquez, 2003). Moreover, some hypermutator bacteria are multidrug-resistant, hence, there is an urgent need to develop new therapeutic alternatives.

The use of probiotics has been suggested as a potential new strategy to limit the development and/or severity of digestive bacterial infection by decreasing pathogen load (Fijan, 2014). Probiotics are typically, although not necessarily, commensal bacteria. They are generally lactic acid bacteria, most commonly *Lactobacilli* and *Bifidobacteria* species, although *Lactococcus*, *Streptococcus*, and *Enterococcus* species, as well as some non-pathogenic *Escherichia* coli strains, are also known probiotics (Chang *et al.,* 2019; Erturk-Hasdemir and Kasper, 2018). As a prominent species of the genus *Bacteroides* in the phylum *Bacteroidetes*, *Bacteroides fragilis* is an important anaerobic gut commensal (Wexler, 2007), despite the fact that some enterotoxigenic *B. fragilis* strains possess a *B. fragilis* pathogenicity island (BfPAI). Recently, non-toxigenic *B. fragilis* (NTBF) was shown to have powerful health benefits to the host and was recommended as a probiotic candidate (Deng *et al.,* 2016; Wang *et al.,* 2017).

Our aim in this study was to evaluate whether *B. fragilis*, a non-toxigenic strain, could be useful to limit the severity of the *S.* Heidelberg hypermutator phenotype infection by analysing their impact on growth and mucosal translocation. For this purpose, we used a multi-cells model *in vitro* associating cells representing human enterocytes (Caco-2 cells), mucus-secreting goblet cells (HT29-MTX) and M cells.

2. Materials and methods

Bacteria and growth conditions

The non-toxigenic *B. fragilis* strain ATCC 25285 (Choi *et al.,* 2016; Sears *et al.,* 2014) was purchased from the American Type Culture Collection (Manassas, VI, USA). *B. fragilis* was isolated on Columbia blood agar and incubated at 37 °C in an anaerobic chamber (MAC500, Don Whitley Scientific, Bingley, UK) with 10% H_2, 10% CO_2 and 80% N_2 during 48 h. Then *B. fragilis* was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 20% of foetal bovine serum, 1% L-glutamine and 1% of L-cysteine, as well as reducing agents added in this medium to provide adequate anaerobiosis. After an incubation of 18 h, *B. fragilis* was centrifuged at 3,000×*g* for 5 min to separate supernatant and pellets. *B. fragilis* supernatant was filter sterilised through a 0.20 µm-pore-size syringe filter before using it on the multicellular model.

S. Heidelberg strain B182, with a hypermutator phenotype (deletion of 12 bp in *mutS*), was isolated on Luria-Bertani (LB) agar and grown in LB medium overnight at 37 °C. *S.* Heidelberg was then subcultured by dilution in fresh LB medium followed by incubation for 90 min at 37 °C (Le Bars *et al.,* 2012b). After that this culture was then centrifuged at 3,000×*g* for 15 min and the pellet was resuspended in DMEM with L-cysteine as *B. fragilis* before adding it to the apical side of the multicellular model.

Cell lines and growth culture

Caco-2 cells, obtained from American Type Culture Collection, were cultivated in DMEM supplemented with 20% foetal bovine serum, 1% L-glutamine and 1% penicillin and streptomycin. HT29-MTX cells were kindly provided by CRB CelluloNet (SFR Biosciences, CNRS UMS 3444, Inserm US 8, Université Claude-Bernard, Lyon, France) and were grown in the same medium as Caco-2 with only 10% of foetal bovine serum under a 5% CO_2 water-saturated atmosphere (Lesuffleur *et al.,* 1991). The Raji B cells (ECACC 85011429), issued from human Burkitt's lymphoma cell-line, were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% foetal bovine serum, 1% non-essential amino acids, 1% L-glutamine and 1% penicillin and streptomycin, at 37 °C in a 5% CO_2 water-saturated atmosphere.

Upon confluence, cells were harvested with trypsin-EDTA and a predetermined amount of cells of each type were mixed prior to seeding to yield cell ratio of 9/1 (Caco-2/HT29-MTX) on the apical chamber of polycarbonate Transwell® inserts (Corning Inc., Corming, NY, USA). After 14 days of culture, Raji B cells are added to the basolateral chamber to induce the differentiation of Caco-2 cells into M cells. Caco-2/HT29-MTX and Raji B co-culture were maintained for 7 days in DMEM.

Evaluation of bacterial translocation

To mimic the *in vivo* scenario of the gut lumen, diluted *S.* Heidelberg and *B. fragilis* were co-cultured with different proportions to obtain low and high numbers thereby simulating a typical number of microorganisms that would be ingested and may result in an infection. A mixture of *S.* Heidelberg (2×10^3 cfu) and *B. fragilis* (1.98×10^5 cfu) was prepared and applied simultaneously to the apical side of the Transwell system containing intestinal cells cultured after 21 days as described above. These bacteria were added at a multiplicity of infection of 10 bacteria/cells and incubated at 37 °C for 30 min or 3 h. Controls including *S.* Heidelberg or *B. fragilis* alone were used to infect the model.

To evaluate the adhesion of *Salmonella* alone or in the presence of *B. fragilis*, triple co-cultured model was incubated with these bacteria as described above. After 30 min of incubation, cells in apical surface were lysed with 0.2% of Triton in phosphate buffered saline (PBS) solution and plated on LB agar for bacterial enumeration (cfu). For invasion assays, the apical and basolateral surfaces were washed (3 times with PBS) and incubated 150 min with DMEM containing gentamicin (100 µ g/ml) to kill extracellular bacteria. Intracellular bacterial were released by 0.2% of Triton in PBS solution and the resulting suspension was plated onto LB agar for enumeration.

To evaluate the impact of *B. fragilis* supernatant on *S.* Heidelberg translocation, the volume of *B. fragilis* supernatant, corresponding to the volume used for *B. fragilis*, was added to *S.* Heidelberg in the upper compartment of the triple co-culture model for 3 h. Following incubation, basal and apical medium were separately collected and colony-forming unit enumeration was performed. *S.* Heidelberg and *B. fragilis* were respectively enumerated on LB agar and Columbia blood agars incubated during 48 h in an anaerobic chamber. The number of translocated bacteria recovered in the lower chambers was expressed as a ratio between this number and the number of bacteria counted in the upper chamber.

TEER measurements and paracellular permeability study

The integrity of the polarised epithelial co-culture (Caco-2/HT29-MTX/M cells) was evaluated by measuring the transepithelial electrical resistance (TEER) using an Ohm/voltmeter (EVOM2; World Precision Instruments, Sarasota, FL, USA). The resistance obtained from a cell-free culture insert was subtracted from the resistance measured across each well and resistance values were calculated in Ohms (Ω).cm^2 by multiplying the resistance values by the filter surface area.

The integrity of polarised cells was also checked by measuring the Lucifer yellow (LY) transport rate. Regarding the paracellular permeability study, LY solution was prepared in DMEM at 10 µM to be added to the apical side of the insert while only DMEM was added in the basolateral side. After incubation for the periods indicated for TEER study, the solution in the basal compartment was collected and the fluorescence intensity of LY was measured using POLARstar Omega Microplate Reader (Labtech GmbH, Ortenberg, Germany). Results were expressed in pmol/cm^2/s in kinetic differentiation or as percentage of LY permeability inhibition compared to insert without cells.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed on polarised cells after 21 days of growth on polycarbonate Transwell cell culture inserts. After several washings with PBS, samples were fixed for 2 h in room temperature in 2.5% glutaraldehyde dissolved in 0.1 M cacodylate, postfixed in 1% osmium tetroxide for 1 h at room temperature, rinsed in cacodylate buffer, and dehydrated in an ascending series of ethanol (70, 90 and 100%). The polycarbonate membrane contained in the inserts and on which the cells were grown was recovered, then cut into thin strips. Samples were then infiltrated with an ascending concentration of Epon resin in ethanol mixtures. Finally, they were placed in fresh Epon for several hours and then embedded in Epon for 48 h at 60 °C. Resin blocks were sectioned into 80 nm ultrathin sections using LEICA UC7 ultramicrotome (LEICA Systems, Vienna, Austria): cut sections were performed so that it was allowed to visualise transversally Transwell membrane with cells layer. These sections were mounted on copper grids and stained. Grids were observed using a TEM JEOL-JEM 1400 (JEOL Ltd, Tokyo, Japan) at an accelerating voltage of 120 kV and equipped with an Orius 1000 camera (Gatan Inc., Pleasanton, CA, USA).

Expression level of selected genes by RT-qPCR

After 21 days of co-culture, RNA was extracted from the upper chamber using Total RNA and Protein isolation kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions. Afterward, High-Capacity

cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to reverse-transcribe the RNA into cDNA. Then, the genes that were selected specifically for each cells were relatively quantified using StepOnePlus (Applied Biosystems) with the SYBR Green PCR Master Mix (Applied Biosystems) (Le Bars *et al.,* 2012b). Genes playing essential roles for each cell were selected: SI which is specific of Caco-2, mucin-2 (*muc2*) secreted by HT29-MTX, and glycoprotein 2 (*GP2*) associated to M cells. To investigate genes of tight-junction components, we selected occludin and zona occludens (ZO-1) genes.

Primers used for these selected genes were described in Table 1. Each gene was normalised to the TBP (TATA box binding protein) mRNA expression level before calculating the fold-change values. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Experiments were performed at least three times and data were analysed using Student's t-test. The data were presented as mean ± standard deviation and *P*-values less than 0.05 were considered as significant (* $P<0.05$; ** $P<0.01$; *** $P<0.001$).

3. Results

Characterisation of Caco-2/HT29-MTX/M cells co-culture

In this study, we used an original *in vitro* triple co-culture model composed of enterocytes (Caco-2), goblets cells (HT29-MTX) secreting mucus and M cells after 21 days of culture. We investigated the cell morphologies by TEM in a triple co-culture of 21 days in a Transwell membrane. TEM allowed to recognise M cells through the particular shape of their apical membrane exhibiting short and irregular microvilli, whereas Caco-2 cells showed a typical brush border (Figure 1A). TEM revealed that 7% of Caco-2 cells expressed characteristics of M-like in the triple co-culture model. Goblet cells, randomly distributed, were visualised by their typical mucus containing vesicles (Figure 1B). Figure 1B also shows that cells are linked together by tight junctions as indicated by black arrows.

To further characterise the triple co-culture model, we checked the level of expression of genes that are molecular markers for each cell type at the differentiation state (21 days). Muc2 expression, specific of goblet cells, was significantly upregulated in the double (Caco-2/HT29-MTX) and triple (Caco-2/HT29-MTX/M) co-culture models compared to Caco-2, 4.36±1.01 and 3.9±0.37 fold higher than Caco-2 respectively ($P<0.05$). However, no significant difference was observed between double and triple co-culture models (Figure 1C). The gene expression of the M-like cells markers GP2 increased significantly in the triple co-culture model, 9.6±0.85 fold higher than Caco-2 ($P<0.01$). This GP2 expression in the triple co-culture model was also significantly different from the double co-culture model ($P<0.01$). Concerning sucrase-somaltase (SI) mRNA relative expression, a decrease was observed in the triple co-culture compared to Caco-2 (0.6±0.3 fold), however, this difference was not significant.

To investigate the permeability of this *in vitro* triple co-culture model, we have evaluated the epithelial barrier integrity by measuring TEER. The TEER values increased significantly with time for all models compared to 3 days of culture ($P<0.05$) (Figure 2A). At the end of the 21 days of differentiation process, Caco-2 monoculture presented the highest value (390±37 $\Omega.cm^2$) followed by double co-culture (Caco-2/HT29-MTX) (364±20 $\Omega.cm^2$) while triple co-culture (Caco-2/HT29-MTX/RajiB) showed a value of 264±99 $\Omega.cm^2$. However, these values were not significantly different from Caco-2 alone at 21 days of differentiation. We have also analysed the expression of tight junction genes (Figure 1C). The results showed that the gene expression of ZO-1 and occludin in the double and triple co-culture model was not significantly different compared to Caco-2 after 21 days of culture. The differentiation of Caco-2 in M cells in the triple co-culture model did not impact ZO-1 and occludin expression.

The cell permeability was investigated by measuring the paracellular efflux of a fluorescent tracer, Lucifer yellow (LY), across our models (Figure 2B). After 7 days of culture,

Table 1. Primers used in this study.

Gene	Genes encoded	Left primer	Right primer
TBP	TATA box protein	CCGGAATCCCTATCTTTAGTCC	GGGTCAGTCCAGTGCCATAAG
Muc2	mucin-2	CAGCACCGATTGCTGACTTG	GCTGGTCATCTCAATGGCAG
IS	isomaltase sucrase	CATCCTACCATGTCAAGAGCCA	GCTTGTTAAGGTGGTCTGGTTT
GP2	glycoprotein 2	AATCAAGGCCAGGCTAGGTT	TGGCCAGGCTGATTTTGAAC
ZO-1	zonula occludens 1	ATCCCTCAAGGAGCCATTC	CACTTGTTTTGCCAGGTTTTA
Occludin	occludin	CCAATGTCGAGGAGTGGG	CGCTGCTGTAACGAGGCT

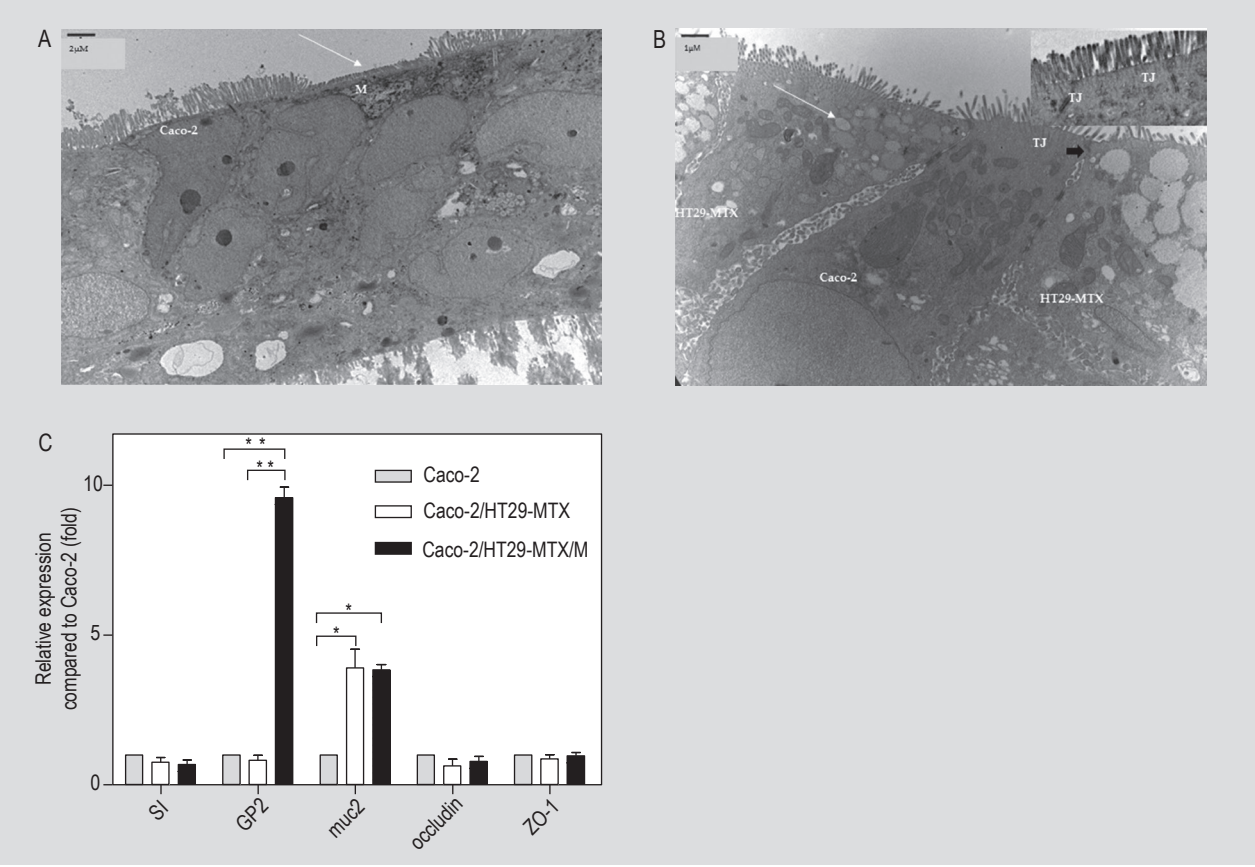

Figure 1. Epithelial cells differentiation after 21 days of culture. (A,B) Electron microscopy analysis of triple co-culture (HT29-MTX, Caco-2 and M cells: (A) Caco-2 and M cells; white arrows show M cells. (B) HT29-MTX and Caco-2; white arrows show mucus secretory vesicles and black arrows tight junctions (TJ). (C) Relative gene expression of markers of intestinal cells by real time PCR: the expression of mucin-2 (muc2) in HT29-MTX, sucrase isomaltase (SI) in Caco-2 and glycoprotein 2 (GP2) in M cells in double (Caco-2/HT29-MTX) or in triple co-culture (Caco-2/HT29-MTX/M) models compared to Caco-2. The expression of tight junctions (TJ) related genes, occludin and ZO-1, was also evaluated. The data are expressed as the mean fold change (± standard error of the mean) of three replicates across three independent experiments. * *P*<0.05; ** *P*<0.01.

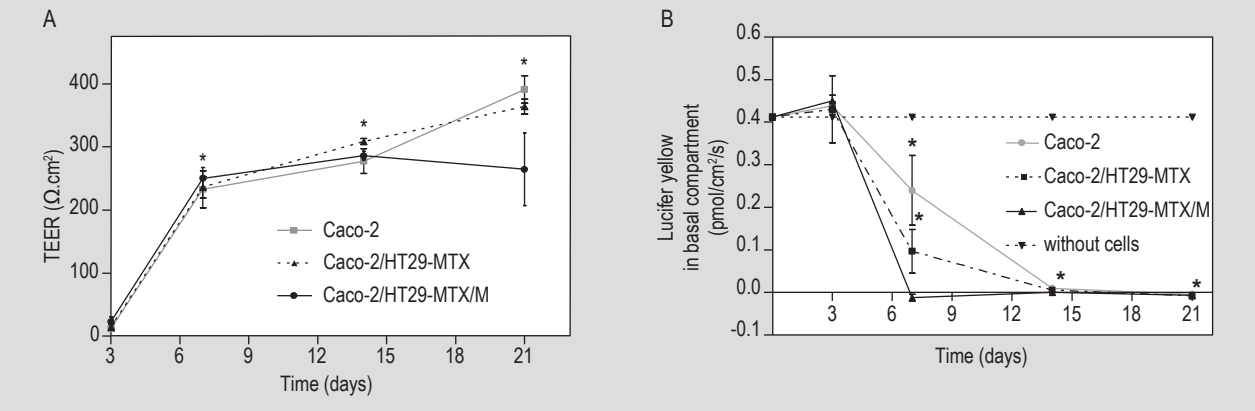

Figure 2. Epithelial integrity during cells differentiation of Caco-2, Caco-2/HT29 MTX (double co-culture), and Caco-2/HT29 MTX/M (triple co-culture). (A) Transepithelial electrical resistance (TEER) analysis; (B) Lucifer yellow (LY) permeability crossing different monolayers, expressed in pmol/cm^2/s in basal compartment (* *P*<0.05, compared to 3 days of culture).

LY permeability decreased significantly for all conditions compared to 3 days of culture (P<0.05). After 14 and 21 days of differentiation, there was no detectable amount of LY in the basal chamber whereas LY could be found in insert without cells (Figure 2B).

Bacteroides fragilis and *Salmonella* Heidelberg translocation across triple co-culture model

To evaluate the impact of M cells on bacterial translocation in the triple co-culture model, we evaluated the translocation rate of two different bacterial strains across this model. Those were a commensal, *B. fragilis* a non-toxigenic strain, and a pathogen, *S.* Heildeberg (Le Gall-David *et al.*, 2015). We compared data with measurement within the Caco-2 model and the double cell type model (Caco-2 and HT29-MTX without M Cells). For this purpose, we enumerated each strain of bacteria in the basal compartment. *S.* Heidelberg translocated with the highest efficiency across the triple co-culture model (5.9±1.9%) after 3 h of incubation whereas the translocation rate was 0.0003±0.00006% in Caco-2 alone and 0.002%±0.001 in the double co-culture model (Figure 3A). *S.* Heidelberg translocation in the triple co-culture model was significantly different from double co-culture and from Caco-2 (P<0.01). Concerning *B. fragilis,* the translocation was very weak through all cell models even in the triple co-culture model (0.005±0.006%) and no significant difference was determined compared to double co-culture or to Caco-2 infections (Figure 3B).

The impact of bacteria exposure on the integrity of the different models was evaluated by measuring TEER (Figure 3C). When the double and triple co-culture models were exposed to *B. fragilis*, no significant modification of the TEER was shown in double and triple co-culture with a TEER of 303±42 Ω.cm^2 and 327±9 Ω.cm^2 respectively, compared to the model without bacteria. However, in the presence of *S.* Heidelberg, TEER decreased significantly in double (160±36 Ω.cm^2, P<0.05) and triple co-culture (121±30 Ω.cm^2, P<0.001) compared to the models without bacteria (302±36 Ω.cm^2 and 296±9 Ω.cm^2 respectively). In the double co-culture model, TEER was of 160±36 Ω.cm^2 but only 0.002%±0.001 of *S.* Heidelberg translocated in basal compartment whereas in the triple co-culture model,

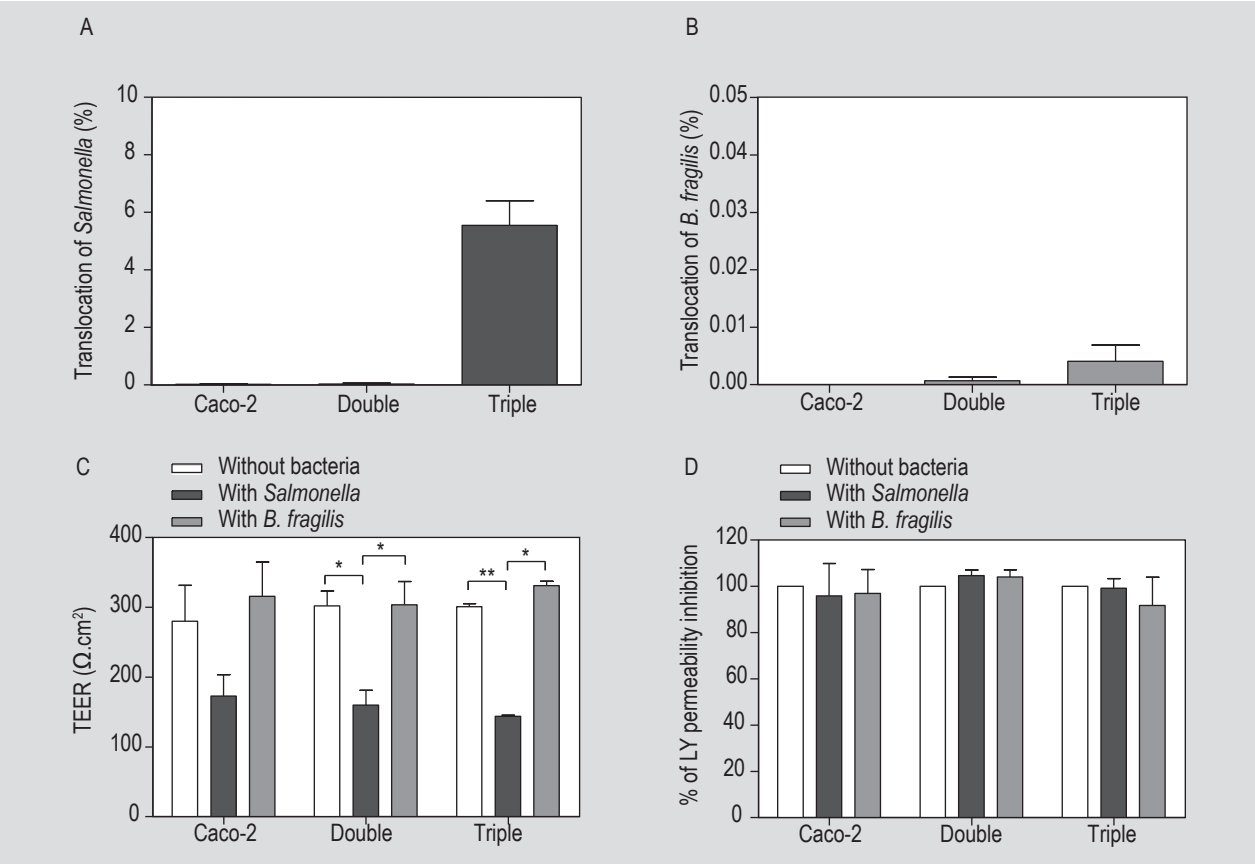

Figure 3. Interaction between the *in vitro* co-culture models (Caco-2, Caco-2/HT29 MTX (double) and Caco-2/HT29 MTX/M (triple)) and bacteria (*Salmonella* Heidelberg or *Bacteroides fragilis*). Translocation of (A) *Salmonella* and (B) *B. fragilis* in basal compartment. (C) Impact of *Salmonella* and *B. fragilis* on transepithelial electrical resistance (TEER). (D) Lucifer yellow (LY) permeability after 21 days of culture. * *P*<0.05, ** *P*<0.01.

TEER was 121±30 $\Omega.cm^2$ and the translocation was of 5.9±1.9%. Measuring the paracellular transport of LY across the different models infected by *S.* Heidelberg or *B. fragilis*, no significant increase of LY permeability was observed, compared to untreated cells (Figure 3D). Thus, it appears that infection with *S.* Heidelberg decreased the TEER of the triple model, but not enough to compromise the barrier integrity.

Salmonella Heidelberg translocation was inhibited in triple co-culture model in the presence of *Bacteroides fragilis* or its supernatant

To evaluate whether *B. fragilis* can modify the translocation rate of *S.* Heidelberg, we co-cultured *S.* Heidelberg with *B. fragilis* in the upper chamber of the triple co-culture model. Significant reduction in *S.* Heidelberg translocation was observed ($P<0.05$ from *S.* Heidelberg alone) (Figure 4A). In

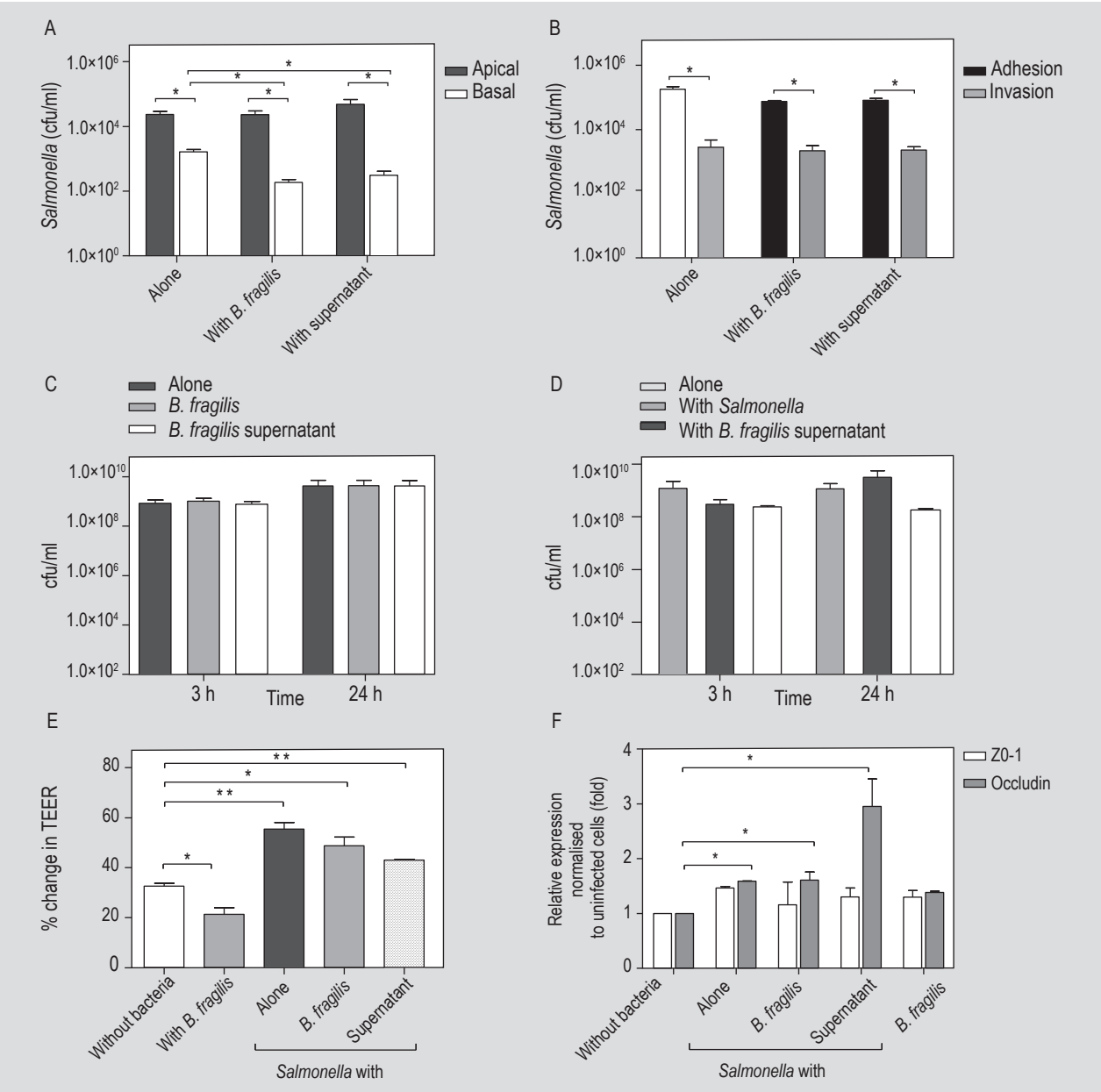

Figure 4. Impact of *Bacteroides fragilis* or its cell-free supernatant on *Salmonella* Heidelberg host cells interaction. (A) Translocation of *Salmonella* alone or after 3 h of infection in co-culture with *B. fragilis* or its cell-free supernatant, media taken from the apical or basal compartment were enumerated (cfu/ml); (B) *Salmonella* adhesion and invasion (cfu/ml); (C) *Salmonella* growth (cfu/ml) after 3 and 24 h of co-culture; (D) *B. fragilis* growth (cfu/ml) after 3 and 24 h of co-culture; (E) transepithelial electrical resistance (TEER) on triple co-culture model (% are expressed compared to cells before infection); (F) mRNA expression changes in tight junction protein genes (occludin and ZO-1) in the triple co-culture model infected by *Salmonella*, *B. fragilis* or both. * $P<0.05$, ** $P<0.01$.

the presence of *B. fragilis*, *S.* Heidelberg translocation was decreased significantly in the basal compartment of triple co-culture model, with 186±75 cfu/ml in the presence of *B. fragilis* and 1,633±568 cfu/ml when *S.* Heidelberg was alone.

In order to study the role of bacterial-secreted substances on the inhibition of *S.* Heidelberg translocation, we examined whether *B. fragilis* supernatant could abrogate the effects of *S.* Heidelberg on intestinal cells (Figure 4A). The volume of *B. fragilis* supernatant, corresponding to the volume used for *B. fragilis*, was added to *S.* Heidelberg in the upper compartment of the triple co-culture model. Enumeration of *S.* Heidelberg with *B. fragilis* supernatant showed that *S.* Heidelberg translocation was also significantly inhibited, with 309±180 cfu/ml in the basal compartment. We observed no significant difference compared to the co-culture of *S.* Heidelberg with *B. fragilis*.

Because adhesion and invasion of bacteria to intestinal cells are an important step for intestinal translocation, we determined whether *B. fragilis* or its cell-free surpernatant had an inhibitory effect on adhesion and invasion of *Salmonella*. Cell-associated bacteria and intracellular bacteria were enumered. Figure 4B showed a significant difference between adhesion and invasion when cells were exposed to *Salmonella,* alone, with *B. fragilis* or its cell-free supernatant (P<0.05). However, no significant difference was noticed between adhesion of *Salmonella* alone and *Salmonella* co-cultured with of *B. fragilis* or its cell-free supernantant. Invasion of *Salmonella* alone was not significantly different from invasion of *Salmonella* in the presence of *B. fragilis* or its cell-free supernatant (Figure 4B).

To verify that this decrease in translocation was not due to a decrease in bacterial growth in co-culture, each strain at 3 and 24 h were enumerated in upper and lower chambers. Figure 4C showed that there was no significant impact of *B. fragilis* on *S.* Heidelberg growth. After 3 h of co-culture between *S.* Heidelberg and *B. fragilis,* $1\times10^9\pm5\times10^8$ cfu/ml of *Salmonella* were enumerated and they were not significantly different from *Salmonella* alone ($8.3\times10^8\pm5\times10^8$ cfu/ml). *B. fragilis* growth was also not impacted by the presence of *S.* Heidelberg (Figure 4D). At 24 h of co-culture, the two bacteria were also detected demonstrating that *B. fragilis* did not inhibit *S.* Heidelberg growth. We have also evaluated the role of this bacterial-secreted substances on *S.* Heidelberg growth and the results presented in figure 4C showed that *B. fragilis* supernatant did not affect the growth of *S.* Heidelberg. The cell-free supernatant of *B. fragilis* did not modify *B. fragilis* growth after 3 or 24 h of incubation (Figure 4D).

To investigate the effect on the intestinal epithelial barrier integrity, we analysed the impact of *S.* Heidelberg and *B. fragilis* co-culture on TEER and compared it to the model infected with only one of these bacteria. Figure 4E showed that TEER, in triple co-culture model infected for 3 h by both bacteria, decreased significantly of 48.7±6% compared to untreated cells (P<0.05). This decrease was not significantly different from the TEER measured when cells were infected with *S.* Heidelberg alone (a change of 55.4±4.4%). Figure 4E also showed a significant change of 43±1.5% of the TEER when *S.* Heidelberg was co-cultured with *B. fragilis* supernatant (P<0.01 compared to cells without bacteria). When the triple co-culture model was infected by only *B. fragilis*, a change of 21% was observed compared to cells without bacteria, where the change of TEER was 32% (P<0.05). The LY transfer in the basal compartment of the triple co-culture model was not modified when *S.* Heidelberg was co-cultured with *B. fragilis* or its supernatant confirming that the integrity of the epithelium was not altered in the presence of *S.* Heidelberg (data not shown).

To complete the effect on the intestinal epithelial barrier, we have evaluated the expression of two tight junctions related genes, occludin and ZO-1. Occludin and ZO-1 mRNA analysis by RT-qPCR showed that only occludin gene was significantly increased compared to uninfected cells in the presence of *S.* Heidelberg (1.9±0.25 fold higher than untreated cells; P<0.01) (Figure 4F). When *S.* Heidelberg was co-cultured with *B. fragilis* or its supernatant, occludin expression was of 1.9±0.25 (P<0.05) and 2.9±1.2 fold (P<0.05), respectively compared to untreated cells. However, in the presence of *B. fragilis*, the ZO-1 and occludin expressions were not significantly different from uninfected cells (1.3±0.04 and 1.4±0.03 fold, respectively).

4. Discussion

Our data showed that *B. fragilis* and its cell-free supernatant inhibited the translocation of *S.* Heidelberg, in a complex multicellular model mimicking intestinal epithelium. The data characterising the functionality of the multicellular model that we have used, corroborated that co-culture of Caco-2 with Raji B cells induced a specific phenotypic switch towards M cells. TEM analysis showed that this triple co-culture model contained M cells with short and irregular microvilli, Caco-2 with a typical brush border and HT29-MTX with mucus-containing vesicles. Additionally, expression of a specific gene by each cells, GP2 (M cells), IS (Caco-2) and muc2 (HT29-MTX), was also detected in the triple co-culture model. LY permeability, TEER measurements, and tight junctions related genes (ZO-1 and occluding) expression during the co-culture formation confirmed the epithelial integrity and its role as an intestinal barrier as described by several studies (García-Rodríguez *et al.,* 2018).

The model developed in our study provides a valuable tool to study the transport of bacteria, such as *S.* Heidelberg (a pathogenic strain) or *B. fragilis* (a commensal strain),

to better understand their impact on the gastrointestinal tract physiology. A multicellular model containing M cells is important for *Salmonella* infection. Indeed, after oral ingestion, *Salmonella* can target the specialised M cell population overlying lymphoid structures called Peyer's patches (Jones, 1994). Finlay and Falkow (1990) have found that invasive *S.* Typhimurium preferentially associates with and invades M cells within 30 min of infection. Within the same time period, they were unable to detect interactions between invasive *S.* Typhimurium and enterocytes. We demonstrated that *S.* Heidelberg was able to cross the epithelial barrier, whereas the translocation rate of *B. fragilis* was very low. As demonstrated by Roberts *et al.* (2010), we showed that Caco-2 derived M-cells can be used to investigate the translocation of gut microbiota and that the translocation of *S.* Heidelberg was markedly increased in comparison with its translocation through parent Caco-2 monocultures. Steffen *et al.* (1988) suggested that enteric bacilli, such as *Escherichia coli*, *Proteus* and *Enterobacter* translocate more efficiently from the gut than other bacteria, especially obligate anaerobes. Lapthorne *et al.* (2012) also showed that M cells were capable of discriminating between different commensal bacteria (Ohno, 2016). It is conceivable that pathogenic microorganisms might have evolved specific ligands for the outer surface of M cells in order to enhance M cell targeting and translocation.

It was shown in several studies that bacterial translocation can be related to the disruption of intestinal barrier integrity which can be determined *in vitro* by measuring TEER (Hsu *et al.,* 2015; Nagpal and Yadav, 2017). In our study, no correlation between TEER and translocation was observed. Indeed, *S.* Heidelberg decreased TEER in Caco-2, Caco-2/HT29 MTX and Caco-2/HT29 MTX models with the same level (with no significant difference observed between these models) but *S.* Heidelberg translocation occurred only in triple co-culture model. Köhler *et al.* (2007) found that infection of human intestinal T84 cells by *S.* Typhimurium over 2 h resulted in an 80% loss of transepithelial electrical resistance, increased bacterial translocation and increased inulin paracellular influx.

A number of enteric pathogens are known to perturb the intestinal epithelial barrier and impact TEER most often with an alteration in the arrangement of tight junctional component proteins: peripheral membrane proteins, such as ZO and different classes of transmembrane proteins, such as occludin (Sears, 2000). In our work, only occludin mRNA expression was increased whereas that of ZO-1 was not impacted in the presence of *S.* Heidelberg and there was no modification with *B. fragilis*. Yu *et al.* (2012) and Shao *et al.* (2017) reported that *S.* Typhimurium infection decreased expression of ZO-1 protein and caused a large increase in permeability. This suggested that within *Salmonella* species, effects on TEER and tight-junctions are strain-dependent, and related maybe to the genetic background of *S.* Heidelberg, with hypermutator phenotype, which contains a 12-bp deletion in *mutS* generating a defective methyl-mismatch repair system (Le Bars *et al.*, 2012a). Hypermutator genotype generates favorable mutations and drives adaptation to a new environment. Strong mutator population may acquire different properties by expressing different metabolic pathways, by inducing distinct stress adaptation traits or producing different virulence factors and thus display new functionalities. Khil *et al.* (2019) demonstrate that mismatch repair deficiencies may be exploited by *Pseudomonas aeruginosa* to facilitate rapid acquisition of antimicrobial resistance in acute infection.

To treat an infection with this hypermutator strain, potential probiotics, such as *B. fragilis,* could be used. The addition of *B. fragilis* to *S.* Heidelberg in triple co-culture model resulted in a decrease of *S.* Heidelberg translocation without modifying TEER or occludin gene expression. Zhang *et al.* (2018) showed that a non-toxigenic strain of *B. fragilis*, *B. fragilis* ZY-312, can modulate specific components of the commensal microbiota in antibiotic-associated diarrhoea in rats. Several studies demonstrated that interference competition occurs among human gut bacteria by the production of factors that directly harm other members (Chatzidaki-Livanis *et al.,* 2014; Koropatkin *et al.,* 2012). To evaluate competition in the growth of the two bacteria used in this study, bacterial growth was monitored when both strains were added on the triple co-culture model. *S.* Heidelberg, alone or in co-culture with *B. fragilis*, exhibited similar growth rates after 3 h of co-culture. There were no significant differences in the number of microorganisms at 24 h also, indicating that these organisms did not appear to compete with one another. Hentges and Maier (1970) showed that no inhibition occured when *Shigella* and *Bacteroides* were simultaneously inoculated into liquid culture medium. However, Li *et al.* (2017) showed that the *Vibrio parahaemolyticus* was inhibited by *B. fragilis* coinfection *in vitro* on LB agar or when they mixed *V. parahaemolyticus* with LB broth containing the culture of 16 h of *B. fragilis*. The experimental conditions may influence the inhibitory effect of *B. fragilis* on a pathogenic bacteria by modulating *B. fragilis* secreted molecules. In this study, we have also demonstrated that *B. fragilis* secreted cell-free supernatant had no direct antibacterial activity toward either *S.* Heidelberg or *B. fragilis.* However, this supernatant inhibited *S.* Heidelberg translocation without modifying TEER, ZO-1 or occludin expression. These results suggested that *B. fragilis* activities against *S.* Heidelberg translocation are due to molecules present in the supernatant.

Several probiotic and commensal bacteria, including *Lactobacillus rhamnosus* GG and *Bifidobacterium breve*, have been shown to secrete metabolites exhibiting a beneficial effect against several intestinal disorders (traveler's diarrhoea, antibiotic-associated diarrhoea, and

acute infectious diarrhoea) (Reid *et al.,* 2003). Probiotics were found to markedly reduce sepsis in infants through competition with pathogens, maintenance of intestinal barrier integrity and promoting immune function (Panigrahi *et al.,* 2017; Sanders *et al.*, 2019). Panigrahi *et al.* (2017) have shown that using a mix containing *Lactobacillus plantarum* and prebiotic fructooligosaccharides has reduced neonatal sepsis and death among infants in rural India by 40%, raising the question of adding prebiotics to enhance probiotics effects. However, before using *B. fragilis* as probiotic, the safety of this organism should be evaluated in clinical practice. For safety, the use of probiotic-derived products rather than live bacteria to increase barrier resistance and maintain the intestinal barrier may have significant clinical implications.

Conflict of interest

The authors declare no conflict of interest.

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