Journal of Crohn's and Colitis, 2017, 229–236
doi:10.1093/ecco-jcc/jjw142
Advance Access publication August 1, 2016
Original Article

Original Article

Reduced Mucosa-associated *Butyricicoccus* Activity in Patients with Ulcerative Colitis Correlates with Aberrant Claudin-1 Expression

Sarah Devriese,[a] Venessa Eeckhaut,[b] Annelies Geirnaert,[c] Lien Van den Bossche,[a] Pieter Hindryckx,[a] Tom Van de Wiele,[c] Filip Van Immerseel,[b] Richard Ducatelle,[b] Martine De Vos,[a] Debby Laukens[a]

[a]Department of Gastroenterology, Ghent University, Ghent, Belgium [b]Department of Pathology, Bacteriology and Avian Diseases, Ghent University, Merelbeke, Belgium [c]Laboratory of Microbial Ecology and Technology [LabMET], Ghent University, Ghent, Belgium

Corresponding author: Debby Laukens, PhD, Department of Gastroenterology, Ghent University Hospital, De Pintelaan 185, 3K12-IE, B-9000 Ghent, Belgium. Tel.: +32 9 332 20 64; fax: +32 9 332 49 84; email: debby.laukens@ugent.be

Abstract

Background and Aims: *Butyricicoccus* is a butyrate-producing clostridial cluster IV genus whose numbers are reduced in the stool of ulcerative colitis [UC] patients. Conditioned medium of *Butyricicoccus [B.] pullicaecorum* prevents tumour necrosis factor alpha [TNFα]-induced increase in epithelial permeability *in vitro*. Since butyrate influences intestinal barrier integrity, we further investigated the relationship between the abundance of mucosa-associated *Butyricicoccus* and the expression of butyrate-regulated tight junction [TJ] genes.

Methods: Tight junction protein 1 [*TJP1*], occludin [*OCLN*], claudin-1 [*CLDN1*], and *Butyricicoccus* 16S rRNA expression was analysed in a collection of colonic biopsies of healthy controls and UC patients with active disease. The effect of butyrate and *B. pullicaecorum* conditioned medium on TJ gene expression was investigated in TNFα-stimulated Caco-2 monolayers and inflamed mucosal biopsies of UC patients.

Results: *TJP1* expression was significantly decreased in inflamed UC mucosa, whereas *CLDN1* mRNA levels were increased. *OCLN* did not differ significantly between the groups. Mucosa-associated *Butyricicoccus* 16S rRNA transcripts were reduced in active UC patients compared with healthy controls. Interestingly, *Butyricicoccus* activity negatively correlated with *CLDN1* expression. Butyrate reversed the inflammation-induced increase of CLDN1 protein levels, and stimulation of inflamed UC biopsies with *B. pullicaecorum* conditioned medium normalized *CLDN1* mRNA levels.

Conclusions: *Butyricicoccus* is a mucosa-associated bacterial genus under-represented in colonic mucosa of patients with active UC, whose activity inversely correlates with *CLDN1* expression. Butyrate and *B. pullicaecorum* conditioned medium reduce CLDN1 expression, supporting its use as a pharmabiotic preserving epithelial TJ integrity.

Key Words: Tight junctions; pharmabiotic; butyrate

1. Introduction

In the healthy gut, a symbiotic relationship exists between the host and commensal bacteria, which is of paramount importance to our general well-being. Commensals are crucial to the maintenance of immunological gut homeostasis. During adult life, the composition of the intestinal microbiota is fairly stable but may fluctuate due

to infections or oral antibiotic treatment. This fluctuation generally entails a decrease in both bacterial abundance and diversity—a condition termed dysbiosis.[1] Several chronic diseases, including inflammatory bowel disease [IBD], are associated with intestinal dysbiosis. IBD, comprising Crohn's disease [CD] and ulcerative colitis [UC], is characterized by a chronic, relapsing inflammation of the gastrointestinal tract. The aetiology of IBD is unknown but inflammation most likely results from an abnormal mucosal immune response to antigens derived from the commensal microbiota in a genetically susceptible host. One of the most important microbial communities affected by intestinal inflammation are specific butyrate-producing members of the *Firmicutes* phylum like the *Lachnospiraceae* subgroup [which comprises *Clostridium* XIVa and IV groups within the order *Clostridiales*] which are under-represented in the mucosal microbiota of patients with IBD compared with healthy subjects.[2] Butyrate is a short-chain fatty acid produced during fermentation of dietary fibre in the colon. Besides being the main energy source for colonocytes, butyrate is also responsible for the maintenance of colonic homeostasis by modulating a wide variety of cellular functions including proliferation, differentiation, apoptosis, and the control of intestinal epithelial permeability.[3,4] Butyrate is a potent anti-inflammatory mediator given its role in promoting epithelial barrier function,[5] its inhibitory effect on cytokine expression,[6] and its ability to induce differentiation of colonic regulatory T-cells.[7] Most of the butyrate-producing bacteria cultured so far belong to the clostridial clusters XIVa and IV that include *Roseburia*[8] and *Faecalibacterium*[9] species, respectively, two abundant colonizers of the human gut. A decrease in both *Roseburia hominis* and *Faecalibacterium prausnitzii* has been documented in stools of UC patients, with both species showing an inverse correlation with disease activity.[10]

Consequently, there is increasing interest in using butyrate to restore homeostasis in IBD. However, its routine clinical application has been impeded by practical issues. When administered orally, butyrate might not reach the colon in sufficient amounts due to its rapid gastric and duodenal absorption. Besides, butyrate has a very unpleasant taste and smell. Rectal butyrate enemas have proven to be effective in treating distal UC[11,12] but are cumbersome for the patient and exposure of the colonic mucosa to the butyrate is brief and discontinuous. The administration of naturally occurring butyrate-producing bacteria that would continuously secrete butyrate into the colonic lumen is an alternative strategy to locally increase butyrate concentrations. One of these high-level butyrate-producing bacteria is *Butyricicoccus pullicaecorum* [*B. pullicaecorum*], an anaerobic Gram-positive clostridial cluster IV species, first isolated from the caecal content of a broiler chicken.[13] The average number of *Butyricicoccus* is decreased in stool samples of IBD patients, and a specific *B. pullicaecorum* strain is able to reduce intestinal inflammation in a rat colitis model. Furthermore, its conditioned medium prevents cytokine-induced increase in epithelial permeability *in vitro*.[14]

A dysfunctional epithelial barrier is one of the key characteristics of IBD.[15] This barrier consists of a single layer of epithelial cells linked together by tight junctions [TJs] which seal off the intercellular space and regulate selective paracellular ionic solute transport. TJs are composed of four different intergral membrane proteins: occludin [OCLN], tricellulin, junctional adhesion molecules, and claudins, which are linked to the actin cytoskeleton through scaffolding proteins like tight junction protein 1 [TJP1].[16] In IBD, expression of most claudins—like other tight junction proteins—is reduced.[17] Remarkably, claudin-1 [CLDN1] protein levels are increased in areas of active inflammation.[18] Given the reduction of *Butyricicoccus* numbers in IBD and the ability of its conditioned medium to prevent cytokine-induced epithelial dysfunction, B. *pullicaecorum* bacteria therefore seem conceptually attractive as pharmabiotics to reduce intestinal inflammation or to prevent disease relapse in IBD patients. However, it is currently unknown how *Butyricicoccus* could affect barrier integrity in human IBD. Therefore, the purpose of this study was to detect and quantify *Butyricicoccus* in human colonic mucosa and to further investigate the host response to butyrate and conditioned medium of *B. pullicaecorum* in terms of epithelial barrier function.

2. Materials and Methods

2.1. Ethics statement

The use of patient material was approved by the Ethics Committee of the Ghent University Hospital [permit number UZG 2004/242]. Written informed consent was obtained from all participants. Mice were housed in the laboratory animal facility at Ghent University Hospital according to the institutional animal healthcare guidelines. This study was approved by the Institutional Review Board of the Faculty of Medicine and Health Sciences of Ghent University [ECD2014-25].

2.2. Patient samples

Biopsies from healthy controls [*N* = 36], UC patients with active disease [*N* = 37], and UC patients in clinical and endoscopic remission [*N* = 16] were obtained during routine colonoscopy. Active disease in UC patients was defined as the presence of endoscopic signs of disease activity [Mayo score of 1 or higher] and biopsies were taken from the inflamed site [sigmoid or rectum]. UC patients in remission had no clinical or endoscopic signs of inflammation [Mayo score of 0]. Control biopsies were taken from the sigmoid of healthy patients who underwent colonoscopy to screen for cancer. Patient characteristics are provided in Table 1. All biopsies were immediately placed in RNAlater [Ambion, Foster City, CA] and stored at -80°C.

2.3. Mice and experimental protocol

Heterozygous C57BL/6 $TNF^{\Delta ARE/WT}$ mice and $TNF^{WT/WT}$ littermates were conventionally raised in a temperature-controlled room at 20°C with a light/dark cycle of 12/12 h. Water and a commercial chow [mice maintenance chow, Carfil Labofood, Pavan Service, Belgium] were provided *ad libitum*. At 24 weeks of age, eight $TNF^{\Delta ARE/WT}$ mice and eight WT littermate controls were sacrificed by cervical dislocation. The terminal ileum was removed, opened longitudinally and washed with phosphate-buffered saline [PBS]. Tissue samples were cut, snap-frozen and stored at -80°C until analysis.

2.4. Bacterial strain and growth conditions

B. pullicaecorum 25-3^T [LMG 24109^T] was grown overnight at 37°C in an anaerobic [90% N_2, 10% CO_2] workstation [GP-Campus, Jacomex, TCPS NV, Rotselaar, Belgium] in anaerobic modified M2GSC medium at pH 6, prepared as described by Miyazaki *et al.*[19] but without clarified rumen fluid. The bacterial cells were collected by centrifugation [10 min, 5000 *g*, 37°C] and discarded. The resulting supernatant was sterile-filtered [0.22 µml] and the concentration of short-chain fatty acids [acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate, and isocaproate] was determined using a gas chromatograph as described previously.[20] Only butyrate was detected in supernatant of *B. pullicaecorum* 25-3^T at a concentration of 4 mM.

Table 1. Patient characteristics.

	Healthy controls	UC inflamed	UC remission
For baseline expression of *Butyricicoccus* and tight junction complex genes in colonic mucosa			
N [patients]	36	37	16
Gender [male/female]	16/20	25/12	12/4
Age [years, mean]	52	36	42
Age [years, range]	18–80	6–68	16–74
Disease location [E1/E2/E3]	N/A	[8/17/12]	N/A
Medication			
No	36	11	1
5-aminosalicylates	0	15	10
Corticosteroids	0	11	1
Immunomodulators	0	7	6
Biologicals	0	7	4
For stimulation of inflamed UC biopsies with *B. pullicaecorum* conditioned medium			
N [patients]	N/A	25	
Gender [male/female]	N/A	15/10	
Age [years, mean]	N/A	40	
Age [years, range]	N/A	20–68	
Disease location [E1/E2/E3]	N/A	[13/10/2]	
Medication			
No	N/A	2	
5-aminosalicylates	N/A	16	
Corticosteroids	N/A	7	
Immunomodulators	N/A	9	
Biologicals	N/A	10	

UC, ulcerative colitis; E1, proctitis; E2, distal colitis; E3, pancolitis; N/A, not applicable.

2.5. Stimulation of colonic mucosal biopsies with *B. pullicaecorum* conditioned medium

Colonic biopsies from patients with active UC [*N* = 25] were obtained as described above and harvested in RPMI 1640 medium supplemented with 10% fetal bovine serum [FBS], 10.000 units/ml penicillin, 10.000 µg/ml streptomycin, and 200 µg/ml gentamycin [all Life Technologies, Ghent, Belgium]. Patient characteristics are provided in Table 1. Biopsies were cultured for 24 h with *B. pullicaecorum* conditioned medium diluted to contain 2 mM of butyrate or M2GSC anaerobic medium as control. Biopsies were recovered in RNAlater and stored at -80°C.

2.6. Cell culture and induction of barrier dysfunction *in vitro*

Caco-2 cells [HTB-37, ATCC Cell Biology Collection, Manassas, VA, USA] were seeded on 24-well semipermeable inserts [0.4 µm, translucent ThinCerts™, Greiner Bio-One, Vilvoorde, Belgium] at a density of 10^5 cells per well and cultured for a minimum of 2 weeks in Dulbecco's modified Eagle medium supplemented with 10% FBS [both Life Technologies]. After this period, the integrity of the monolayer was evaluated by measuring the transepithelial electrical resistance [TEER] using a Millicell ERS-2 Voltohmmeter [Merck Millipore, Billerica, MA, USA] to ensure that functional polarized epithelial monolayers with absolute TEER-values of more than 3000 Ohm were obtained. The Caco-2 differentiated monolayer was then incubated apically with 8 mM sodium butyrate [NaB] [Sigma-Aldrich, Diegem, Belgium] and basolaterally with recombinant human 100 ng/ml tumour necrosis factor [TNF] α and 300 ng/ml interferon [IFN] γ [both Life Technologies] to induce barrier dysfunction measured as a drop in TEER. After 48 h, absolute TEER values were normalized to their pretreatment values and expressed as percentage of the initial TEER values. After the TEER measurements, Caco-2 inserts were used for CLDN1 detection by either western blotting or immunofluorescence. Medium from the basolateral compartments was used for interleukin-8 [IL-8] detection.

2.7. IL-8 measurements

IL-8 secretion into the basolateral medium was measured using an enzyme-linked immunosorbent assay [ELISA] [R&D Systems, Abingdon, UK] according to the manufacturer's instructions. IL-8 concentration was expressed as picograms of cytokine per millilitre of medium.

2.8. Western blot analysis

Caco-2 monolayers were lysed using sonication on ice for 1 min in 200 µl of Radio Immunoprecipitation Assay [RIPA] buffer supplemented with a phosphatase and protease inhibitor cocktail [Sigma-Aldrich]. The concentrations of protein lysates were determined using the Bio-Rad Protein Assay [Bio-Rad, Temse, Belgium] according to the manufacturer's instructions with bovine serum albumin [BSA] as a protein standard. Thirty µg of each sample was mixed with 1:4 loading buffer [Life Technologies] and 1 mM dithiothreitol [Roche, Vilvoorde, Belgium]. Samples were denatured by boiling for 10 min at 95°C, separated on a 4–12% gradient Bis-Tris SDS-PAGE gel and transferred to a nitrocellulose membrane using a wet transfer [all Life Technologies]. Afterwards, membranes were blocked with 5% BSA in Tris-buffered saline with 0.1% Tween-20 [TBST] [Sigma-Aldrich] and incubated overnight at 4°C with 1:1000 rabbit anti-CLDN1 antibody [Cell Signalling, Leiden, The Netherlands] in 5% BSA in TBST. Next, blots were incubated for 1 h at room temperature with 1:2000 secondary goat anti-rabbit IgG, HRP-conjugated secondary antibody [Cell Signalling]. Bound antibodies were visualized using the BM Chemiluminescence Western Blotting Substrate POD [Roche] according to manufacturer's instructions and membranes were exposed to X-ray films. Equal loading of proteins was confirmed by immunoblotting with 1:5000 anti-tubulin [Abcam, Cambridge, UK] antibody in 5% BSA in TBST.

2.9. Immunofluorescence staining

Caco-2 inserts were fixed in cold methanol: acetone [50%: 50%] for 1 min. Blocking step was performed using 10% [v/v] normal goat serum [Sigma-Aldrich] in PBS. Then cells were incubated for 1 h with 1:200 rabbit anti-CLDN1 antibody [Life Technologies] in 2% normal goat serum in PBS. After washing, the cells were stained with 1:200 Alexa Fluor 488 anti-rabbit secondary antibody [Life Technologies] in 2% normal goat serum in PBS for 30 min in the dark at room temperature. Cells were incubated with DAPI [Life Technologies] for 5 min to allow nuclear staining. Inserts were mounted using mounting medium containing an anti-fading agent [Dako, Heverlee, Belgium]. Cells were examined under a BD Pathway 435 confocal laser microscope [BD Biosciences, San Diego, CA, USA] and images were processed using Adobe Photoshop software 5.5 [Adobe systems, Dublin, Ireland].

2.10. RNA extraction

Total RNA from Caco-2, human mucosal biopsies and mouse terminal ileum tissue samples was extracted using the Qiagen RNeasy

Mini Kit [Qiagen, Venlo, The Netherlands] with on-column DNase treatment. Concentration and purity were determined using nanodrop technology [BioPhotometer Plus, Eppendorf, Rotselaar, Belgium]. All samples exhibited an OD260/OD280 ratio between 1.8 and 2.1.

2.11. Quantitative real-time polymerase chain reaction

One microgram of total RNA was converted to single-stranded cDNA by reverse transcription using the iScript™ cDNA synthesis kit [Bio-Rad] according to the manufacturer's instructions. The cDNA was diluted to a concentration of 5 ng/µl and 15 ng was used in quantitative real-time polymerase chain reaction [qRT-PCR] with SYBR Green [SensiMix™ SYBR No-ROX kit, GC Biotech, Alphen a/d Rijn, The Netherlands] and 250 nM of each primer. A two-step programme was performed on the LightCycler 480 [Roche]. Cycling conditions were 95°C for 10 min, 45 cycles of 95°C for 10 s and 60°C for 1 min. Melting curve analysis confirmed primer specificities. All reactions were performed in duplicate. Expression of all eukaryotic genes was normalized to the stably expressed reference gene levels of glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*] and succinate dehydrogenase complex A subunit [*SDHA*] for the Caco-2 cDNA, to *GAPDH*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein [*YWHAZ*] and hydroxymethyl-bilane synthase [*HMBS*] for *B. pullicaecorum*-stimulated active UC biopsy cDNA, to *GAPDH*, *YWHAZ* and *HMBS* for all other human biopsy cDNA and to *HMBS* and *GAPDH* for mouse terminal ileum tissue samples. To enable normalization to biopsy size, the number of cDNA copies corresponding to the *Butyricicoccus* 16S rRNA gene were normalized to the expression of human *GAPDH*, *HMBS* and *YWHAZ* as described previously.[21] The PCR efficiency of each primer pair was calculated using a standard curve of reference cDNA. Amplification efficiency was determined using the formula $10^{-1/slope}$. Sequences of the primer sets are listed in Table 2.

2.12. Statistical analysis

Statistical analysis was performed using GraphPad Prism software [GraphPad, La Jolla, CA, USA] and SPSS Statistics version 22.0 [IBM SPSS Statistics, Armonk, NY, USA]. Values are presented as the mean ± standard error of the mean [SEM]. Normality of the data was checked using the Kolmogorov-Smirnoff test. Differences between groups were analysed using an unpaired Student's t-test for independent samples in the case of normally distributed data [applying the Welch's correction in the case of unequal variances] or the Mann-Whitney statistic if the data were not normally distributed. Two-tailed probabilities were calculated and *p*-values less than 0.05 were considered statistically significant. Prevalence of the genus *Butyricicoccus* in healthy controls versus active UC patients was calculated using a Fisher's exact test. The Kolmogorov-Smirnoff test also determined the use of either a parametric [Pearson] or a non-parametric [Spearman] correlation test.

3. Results

3.1. Tight junction gene expression is deregulated during intestinal inflammation

In order to link the presence of *Butyricicoccus* with barrier integrity *in vivo*, mRNA expression levels of three major TJ genes—*CLDN1*, *TJP1* and *OCLN*—were measured in a collection of colonic mucosal biopsies from healthy controls [*N* = 36] and patients with active UC [*N* = 37] using qRT-PCR. Expression of *TJP1* was significantly decreased [$p < 0.001$, Figure 1A] in UC biopsies, whereas *CLDN1* expression was significantly increased [$p < 0.0001$, Figure 1B]. Expression of *OCLN* did not differ significantly between active UC patients and healthy controls [$p = 0.091$, Figure 1C]. In remission, no significant differences were observed [Figure 1A–C]. The inflammation-associated upregulation of CLDN1 observed in active UC seems counter-intuitive; however, increased CLDN1 levels have been reported in experimental models for colitis.[22] Also, inflammation of the ileum in $TNF^{\Delta ARE/WT}$ mice[23] exhibits an increased expression of *Cldn1* [$p = 0.0002$, Figure 1D].

3.2. *Butyricicoccus* activity is decreased in mucosal samples of patients with active UC

Next, we used this biopsy cohort to determine whether *Butyricicoccus* bacteria were present using genus-specific 16S rRNA primers. *Butyricicoccus* 16S rRNA levels could be measured adequately, ranging from 0 to 100.000 copies. Functionally active *Butyricicoccus* bacteria were detectable in all healthy control samples but below detection in eight out of 37 active UC samples [Fisher's exact test $p = 0.0052$]. In UC samples with detectable 16S rRNA transcripts, the absolute numbers were reduced compared with those of healthy controls [$p = 0.046$, Figure 2A]. Interestingly, the amount of *Butyricicoccus* inversely correlated with *CLDN1* mRNA levels [Pearson R = -0.239, $p = 0.024$, Figure 2B].

Table 2. Primers for quantitative real-time polymerase chain reaction [qRT-PCR] analysis.

Gene symbol	Species	Forw [5'-3']	Rev [3'-5']	E [%]
N/A	*Butyricicoccus*	ACCTGAAGAATAAGCTCC	GATAACGTTGCTCCCTACGT	74
Gapdh	Mouse	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA	88
Hmbs	Mouse	AAGGGCTTTTCTGAGGCACC	AGTTGCCCATCTTTCATCACTG	95
Cldn1	Mouse	TGCCCCAGTGGAAGATTTACT	CTTTGCGAAACGCAGGACAT	97
CLDN1	Human	GGGATGGATCGGCGCCATCG	CGTACATGGCCTGGGCGGTC	104
TJP1	Human	CTCACCACAAGCGCAGCCACAA	ACAGCAGAGGTTGATGATGCTGGG	98
OCLN	Human	AGACGTCCCCAGCCCAGTCC	CGTACATGGCCTGGGCGGTC	111
GAPDH	Human	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	91
SDHA	Human	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG	92
YWHAZ	Human	ACTTTTGGTACATTGTGGCTTCAA	CCGCCAGGACAAACCAGTAT	93
HMBS	Human	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC	101

N/A, not applicable; E, efficiency.

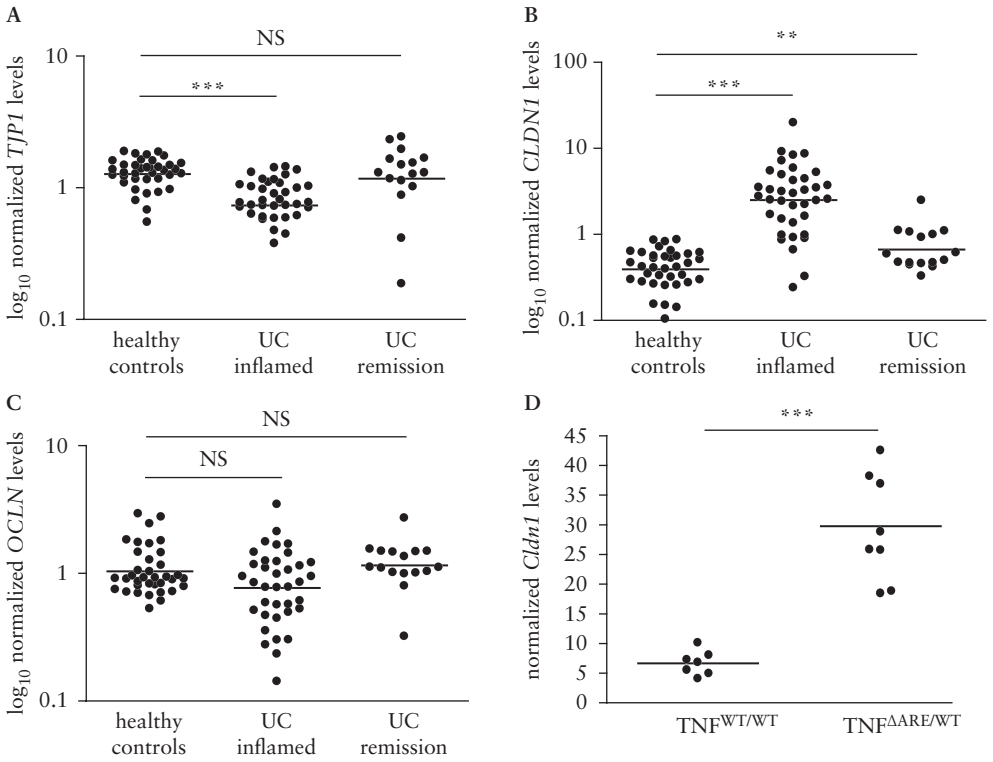

Figure 1. Tight junction gene expression in biopsies of UC patients versus healthy controls and in TNF$^{\Delta ARE/WT}$ mice versus TNF$^{WT/WT}$ littermate controls. [A–C] *TJP1*, *OCLN*, and *CLDN1* mRNA levels in colonic mucosal biopsies of healthy controls [$N = 36$], UC patients with active disease [$N = 37$] and UC patients in remission [$N = 16$]. [D] *Cldn1* expression in terminal ileum samples of 24-week old TNF$^{\Delta ARE/WT}$ mice [$N = 8$] versus TNF$^{WT/WT}$ littermate controls [$N = 8$]. $^{**}p < 0.01$, $^{***}p < 0.001$. NS, not significant.

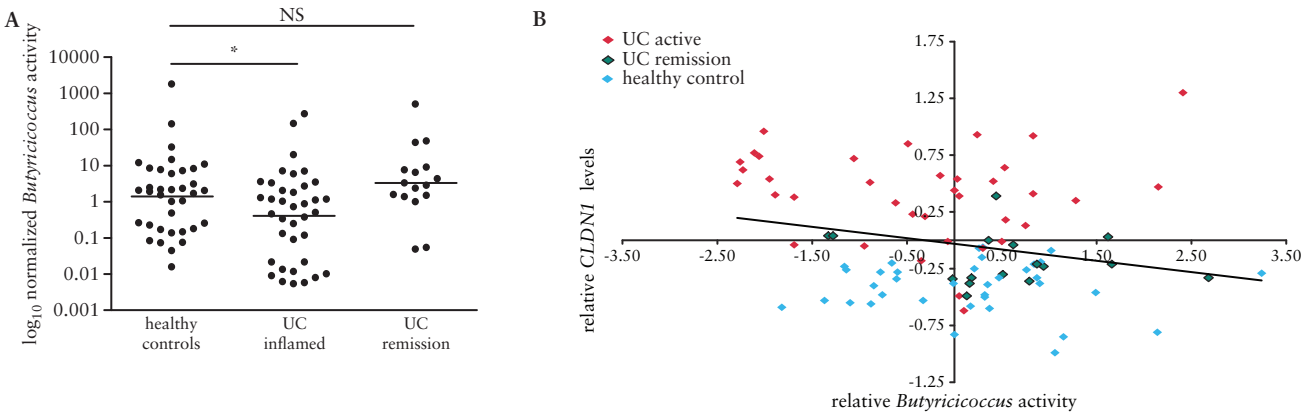

Figure 2. Quantification of *Butyricicoccus* activity in colonic mucosal biopsies of healthy controls and UC patients. [A] *Butyricicoccus* activity in colonic mucosal biopsies of healthy controls [$N = 36$], UC patients with active disease [$N = 37$], and UC patients in remission [$N = 16$]. Bacterial activity is expressed as $\log_{10}$ copy number of normalized 16S rRNA genes for *Butyricicoccus*. [B] Correlation plot between *CLDN1* and *Butyricicoccus* activity in biopsies of healthy controls [blue], patients with active UC [red], and UC patients in remission [green]. $^{*}p < 0.05$. NS, not significant.

3.3. Butyrate counteracts TNFα-induced barrier disruption and TJ deregulation

Next, we questioned whether the *in vitro* model for barrier disruption using TNFα-stimulated Caco-2 monolayers mimics the deregulated TJ gene expression observed in UC biopsies. A significant drop in TEER was observed [$p < 0.0001$, Figure 3A] 48 h following the addition of TNFα, which coincided with increased IL-8 production [$p = 0.0043$, Figure 3B] and a significant reduction of *TJP1* and *OCLN* mRNA levels [$p = 0.0065$ and 0.0014, respectively, Figure 3C–D], while *CLDN1* mRNA [$p = 0.012$, Figure 3E] and CLDN1 protein expression [Figure 3F] were increased. In addition, CLDN1, which localizes to the plasma membrane in functionally intact Caco-2 monolayers, internalized following TNFα stimulation [Figure 3G]. The addition of 8 mM butyrate to the transwell culture inhibited the TNFα-induced TEER drop [$p = 0.001$], diminished IL-8 secretion [$p > 0.05$] and increased the expression of TJ genes *TJP1* [$p = 0.0223$] and *OCLN* [$p = 0.0012$] [Figure 3A–D]. Although the TNFα-induced increase in mRNA expression of *CLDN1* was not influenced by butyrate [Figure 3E], total protein levels of CLDN1 [Figure 3F] were markedly decreased. However, cytoplasmic expression of CLDN1 was still observed [Figure 3G].

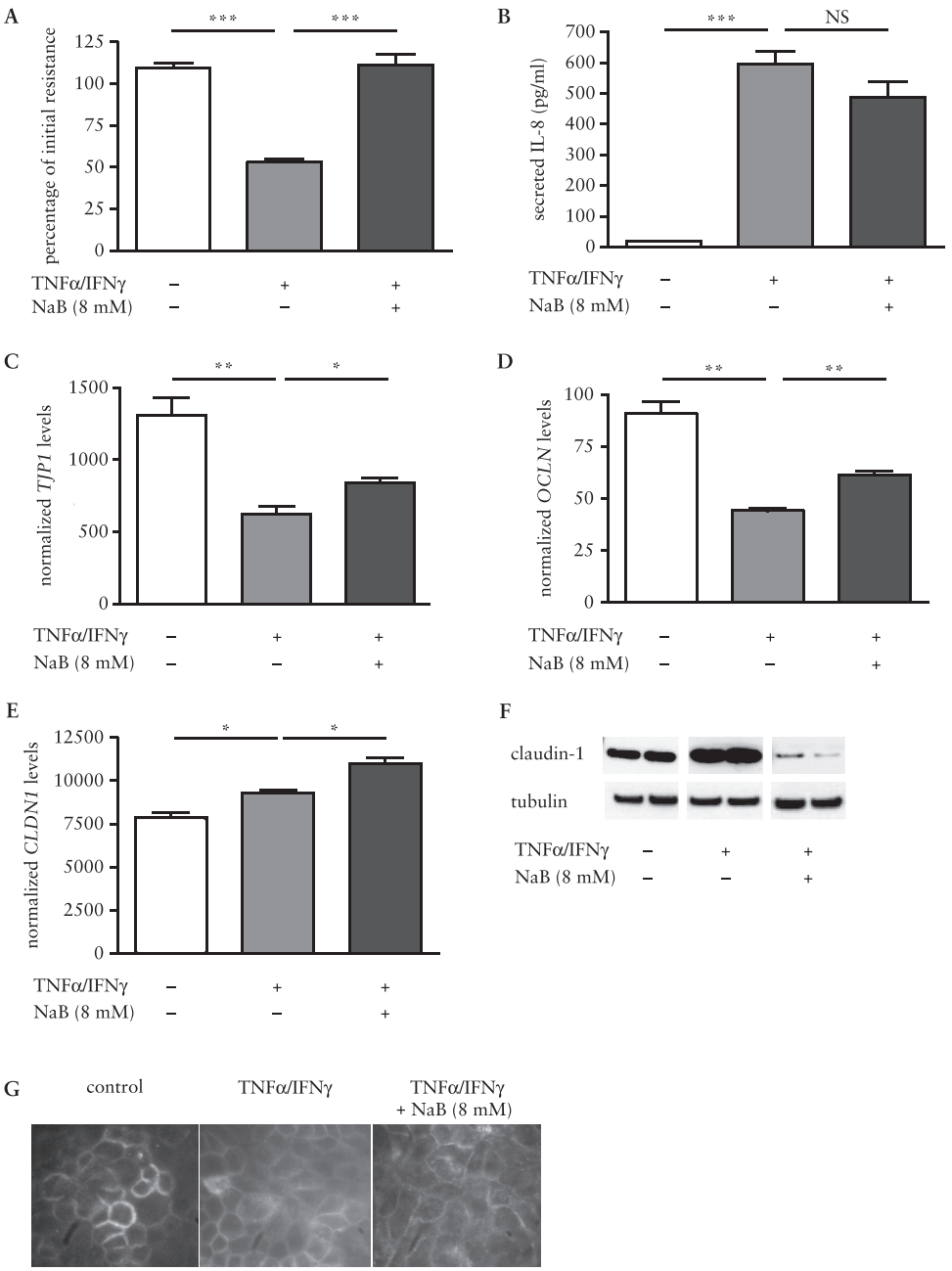

Figure 3. Effect of butyrate on TNFα-induced barrier disruption and TJ deregulation in Caco-2 monolayers. Caco-2 differentiated monolayers were stimulated basolaterally with a combination of TNFα and IFNγ and treated apically with 8 mM NaB. After 48 h, TEER [A], basolateral IL-8 secretion [B], *TJP1* [C], *OCLN* [D], and *CLDN1* [E] mRNA levels and CLDN1 protein expression [F] were determined. [G] Immunofluorescent microscopic images [63x] of CLDN1 internalization in a Caco-2 monolayer stimulated with TNFα and 8 mM NaB. Data represent the mean ± SEM of 3 [A–E], 2 [F], or 1 [G] replicate[s]/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, NS, not significant.

3.4. *B. pullicaecorum* conditioned medium reduces *CLDN1* expression in mucosa of patients with active UC

Finally, to investigate whether *B. pullicaecorum* affects TJ expression *ex vivo*, mucosal biopsies of UC patients with active disease [$N = 25$] were stimulated with *B. pullicaecorum* conditioned medium diluted to contain 2 mM of butyrate or an equal dilution of the growth medium control. After 24 h, a decrease in *CLDN1* mRNA levels compared with medium control [$p = 0.001$, Figure 4A] was observed whereas *TJP1* and *OCLN* expression were not significantly affected by the conditioned medium [Figure 4B, C].

4. Discussion

Intestinal epithelial TJ integrity is compromised in IBD. The observed abnormalities include reduced strand numbers, strand discontinuities, and reduced depth of the TJ complex. Changes in expression and localization of specific TJ molecules have also been described.[24,25] In our collection of colonic mucosal biopsies, we also observed a severely deregulated expression of two major TJ genes—*TJP1* and *CLDN1*—in samples of patients with active UC compared with healthy controls; *TJP1* is under-represented in inflamed tissue, whereas *CLDN1* is highly upregulated. A similar increase in *Cldn1* was observed in a model for Crohn's-like ileitis due to constitutive overexpression of TNFα, a key pro-inflammatory cytokine in IBD. Likewise compromised TJ integrity,

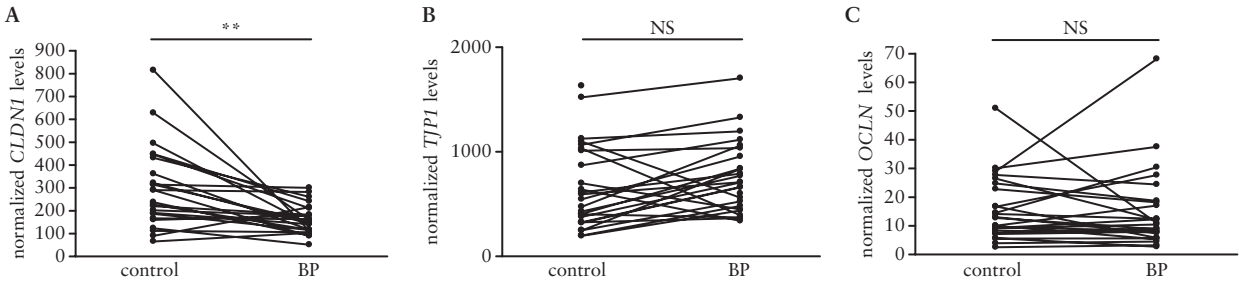

Figure 4. Effect of *B. pullicaecorum* conditioned medium on tight junction [TJ] expression in colonic mucosal biopsies of active ulcerative colitis [UC] patients. *CLDN1* [A], *TJP1* [B], and *OCLN* [C] expression in paired colonic biopsies from the same UC patients [$N = 25$] treated with *B. pullicaecorum* conditioned medium diluted to contain 2 mM of butyrate or M2GSC anaerobic medium as control for 24 h. $^{**}p < 0.01$. BP; *B. pullicaecorum*; NS, not significant.

mimicked *in vitro* by stimulating differentiated Caco-2 monolayers with TNFα, leads to loss of *TJP1* and *OCLN* and an increase in *CLDN1*. In addition, CLDN1, which is localized solely at the plasma membrane in functionally intact Caco-2 monolayers, internalizes following TNFα-stimulation. Under normal physiological conditions, CLDN1 is a key pore-sealing TJ protein crucial to epithelial barrier integrity; its genetic deletion results in rapid postnatal death due to severe epidermal permeability defects.[26] Also, baseline *CLDN1* overexpression *in vitro* results in increased barrier tightness, whereas its concomitant knockdown decreases it.[5] Under inflammatory conditions however, the role of CLDN1 is much less straightforward. In agreement with upregulated *CLDN1* mRNA levels in our cohort, increased CLDN1 protein levels have been documented in IBD and in acute dextran sodium sulphate-induced colitis.[27,22] Also, its intestinal epithelial overexpression renders mice more susceptible to colitis and impairs their recovery.[28] Interestingly, *in vitro* pro-inflammatory cytokine-induced upregulation of CLDN1 coincides with a redistribution of the protein away from the TJs[29,30] and a concomitant increase in intestinal permeability. So, it appears that elevating CLDN1 expression under baseline conditions increases epithelial barrier integrity, whereas under inflammatory conditions its internalization causes a weakening of the intestinal barrier despite an increase in overall expression.

Next, we found that this aberrant *CLDN1* expression in colonic mucosa of active UC patients negatively correlates with reduced *Butyricicoccus* activity. The detection of the genus *Butyricicoccus* in mucosal tissue is in agreement with a study published by Nava and Stappenbeck who identified *Butyricicoccus* as an autochthonous microbe predominantly colonizing the mucosa-associated surface of the colon.[31] This close proximity of *Butyricicoccus* bacteria to the apical surface of the colonic epithelium facilitates host access to its metabolites, like butyrate, which are essential to cellular homeostasis. Since butyrate is known to promote intestinal epithelial barrier function, we analysed its effect on TNFα-induced deregulation of TJ gene expression *in vitro*. Butyrate completely prevented loss of TEER in TNFα-stimulated Caco-2 monolayers while increasing *TJP1* and *OCLN* expression. Neither increased *CLDN1* mRNA levels nor CLDN1 internalization was reduced by butyrate; however, a drop in total CLDN1 protein levels was observed. Finally, e*x vivo* stimulation of inflamed UC biopsies showed that the observed *in vitro* effects of butyrate could be mimicked by *B. pullicaecorum* conditioned medium, i.e. a marked reduction in *CLDN1* levels.

In conclusion, this study demonstrates that the functions of CLDN1 are not restricted to its traditional role of maintaining intestinal barrier function and that its elevation during mucosal inflammation is detrimental to both epithelial integrity and further downstream pathways responsible for colonic homeostasis. Given the effect of both butyrate and *B. pullicaecorum* conditioned medium on CLDN1 expression, these results further substantiate the use of *Butyricicoccus* as a pharmabiotic in order to preserve epithelial TJ integrity.

Funding

This work was supported by the Institute of Science and Technology, Flanders [IWT], under the contract number SBO-100016. DL and LVdB are paid by Research Foundation Flanders [FWO] grants [G.12982.13 and G.11J99.13N].

Conflicts of Interest

VE, RD, and FVI are listed as co-inventors on a patent application for use of butyrate-producing bacterial strains related to *B. pullicaecorum* in the prevention and/or treatment of intestinal health problems [International Application Number PCT/EP2010/052184 and International Application Number WO2010/094789 A1]. For the remaining authors, no conflicts of interest are declared.

Acknowledgments

The authors are particularly grateful to George Kollias at the Biomedical Sciences Research Center 'Alexander Fleming' [Vari, Greece] for providing the $TNF^{\Delta ARE/WT}$ mice. We would also like to thank Elien Glorieus for the excellent assistance in obtaining patient samples and Elke Decrock and Marijke De Bock for assisting with immunofluorescent staining and confocal microscopy.

References

1. Gareau MG, Sherman PM, Walker WA. Probiotics and the gut microbiota in intestinal health and disease. *Nat Rev Gastroenterol Hepatol* 2010;**7**:503–14.
2. Frank DN, St. Amand AL, Feldman RA, *et al*. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A* 2007;**104**:13780–.
3. Guilloteau P, Martin L, Eeckhaut V, *et al*. From the gut to the peripheral tissues: the multiple effects of butyrate. *Nutr Res Rev* 2010;**23**:366–84.
4. Hamer HM, Jonkers D, Venema K, *et al*. Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther* 2008;**27**:104–19.
5. Wang HB, Wang PY, Wang X, *et al*. Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein claudin-1 transcription. *Dig Dis Sci* 2012;**57**:3126–35.
6. Segain J-P, Raingeard de la Blétière D, Bourreille A, *et al*. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. *Gut* 2000;**47**:397–403.
7. Furusawa Y, Obata Y, Fukuda S, *et al*. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* 2013;**504**:446–50.
8. Duncan SH, Aminov RI, Scott KP, *et al*. Proposal of *Roseburia faecis* sp. nov., *Roseburia hominis* sp. nov. and *Roseburia inulinivorans* sp. nov., based on isolates from human faeces. *Int J Syst Evol Microbiol* 2006;**56**:2437–41.
9. Suau A, Rochet V, Sghir A, *et al*. *Fusobacterium prausnitzii* and related species represent a dominant group within the human fecal flora. *Syst Appl Microbiol* 2001;**24**:139–45.

10. Machiels K, Joossens M, Sabino J, *et al*. A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. *Gut* 2014;**63**:1275–83.
11. Breuer RI, Soergel KH, Lashner BA, *et al*. Short chain fatty acid rectal irrigation for left-sided ulcerative colitis: a randomised, placebo controlled trial. *Gut* 1997;**40**:485–91.
12. Scheppach W, Sommer H, Kirchner T, *et al*. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. *Gastroenterology* 1992;**103**:51–6.
13. Eeckhaut V, Van Immerseel F, Teirlynck E, *et al*. *Butyricicoccus pullicaecorum* gen. nov., sp. nov., an anaerobic, butyrate-producing bacterium isolated from the caecal content of a broiler chicken. *Int J Syst Evol Microbiol* 2008;**58**:2799–802.
14. Eeckhaut V, Machiels K, Perrier C, *et al*. *Butyricicoccus pullicaecorum* in inflammatory bowel disease. *Gut* 2013;**62**:1745–52.
15. Turner JR. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol* 2009;**9**:799–809
16. Groschwitz KR, Hogan SP. Intestinal barrier function: molecular regulation and disease pathogenesis. *J Allergy Clin Immunol* 2009;**124**:3–20.
17. Hering NA, Fromm M, Schulzke J-D. Determinants of colonic barrier function in inflammatory bowel disease and potential therapeutics. *J Physiol* 2012;**590**:1035–44.
18. Weber CR, Nalle SC, Tretiakova M, *et al*. Claudin-1 and claudin-2 expression is elevated in inflammatory bowel disease and may contribute to early neoplastic transformation. *Lab Invest* 2008;**88**:1110–20.
19. Miyazaki K, Martin JC, Marinsek-Logar R, *et al*. Degradation and utilization of xylans by the rumen anaerobe *Prevotella bryantii* [formerly P. *ruminicola* subsp. *brevis*] B[1]4. *Anaerobe* 1997;**3**:373–81.
20. De Weirdt R, Possemiers S, Vermeulen G, *et al*. Human faecal microbiota display variable patterns of glycerol metabolism. *FEMS Microbiol Ecol* 2010;**74**:601–11.
21. Rokbi B, Seguin D, Guy B, *et al*. Assessment of *Helicobacter pylori* gene expression within mouse and human gastric mucosae by real-time reverse transcriptase PCR. *Infect Immun* 2001;**69**:4759–66.
22. Poritz LS, Garver KI, Green C, *et al*. Loss of the tight junction protein ZO-1 in dextran sulfate sodium induced colitis. *J Surg Res* 2007;**140**:12–9.
23. Kontoyiannis D, Pasparakis M, Pizarro TT, *et al*. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity* 1999;**10**:387–98.
24. Zeissig S, Bürgel N, Günzel D, *et al*. Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut* 2007;**56**:61–72.
25. Heller F, Florian P, Bojarski C, *et al*. Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. *Gastroenterology* 2005;**129**:550–64.
26. Furuse M, Hata M, Furuse K, *et al*. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J Cell Biol* 2002;**156**:1099–111.
27. Poritz LS, Harris LR, Kelly AA, *et al*. Increase in the tight junction protein claudin-1 in intestinal inflammation. *Dig Dis Sci* 2011;**56**:2802–9.
28. Pope JL, Bhat AA, Sharma A, *et al*. Claudin-1 regulates intestinal epithelial homeostasis through the modulation of Notch-signalling. *Gut* 2014;**63**:622–34.
29. Amasheh M, Fromm A, Krug SM, *et al*. TNFalpha-induced and berberine-antagonized tight junction barrier impairment via tyrosine kinase, Akt and NFkappaB signaling. *J Cell Sci* 2010;**123**:4145–55.
30. Bruewer M, Utech M, Ivanov AI, *et al*. Interferon-gamma induces internalization of epithelial tight junction proteins via a macropinocytosis-like process. *FASEB J* 2005;**19**:923–33.
31. Nava GM, Stappenbeck TS. Diversity of the autochthonous colonic microbiota. Gut Microbes 2011;2:99–104.