



# Higher Prevalence of *Bacteroides fragilis* in Crohn's Disease Exacerbations and Strain-Dependent Increase of Epithelial Resistance

Heike E. F. Becker<sup>1,2</sup>, Casper Jamin<sup>3</sup>, Liene Bervoets<sup>1</sup>, Annemarie Boleij<sup>4</sup>, Pan Xu<sup>2</sup>, Marie J. Pierik<sup>2</sup>, Frank R. M. Stassen<sup>1</sup>, Paul H. M. Savelkoul<sup>1,5</sup>, John Penders<sup>1,3\*</sup> and Daisy M. A. E. Jonkers<sup>2\*</sup>

<sup>1</sup> Department of Medical Microbiology, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre+, Maastricht, Netherlands, <sup>2</sup> Division of Gastroenterology/Hepatology, Department of Internal Medicine, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre+, Maastricht, Netherlands, <sup>3</sup> Department of Medical Microbiology, Caphri School for Public Health and Primary Care, Maastricht University Medical Centre+, Maastricht, Netherlands, <sup>4</sup> Department of Pathology, Radboud Institute for Molecular Life Sciences (RIMLS), Radboud University Medical Center, Nijmegen, Netherlands, <sup>5</sup> Department of Medical Microbiology and Infection Control, Amsterdam University Medical Center, Location VUMC, Amsterdam, Netherlands

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### \*Correspondence:

John Penders  
j.penders@maastrichtuniversity.nl  
Daisy M. A. E. Jonkers  
d.jonkers@maastrichtuniversity.nl

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*Bacteroides fragilis* has previously been linked to Crohn's disease (CD) exacerbations, but results are inconsistent and underlying mechanisms unknown. This study investigates the epidemiology of *B. fragilis* and its virulence factors *bft* (enterotoxin) and *ubiquitin* among 181 CD patients and the impact on the intestinal epithelial barrier *in vitro*. The prevalence of *B. fragilis* was significantly higher in active ( $n = 69/88$ , 78.4%) as compared to remissive ( $n = 58/93$ , 62.4%,  $p = 0.018$ ) CD patients. Moreover, *B. fragilis* was associated with intestinal strictures. Interestingly, the intestinal barrier function, as examined by transepithelial electrical resistance (TEER) measurements of Caco-2 monolayers, increased when exposed to secretomes of *bft*-positive (*bft-1* and *bft-2* isotype; increased TEER  $\sim 160\%$ ,  $p < 0.001$ ) but not when exposed to *bft*-negative strains. Whole metagenome sequencing and metabolomics, respectively, identified nine coding sequences and two metabolites that discriminated TEER-increasing from non-TEER-increasing strains. This study revealed a higher *B. fragilis* prevalence during exacerbation. Surprisingly, *bft*-positive secretomes increased epithelial resistance, but we excluded Bft as the likely causative factor.

**Keywords:** *Bacteroides fragilis*, Crohn's disease, barrier function, prevalence, metabolomics, whole genome sequencing, organoids

## INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disease, characterized by patchy inflammation of the intestinal mucosa with or without extra-intestinal manifestations (Baumgart and Sandborn, 2012). The disease course varies largely among patients with alternating periods of remission and exacerbations. Insufficient control of the recurrent mucosal inflammation results in phenotype progression and complications, such as strictures or fistulas, contributing to a high disease and economic burden (Solberg et al., 2007; Floyd et al., 2015).

CD onset is considered to involve genetic predisposition, environmental factors and an adverse immune reaction to the host microbiota (Baumgart and Sandborn, 2012). However, the factors influencing the occurrence of exacerbations, complications and disease phenotype remain largely unclear.

In recent years, microbial dysbiosis gained increasing attention as a factor contributing to exacerbations (Baumgart and Sandborn, 2012; Tedjo et al., 2016). Several studies reported a decreased microbial diversity and altered microbial composition in active CD patients compared to remission (Prosberg et al., 2016; Tedjo et al., 2016; Galazzo et al., 2019). As a consequence of compositional changes, alterations in overall microbial functionality are conceivable (Li et al., 2020). Since the intestinal epithelium limits bacterial attachment by a mucus layer, bacteria often interact with the host via their secretome, consisting of metabolites, proteins and bacterial membrane vesicles (MVs) (Kaparakis-Liaskos and Ferrero, 2015; Sicard et al., 2017; Parker et al., 2018; Caruana and Walper, 2020). On the one hand, secreted glycosidases and mucinases have been shown to degrade the mucus layer and allow microbes to directly interact with epithelial cells (van Passel et al., 2011; Sicard et al., 2017). On the other hand, bacterial metabolites, such as the short chain fatty acid butyrate, have been reported to promote mucus production and the sealing capacity of the intercellular junctional complex (Sun et al., 2016; Zheng et al., 2017). In CD, an impaired intestinal epithelial barrier function has increasingly been recognized as a hallmark of exacerbations (Baumgart and Sandborn, 2012). The impaired barrier function has been associated with alterations in the epithelial junctional complex (Zeissig et al., 2007; Lameris et al., 2013). It remains unknown whether the observed altered microbiota composition and functionality during CD exacerbations can contribute to this impaired epithelial barrier.

In addition to alterations in microbial diversity, specific microbial taxa were detected in CD exacerbations (Eeckhaut et al., 2013; Schäffler et al., 2016; Tedjo et al., 2016) of which *Bacteroides fragilis* is a prominent example (Wills et al., 2014; Schäffler et al., 2016; Tedjo et al., 2016). Several studies investigated the colonization of *B. fragilis* during exacerbation and remission in fecal samples and biopsies. Together, the results are inconclusive and based on rather low sample sizes (Scanlan et al., 2006; Wills et al., 2014; Schäffler et al., 2016; Tedjo et al., 2016). So far, it is still unclear whether the prevalence of *B. fragilis* differs in different disease stages of CD and how it may affect disease activity.

*B. fragilis* is a Gram-negative commensal of the phylum Bacteroidetes and some strains secrete various virulence factors, such as *B. fragilis* toxin (Bft; fragilysin) and an eukaryotic-like ubiquitin (Ubb) (Sears, 2009; Stewart et al., 2018). It is yet unclear whether these virulence factors contribute to CD exacerbations.

To gain more insights into the interaction between the intestinal microbiota and the intestinal barrier in CD, especially during dysbiosis, research essentially needs to focus on microbial functionality, such as reflected by the secretome. In this study, we therefore aim to investigate firstly the prevalence and relative abundance of *B. fragilis* and virulence-factor positive strains in a large, well-defined cross-sectional CD patient cohort and

secondly the impact of the *B. fragilis* secretome on the intestinal epithelial barrier as first site of interaction and pathophysiological factor in CD exacerbations. We hypothesize that *B. fragilis* and its virulence factors *bft* and *ubb* are involved in exacerbations and by disrupting the intestinal epithelial barrier.

## MATERIALS AND METHODS

### Patient Inclusion

Fecal samples of CD patients participating in the IBD South Limburg (IBDSL) biobank project (van den Heuvel et al., 2015) were available to evaluate the colonization of *B. fragilis* and relevant strains in CD patients and the relation with disease activity. Baseline characteristics, including medication use and demographic information, were extracted from the IBDSL database (van den Heuvel et al., 2015).

Patients with a fecal calprotectin level >200 µg/g were defined as active ( $n = 88$ ) and patients with a fecal calprotectin level <100 µg/g as remissive ( $n = 93$ ). Remissive patients without previously reported calprotectin level >200 µg/g were excluded to avoid misclassification unless the previously elevated calprotectin level was combined with a CRP value <10 mg/l.

In addition, from two patients having a colonoscopy scheduled for clinical reasons, biopsies were collected from the macroscopically non-inflamed tissue of the ascending colon for crypt isolation and subsequent organoid culture (Xu et al., 2018).

All patients participated in the IBDSL cohort and gave written informed consent prior to sample collection. The study protocol was approved by the Medical Ethics Committee of the Maastricht University Medical Centre+ (NL31636.068.10) and registered on www.clinicaltrials.gov (NCT02130349).

### Fecal DNA Isolation

Fecal samples stored at  $-80^{\circ}\text{C}$  within 24 h after collection were obtained from the IBDSL biobank (van den Heuvel et al., 2015) and cut frozen to obtain  $\sim 0.5$  g feces. DNA isolation was conducted using Qiagen QIAamp DNA mini kit (Qiagen, ref.: 51306) according to protocol Q of the International Human Microbiome Standards consortium (Costea et al., 2017) with minor adjustments in Fastprep<sup>TM</sup> cycles (three series of 1 min of beating at 5.5 ms and 1 min resting) and vacuum drying at  $37^{\circ}\text{C}$  for 7 min instead of 3 min. Eluted DNA was stored in Buffer AE (Qiagen) at  $-20^{\circ}\text{C}$  until further analysis.

### Enumeration of Fecal *B. fragilis*

Metagenomic DNA obtained from the fecal samples was analyzed for the presence and relative abundance of *B. fragilis* as well as the virulence factors *bft* and *ubb* by means of real-time quantitative PCR. Samples containing *B. fragilis* specific *gyrB* were subsequently analyzed for the presence of *bft* and *ubb*.

The PCR mix for *gyrB* and *bft* contained 5 µl DNA, 12.5 µl Absolute quantitative PCR mix (Abgene, Epsom, United Kingdom), 500 mM forward and reverse primer (Sigma-Aldrich; **Supplementary Table 1**), 250 nM probe and was supplemented with DNase-free water to a final volume of 25 µl. qPCR and analysis were conducted with 7900HT Fast Real-Time

PCR System (Applied Biosystems) and SDS 2.3 software (Applied Biosystems). PCR cycles for *gyrB* and *bft* were 2 min at 50°C, 10 min at 95°C, followed by 42 amplification cycles of 15 s at 95°C and 60 s at 60°C.

PCR mix for *ubb* and *16S rRNA* contained 2 µl DNA, 12.5 µl Absolute qPCR SYBR Green supermix (Bio-Rad, Hercules, CA), 300 nM forward and reverse primer (Sigma-Aldrich; **Supplementary Table 1**) and was supplemented with DNase-free water to a final volume of 25 µl. qPCR and analysis were conducted with MyIQ Single Color Real-Time PCR Detection System (BioRad) and iQ5 software (BioRad). PCR cycles for *ubb* and *16S rRNA* were 3 min at 95°C, followed by 40, respectively, 35 amplification cycles of 95°C, 20 s at 63°C, respectively, 55°C, and 30 s at 72°C. The melting curve was assessed in 60 cycles of 0.5°C for 10 s each.

## B. fragilis Isolation From Fecal Samples

To retrieve toxigenic and non-toxigenic *B. fragilis* strains for *in vitro* experiments, a random selection of fecal samples of three CD patients and two samples of healthy controls (HC) from a previous study that were positive for *B. fragilis* specific *gyrB* were cultured. A portion of ~1 g was dissolved in Brain Heart Infusion broth (BHI; Sigma Millipore, ref.: 53285, Darmstadt, Germany) containing 4 µg/ml Vancomycin hydrochloride (No 15327, Cayman Chemical, Michigan, United States) and incubated in an anaerobic jar (80% N<sub>2</sub>, 10% CO<sub>2</sub>, 20% H<sub>2</sub>) at 37°C for 24 h. Next, 1:100 dilutions were inoculated on Bacteroides Bile Esculin Agar with Amikacin (Becton Dickinson, ref.: 254480, Landsmeer, Netherlands) and incubated for 48 h under the same conditions. Medium to large sized single colonies were subsequently inoculated on Columbia Agar with 5% Sheep Blood (Becton Dickinson, ref.: 254005, Landsmeer, Netherlands) and incubated for 48 h, as described before. This was repeated to guarantee pure cultures. A single colony was then collected in DNase-free water and checked for *gyrB*, *bft* and *ubb* using qPCR as described above. The PCR products of *bft* and *ubb* were confirmed using Sanger sequencing to avoid false positive results. Amplicons were purified from remaining nucleotides using the MSB Spin PCRapace-kit (Stratagene molecular, ref.: 1020220400) according to manufacturer's descriptions. One microliter of DNA was then added to 5.5 µl DNase-free water, 1 µl forward or reverse primer (2 pmol/l), 1.5 µl BDT 1.1 buffer and 1 µl BDT v1.1 enzyme mix. The DNA was amplified with one cycle of 1 min at 96°C and 22 cycles of 10 s at 96°C, 5 s at 58°C and 2–3 min at 60°C. Subsequently, the amplicons were sequenced using an ABI 3730 DNA Analyzer (Thermo Fisher Scientific) and were compared to the Nucleotide Basic Local Alignment Search Tool (BLAST; NCBI) database for sequence similarity with previously sequenced *B. fragilis* derived *bft* and *ubb*.

In total, five *B. fragilis* strains were isolated. As no *ubb*-positive, *bft*-negative genotype was identified, a reference strain from the American Type Culture Collection (ATCC® 25285<sup>TM</sup>) was obtained. Two further reference strains were kindly provided by prof. Sears (John Hopkins University, Baltimore, United States), namely *bft-1*-positive VPI 18786 (VPI) and *bft-2*-positive 86-5443-2-2 (086) and their respective *bft* deletion mutants (VPI  $\Delta bft$ , 086  $\Delta bft$ ) (Myers et al., 1984; Myers and Schoop, 1987;

Rhee et al., 2009; Chung et al., 2018). Finally, the following strains were available for further analysis: one *ubb*-positive strain (ATCC® 25285<sup>TM</sup>), two *bft*-positive strains (CD-2, CD-3), one strain positive for both *bft* and *ubb* (HC-1), two strains negative for both virulence factors (HC-2, CD-1; **Supplementary Table 2**), one *bft-1*-positive strain (VPI) with corresponding deletion mutant (VPI  $\Delta bft$ ), and one *bft-2*-positive strain (086) with corresponding deletion mutant (086  $\Delta bft$ ).

## B. fragilis Supernatant

To study the impact of excreted *B. fragilis* products on the intestinal barrier *in vitro*, bacterial culture supernatant was used. Therefore, three to five colonies of *B. fragilis* strains were inoculated in BHI (Sigma Millipore, ref.: 53285) for 24 h at 37°C in anaerobic jars. Subsequently, bacterial cultures were centrifuged at 4,500 × g for 15 min at 4°C and supernatant was filtered through 0.2 µm pore size syringe filters (Pall Life Sciences, ref: 4652). To increase the sensitivity, filtered supernatant was concentrated 20× using Amicon® Centrifugal Units 10 K (Merck Millipore, ref.: UFC8010) at 4,000 × g for 20 min. Cell-free supernatant was stored at 4°C and used for transepithelial electrical resistance (TEER) experiments within 24 h.

## B. fragilis MVs

To investigate the impact of MVs on intestinal epithelial barrier function we separated the MVs from the *B. fragilis* cell-free supernatant. First, 30 ml of supernatant (see above) was concentrated to ~250 µl during several centrifugation steps at 4,000 × g at 4°C using Ultracentrifugal Filters of 100 kDa (Merck Millipore, ref: UFC9100). After collection of the concentrate, the filter was washed with 250 µl PBS to recover the remaining particles. MVs were isolated by Size Exclusion Chromatography using CL-2B Sepharose (GE healthcare, Little Chalfont, United Kingdom). As described by Benedikter et al. (2017) vesicle-rich fractions were pooled and the concentration was determined by tunable resistive pulse sensing using qNano Gold (Izon Science Ltd., Oxford, United Kingdom) and Izon Control Suite software v3.2.

## Bft Secretion and Activity

To examine the presence of Bft in the cell-free *B. fragilis* supernatant, we isolated the total protein fraction following the protocol of Casterline et al. (2017) with minor modifications. One milliliter of culture supernatant was precipitated with 10% (v/v) trichloric acid in acetone containing 20 mM 1,4-dithiothreitol (DTT) for 1 h on ice and centrifuged at 15,000 × g for 15 min. The pellet was washed twice with acetone, carefully resuspended and centrifuged at full speed for 15 min. The pellet was then recovered in 2× Laemmli buffer, containing 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue and 0.125 M Tris-HCl, and stored at -20°C. For subsequent western blot analysis, the samples were further denatured at 95°C for 10 min and loaded on a 13% SDS gel according to standard protocol. After separation and wet blotting on an Amersham<sup>TM</sup> Protran<sup>TM</sup> 0.2 µm nitrocellulose membrane (GE Healthcare), the membrane was blocked with skim milk in TBST for 1 h at RT. The membrane was then incubated with anti-Bft antibody

(Cusabio, ref.: CSB-PA346537LA01BDP) diluted 1:2,000 for 36 h at 4°C, followed by goat-anti-rabbit antibody conjugated with horseradish peroxidase (Dako p0448) diluted 1:2,000 for 30 min at RT and developed with 0.1 M Tris pH 7.5, 0.2 mg/ml DAB, 0.01% NiCl<sub>2</sub> and 0.03% H<sub>2</sub>O<sub>2</sub>. Pictures were taken with Mini HD 9 (Uvitec, Cambridge, United Kingdom).

Bft activity was confirmed by a slightly adapted HT-29 cell rounding assay (Weikel et al., 1992; Mundy and Sears, 1996). In brief, HT-29 cells were cultured for 5 days in a 96-well plate at a density of 2,000 cells/well with Dulbecco's Modified Eagle Medium (DMEM; Sigma Aldrich, ref.: D6429) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, ref. 10500) and 1% antibiotic-antimycotic (100×; anti-anti; Gibco, ref.: 15240-062). Cells were then washed thrice with PBS (Gibco, Life Technologies, ref.: 10010-031) and incubated overnight with 20× concentrated *B. fragilis* cell-free supernatant diluted 1:10 in HT-29 culture medium without FBS at 37°C. Cell rounding was examined by confocal light microscopy (Leica Microsystems GmbH, Mannheim, Germany), images were taken using LAS-AF software (Leica Microsystems) and analyzed using ImageJ (Abràmoff et al., 2004).

## Barrier Function Analysis

Caco-2 cell monolayers (passage number 47–57), as a well-established *in vitro* model for intestinal epithelial barrier function (Srinivasan et al., 2015), were seeded on Millicell Hanging Cell Culture Inserts (Merck Millipore, ref.: MCHT24H48) at a density of 100,000 cells/insert in DMEM supplemented with 1% FBS, 0.1% non-essential amino acids (Gibco, ref.: 11140050) and 0.1% anti-anti. Culture medium was refreshed every 2–3 days in both compartments. Monolayers were allowed to differentiate during 14–21 days at 37°C and 5% CO<sub>2</sub> and the TEER was evaluated using the EVOM2 Epithelial Volt/Ohm Meter (World Precision Instruments, Sarasota, FL, United States). Mature monolayers (TEER > 600 Ω·cm<sup>2</sup>) were luminally exposed to BHI or bacterial-free concentrated culture supernatant, each diluted 1:10 in Caco-2 culture medium, or to PBS or a final concentration of 5 × 10<sup>7</sup> MVs in PBS, each diluted 1:2 in Caco-2 culture medium, and incubated for 24 h. To investigate whether *B. fragilis* supernatant is able to prevent cytokine induced barrier disruption, experimental conditions were pre-incubated basally with 100 ng/ml tumor necrosis factor α (TNF-α; Sigma, ref.: T6674) in combination with 100 ng/ml interferon γ (IFN-γ; Sigma, ref.: I3265) for 1 h, before *B. fragilis* supernatant was added luminally. After another 24 h of co-incubation, TEER values were assessed and expressed as percentage of the TEER value prior to incubation.

To confirm that relevant findings were not restricted to Caco-2 cells, concentrated *B. fragilis* culture supernatant was also applied to the more physiological model of colonic patient-derived organoids. Therefore, colonic biopsies were collected in cold PBS, washed thrice with 1% anti-anti in PBS, thrice with 10 mM DTT in PBS and incubated with 2 mM EDTA in PBS for 1 h at 4°C and 5 rpm. Biopsies were transferred to PBS and crypts were separated by several rounds of mechanical shaking. The pooled crypt supernatant was then supplemented with 5% FBS and centrifuged at 400 × g for 8 min at 4°C. The pellet was washed twice with 2 ml cold basal medium (DMEM/F12;

Gibco, ref.: 12634-010) supplemented with 1% GlutaMax™ (Life Technologies), 1% HEPES buffer (Life Technologies) and 5% FBS. After centrifugation at 400 × g for 3 min, crypts were plated in GelTrex™ (Gibco, ref.: A1413201) and IntestiCult™ Organoid Growth Medium (StemCell Technology GmbH, Germany, ref.: SC-06010) was added 15 min after incubation at 37°C, 5% CO<sub>2</sub>. Medium was refreshed every 3 days and passaged when the morphology appeared complex, according to previous descriptions (Sato et al., 2011). In brief, GelTrex™ with enclosed organoids was solubilized on ice for 15 min. Culture medium was replaced by ice cold basal medium. Organoids were disrupted mechanically using a 1000P pipette and centrifuged at 150 × g for 5 min. The supernatant was replaced by TrypLE™ Express (ThermoFisher Scientific, ref.: 12605010) containing 10 μM γ27632 (Tebu-bio BV) and incubated at 37°C for 2 min. Organoids were then dissociated mechanically using a firepolished Pasteur pipetted, washed and centrifuged twice with basal culture medium at 400 × g for 5 min. Finally, the pellet was resuspended with GelTrex™ and cultured as described above.

Single lumen organoids (passage numbers 10 and 11) were incubated basolaterally with BHI or *B. fragilis* culture supernatant, diluted 1:10 in organoid medium, or organoid medium only at 37°C for 24 h. Epithelial barrier function was evaluated by 1 mg/ml fluorescein isothiocyanate-labeled dextran of 4 kDa (FITC-d4; Sigma) added 6–8 h before confocal light microscopy (Leica Microsystems GmbH, Mannheim, Germany). Images were taken using LAS-AF software and processed using ImageJ. The experiment was conducted twice and at least eight organoids were evaluated per condition and experiment.

## Analysis of Tight- and Adherens Junction Expression

After TEER measurements of Caco-2 monolayers, RNA was isolated using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions, including DNase treatment for 15 min. RNA quantity and purity were determined using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, United States). cDNA was synthesized using 75% RNA template, 20% iScript reaction mix and 5% reverse transcriptase (by iScript cDNA Syntgeses Kit BioRad) and the recommended cycle of 5 min 25°C, 30 min 42°C and 5 min 85°C.

Real-time qPCR was performed according to manufacturer's instructions using SYBR Green Supermix (BioRad, Veenendaal, Netherlands, ref.: 1708885) and CFX 96 Real Time System C1000 Touch Thermal Cycler (Bio-Rad). Primer sequences are listed in **Supplementary Table 3**. Expression of mRNA was analyzed relative to GAPDH, using the 2<sup>-ΔCt</sup> method (Schmittgen and Livak, 2008).

## Analysis of Tight Junction Localization

After TEER measurements of Caco-2 monolayers, the cells were washed with PBS, fixed with 4% (w/v) paraformaldehyde as described previously (Elamin et al., 2012). The protocol was adjusted by 30 min permeabilization with 0.5% (v/v) Triton X-100 and washing with PBS instead of Hank's balanced salt solution. Monolayers inside the transwells were incubated with

FITC-conjugated Zonulin-1 antibodies (1:200; Invitrogen, ref.: 339111) overnight at 4°C. Confocal images were obtained as described in section “Barrier Function Analysis.”

## Whole Genome Sequencing

To characterize the isolated *B. fragilis* strains and to predict possible differences in secreted proteins, the genome of all six strains was sequenced. Genomic DNA was isolated from single colonies resuspended in PBS using MasterPure™ Complete DNA and RNA purification kit (Epicenter, MC 85200), following the manufacturer's protocol. Library preparation sequencing and *de novo* assembly was performed as described previously (Flipse et al., 2019). Coding sequences were annotated using Prokka (v1.7) (Seemann, 2014). Gene presence/absence was determined using Roary (v3.11.2) (Page et al., 2015). SNP distances among isolates was determined using SKA (v1.0) (Harris, 2018). All bioinformatics tools were run in default settings. Sequencing data are deposited at European Nucleotide Archive (ENA) with accession number PRJEB41450.

## Metabolite Identification Using NMR Spectroscopy

To further examine potential differences in excreted metabolites by the different *B. fragilis* strains, the culture supernatants were analyzed using proton (<sup>1</sup>H) NMR spectroscopy. Therefore, 150 μl of 20× concentrated *B. fragilis* culture supernatant was diluted in 420 μl 100 mM phosphate buffer (pH 7.4 at 25°C) and 30 μl Deuterium oxide (D<sub>2</sub>O, 99 atom%D, 100G, Sigma-Aldrich, Germany) containing 1 mM 3-(trimethylsilyl)propionic-2,2,3,3-d<sub>4</sub> acid (TSP, 98 atom%D TSP, 1G, Sigma-Aldrich, Germany). The samples were then transferred into a 5 mm NMR tube (Bruker samplejet, Sigma-Aldrich, Germany). All <sup>1</sup>H NMR spectra were recorded on a 700 MHz Bruker Avance spectrometer (Bruker, Germany) at 300 K. Spectra were acquired using a one-dimensional NOESY-presat pulse sequence (RD-90°-t-90°-tm-90°-ACQ), an acquisition time of 2 s, a relaxation delay (D1) of 5 s, mixing time (D8) of 100 ms, receiver gain of 32, 64 scans, 45 K data points and a spectral width of 11,161 Hz (15.934 ppm). Spectral preprocessing and preliminary comparisons of the spectra were performed using the Bruker TopSpin 3.2 software.

## Statistical Analyses

In order to compare the prevalence and relative abundance of *B. fragilis* (virulence factors) in active and remissive CD patients' samples, the  $\chi^2$ -test with Yate's correction and Wilcoxon rank sum test (for samples tested positive for the respective gene) were performed. To predict carriage of *B. fragilis*, based on the combination of patient baseline characteristics (age, gender, Montreal classification, and medication use), multivariate logistic regression was conducted using backward stepwise regression based on the likelihood ratio. Analyses were computed in IBM SPSS Statistics 25 and statistical significance was considered when  $p < 0.05$ .

Comparing the effects of *B. fragilis* supernatant and MVs on TEER of Caco-2 epithelial monolayers one-way ANOVA and Tukey's *post hoc* test was performed using GraphPad Prism 5. For

subsequent analysis of differences in tight junction expression, student's *t*-test was applied. Statistical significance was considered when  $p < 0.05$ . Analyses were based on at least duplicate experiments with each three technical replicates.

Partial Least Squares Discriminant Analysis (PLS-DA) was conducted to identify biologically relevant metabolites that differ between TEER-increasing and non-TEER-increasing groups using MetaboAnalyst v2.0 (Chong et al., 2019). The selection of the most discriminating metabolites, that are responsible for the pattern of the PLS-DA score plot, was based on both the interpretation of PLS-loading plot and Variable Importance in Projection (VIP) scores. Metabolites were identified using HMDB 4.0 (The Human Metabolome Database) (Wishart et al., 2018).

## RESULTS

### Patient Population

In total, 181 CD patients, 88 with active disease and 93 in remission, from the IBDL (van den Heuvel et al., 2015) cohort fulfilling the inclusion criteria were available for the present study. Baseline characteristics were comparable between patient groups, although steroid use was slightly higher among patients with active disease (Table 1).

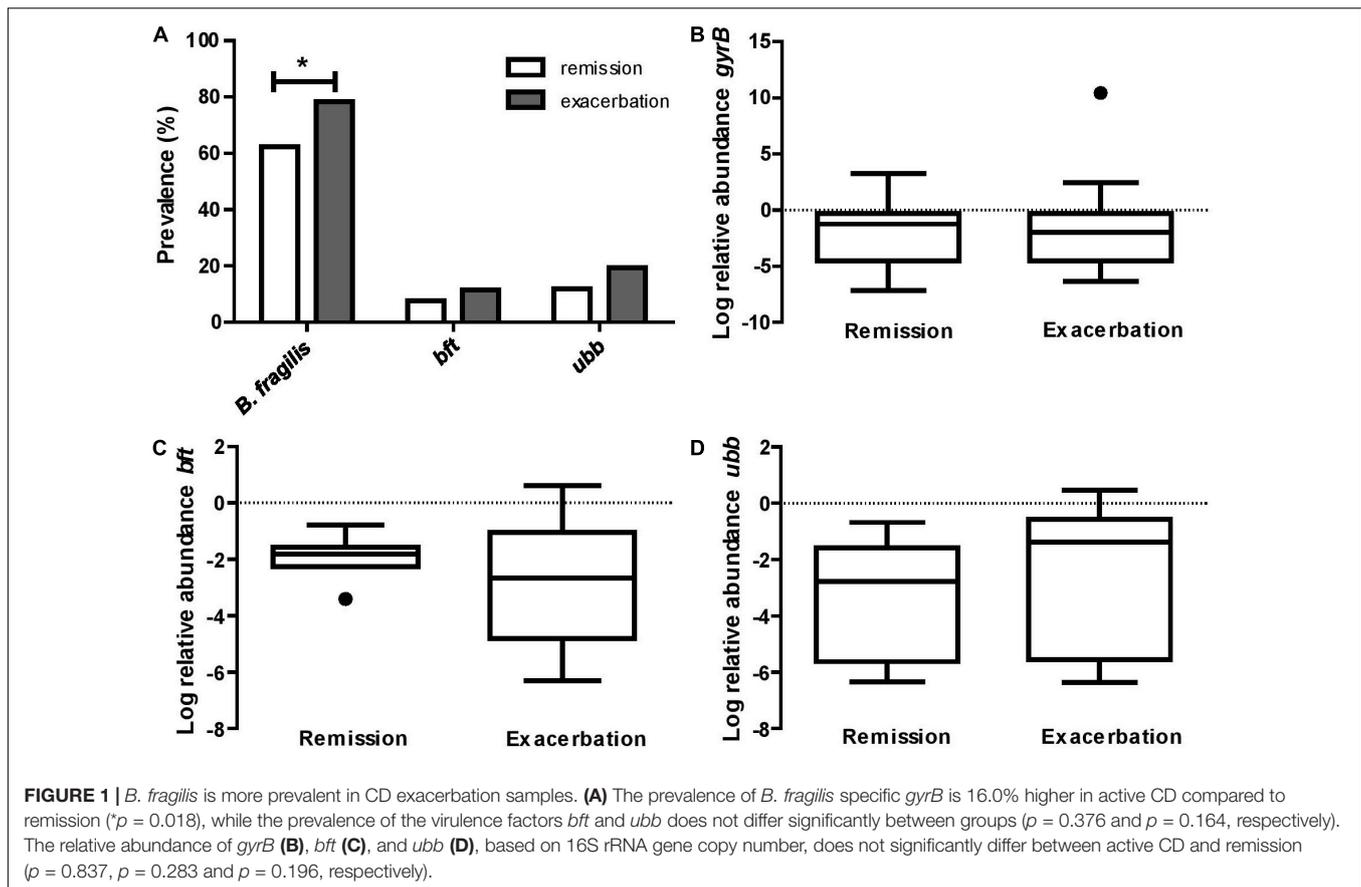
### B. fragilis Prevalence and Relative Abundance

Based on the detection of the *B. fragilis* specific *gyrB* gene, the prevalence of *B. fragilis* was significantly higher among CD patients during an exacerbation (78.4%) when compared to those in remission (62.4%,  $p = 0.018$ , Figure 1A). We subsequently determined the prevalence of the *B. fragilis* virulence factors *bft*

TABLE 1 | Baseline characteristics of the CD patient cohort.

	CD remission (n = 93)	CD exacerbation (n = 88)
Age (median in years; IQR)	43.0 (32.0–56.5)	45.0 (28.0–57.0)
Gender (% female)	63.4	51.1
Calprotectin (median; IQR)	22.0 (14.0–55.5)	476.5 (325.8–703.0)
<b>Montreal classification (%)</b>		
A1; A2; A3	6.5; 67.7; 25.8	10.2; 61.4; 28.4
L1; L2; L3; L4	35.5; 17.2; 47.3; 4.3	23.9; 21.6; 54.5; 11.4
B1; B2; B3; p	59.1; 19.4; 31.2; 17.2	62.5; 25.0; 17.1; 11.4
Surgery	34.4	31.8
<b>IBD medication use (%)</b>		
Thiopurine	34.4	29.6
Biologicals	58.1	48.9
Steroids	17.2	30.7
Other	13.4	18.2

L1, terminal ileum; L2, colon; L3, ileocolon; A1, <16 years, A2, 17–40 years, A3, >40 years at diagnosis; B1, without stricture formation, non-penetrating; B2, stricturing; B3, penetrating.



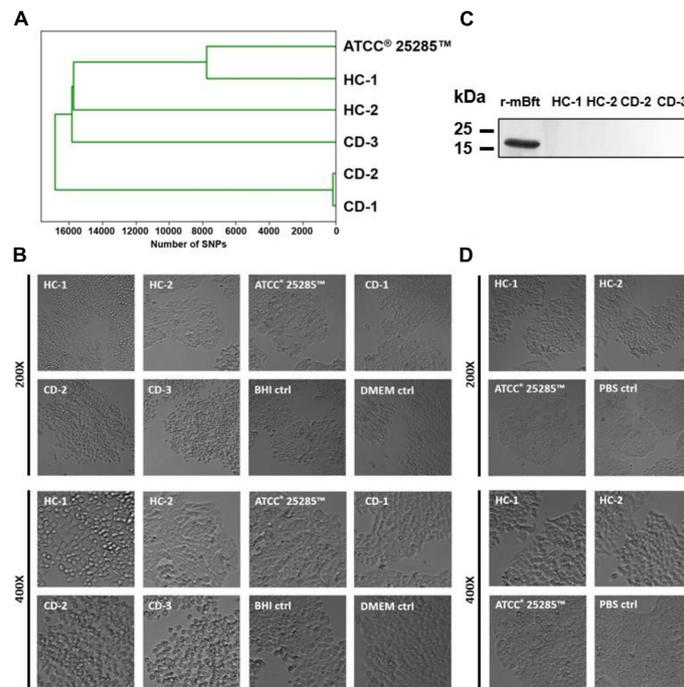
and *ubb*. Only 11.4 and 7.5% ( $p = 0.376$ ) of the samples from the exacerbation and remission group, respectively, were positive for *bft*. When considering only samples that were tested positive for *B. fragilis*, this equals to a proportion of *bft*-positive samples of 14.5 and 12.1% ( $p = 0.689$ ), respectively. For *ubb*, the prevalence was 19.3 and 11.8% ( $p = 0.164$ ) in the entire exacerbation and remission group, respectively, and 24.6 and 19.0% ( $p = 0.442$ ), when considering the *gyrB* positive samples only (Figure 1A). The relative abundance of *gyrB*, *bft* and *ubb* was not significantly different between remission and exacerbation (Figures 1B–D).

Based on multivariable logistic regression, we found the following patient characteristics to be correlated with overall *B. fragilis* colonization: a stricturing disease behavior (Odds ratio (OR) = 3.212, 95% CI = 1.218; 8.469,  $p = 0.018$ ), active Crohn's disease (OR = 2.048, 95% CI = 1.042; 4.027  $p = 0.038$ ), and previous intestinal resections (OR = 0.406, 95% CI = 0.194; 0.848,  $p = 0.016$ ).

## *B. fragilis* Strain Isolation and Characteristics

In order to examine the impact of *B. fragilis* and its virulence factors on barrier function, we selected five *B. fragilis* strains cultured from samples of two healthy controls (HC-1, HC-2) and three CD patients (CD-1, CD-2, CD-3) and additionally included one reference strain (ATCC® 25285™). Based on whole genome sequencing, pairwise genetic distance revealed

only 160 SNPs between the CD-2 (*bft*-positive) and the CD-1 (*bft*-negative) strains, whereas the median distance was 16960 SNPs among all strains (Figure 2A). The genomic similarity was 89.7%, based on shared split kmers between these two strains using Jaccard dissimilarity (mean Jaccard index 59.9%). This indicates a large shared genomic backbone in these two strains. Sanger sequencing confirmed the presence of *bft-1* in HC-1, CD-2, and CD-3 and the presence of *ubb* in HC-1 and ATCC® 25285™ with 93–99 and 96–99% sequence similarity, respectively. HC-2, ATCC® 25285™, and CD-1 were confirmed negative for *bft*, and HC-2, CD-1, CD-2, and CD-3 for *ubb*. Cell rounding of subconfluent HT-29 cells could be detected in response to the supernatants of all *bft*-positive strains, which was interpreted as Bft activity (Figure 2B and Supplementary Figure 1). No cell rounding was detected in *bft*-negative strains, including both *bft* deletion mutants, which supported the assay specificity. However, the concentration of Bft was found to be below the detection limit of western blot analysis (Figure 2C). In addition, no cell rounding was detected when applying concentrated culture supernatant of *bft-1* positive strains (HC-1, CD-2, CD-3) on Caco-2 cells (data not shown) or colonic organoids (CD-3; Figure 3E), which suggests a cell type-dependent response to Bft. No cell rounding was detected on *bft-1* positive strains derived MVs, suggesting that the isolated MVs do not contain or release relevant amounts of (active) Bft (Figure 2D).



**FIGURE 2** | Characteristics of cultured *B. fragilis* strains. **(A)** Based on whole genome sequencing, pairwise genetic distance revealed 160 SNPs between CD-1 and CD-2, whereas the median distance was 16960 SNPs. **(B)** Cell rounding of subconfluent HT-29 cells could be detected in the supernatants of all *bft*-positive strains (HC-1, CD-2, CD-3). **(C)** Mature Bft could not be detected in the supernatant of *bft*-positive strains when compared to 200 ng of recombinant mature Bft (r-mBFT). **(D)** Cell rounding of subconfluent HT-29 cells could not be detected in response to *bft*-positive strain-derived MVs.

## Barrier Modulation by *B. fragilis* Supernatant and MVs

After 24 h exposure to concentrated supernatant of *bft*-positive strains, no decrease in TEER was detected. Instead, TEER values increased up to 160% ( $p < 0.001$ ) when compared to BHI control and to the concentrated culture supernatant of naturally *bft*-negative strains (Figure 3A). A similar increase by *bft*-positive supernatant was also observed after 1 h basal pre-incubation with 100 ng/ml TNF- $\alpha$  and 100 ng/ml IFN- $\gamma$  (+cyt), while *bft*-negative culture supernatant did not alter TEER (Figure 3B). To examine the potential role of *bft-1* in the observed TEER increase, we applied the concentrated culture supernatant of a *bft-1*-positive reference strain and its respective deletion mutant. In addition, we examined whether the effect is isotype specific by the additional inclusion of a *bft-2* reference strain and its respective deletion mutant.

For *bft-1*-positive VPI and *bft-2*-positive 086, as well as their respective deletion mutants, a significant TEER increase was detected (Figure 3C).

Besides culture supernatant, we also examined the effect of MVs, which are vesicles released by Gram positive and Gram negative bacteria and can deliver a variety of products, including toxins, to host cells or neighboring bacteria (Brown et al., 2015; Kaparakis-Liaskos and Ferrero, 2015; Caruana and Walper, 2020). After 24 h incubation with  $5 \times 10^7$  MVs/ml, no alterations in TEER were observed in differentiated Caco-2 cell monolayers (Figure 3D).

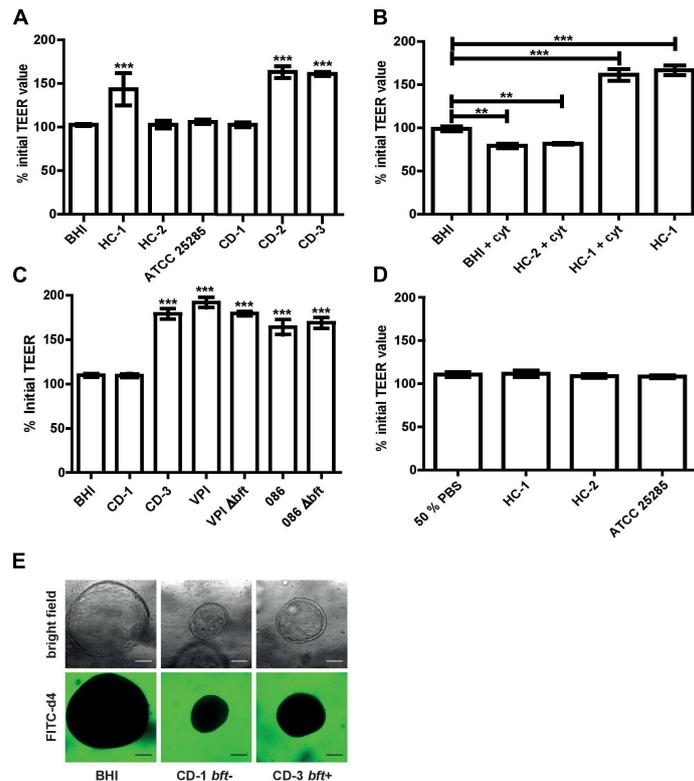
Using a more physiological patient-derived colonic organoid model and a functional FITC-d4 permeation assay, we confirmed that the supernatant of different *B. fragilis* strains did not disrupt the epithelial barrier nor altered the morphology upon 24 h basal incubation (Figure 3E).

## Junctional Alterations in Response to *B. fragilis* Supernatant

To further elucidate the mechanisms underlying the TEER enhancing effect of Bft-positive culture supernatant, we performed qPCR to analyze the mRNA expression of various junctional genes and the proto-oncogene *c-myc* in Caco-2 cells. When comparing one *bft*-positive (CD-3) and one *bft*-negative (CD-1) strain in quadruplicate, we could not detect any significant alterations in gene expression levels of junctional genes or *c-myc* (Figure 4 and Supplementary Figure 2). When evaluating ZO-1 protein localization, no alterations were observed comparing exposure to CD-1 and CD-3 supernatants (Supplementary Figure 3).

## Identification of *B. fragilis* Genome and Metabolome

To identify potential bacterial proteins that may strengthen the epithelial barrier, coding sequences of TEER-elevating strains were compared to those of non-TEER-elevating strains. Herein, nine proteins were identified which were only present in TEER-elevating strains: Bft1, Metalloprotease II



**FIGURE 3 |** Barrier function examination of Caco-2 monolayer and colonic organoids exposed to *B. fragilis* culture supernatant and MVs. **(A)** Luminally applied *B. fragilis* concentrated culture supernatant of HC-1, CD-2, and CD-3 led to increased TEER after 24 h. **(B)** Pre-incubation with 100 ng/ml TNF- $\alpha$  and IFN- $\gamma$  could not impair the TEER enhancing effect of HC-1. **(C)** Luminally applied *B. fragilis* concentrated culture supernatant of 086, 086  $\Delta bft-2$ , VPI, and VPI  $\Delta bft-1$  led to increased TEER after for 24 h. **(D)** Luminally applied MVs, isolated from different *B. fragilis* strains, did not lead to TEER alterations in Caco-2 cell monolayers after 24 h. **(E)** CD patient-derived colonic organoids treated basally with *B. fragilis* CD-1 and CD-3 concentrated supernatant and FITC d4 (green) remain a stable barrier after 24 h incubation. Means  $\pm$  SD was based on triplicates of one representative experiment. cyt, cytokines; BHI, brain heart infusion broth. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

(MPII), putative amidoligase, transposase like protein, putative transposase/insertion sequence protein, and four hypothetical proteins (Table 2).

To further identify metabolites produced by the different *B. fragilis* strains that might contribute to the observed barrier enhancing effect, the concentrated culture supernatants were analyzed using  $^1\text{H}$  Nuclear Magnetic Resonance (NMR) spectroscopy. All strains show comparable metabolic profiles (Figure 5A). Based on PLS-DA, the TEER-enhancing and non-TEER-enhancing groups could be discriminated based on a decreased relative concentration of acetate and lactate in the TEER-enhancing group (Figure 5B).

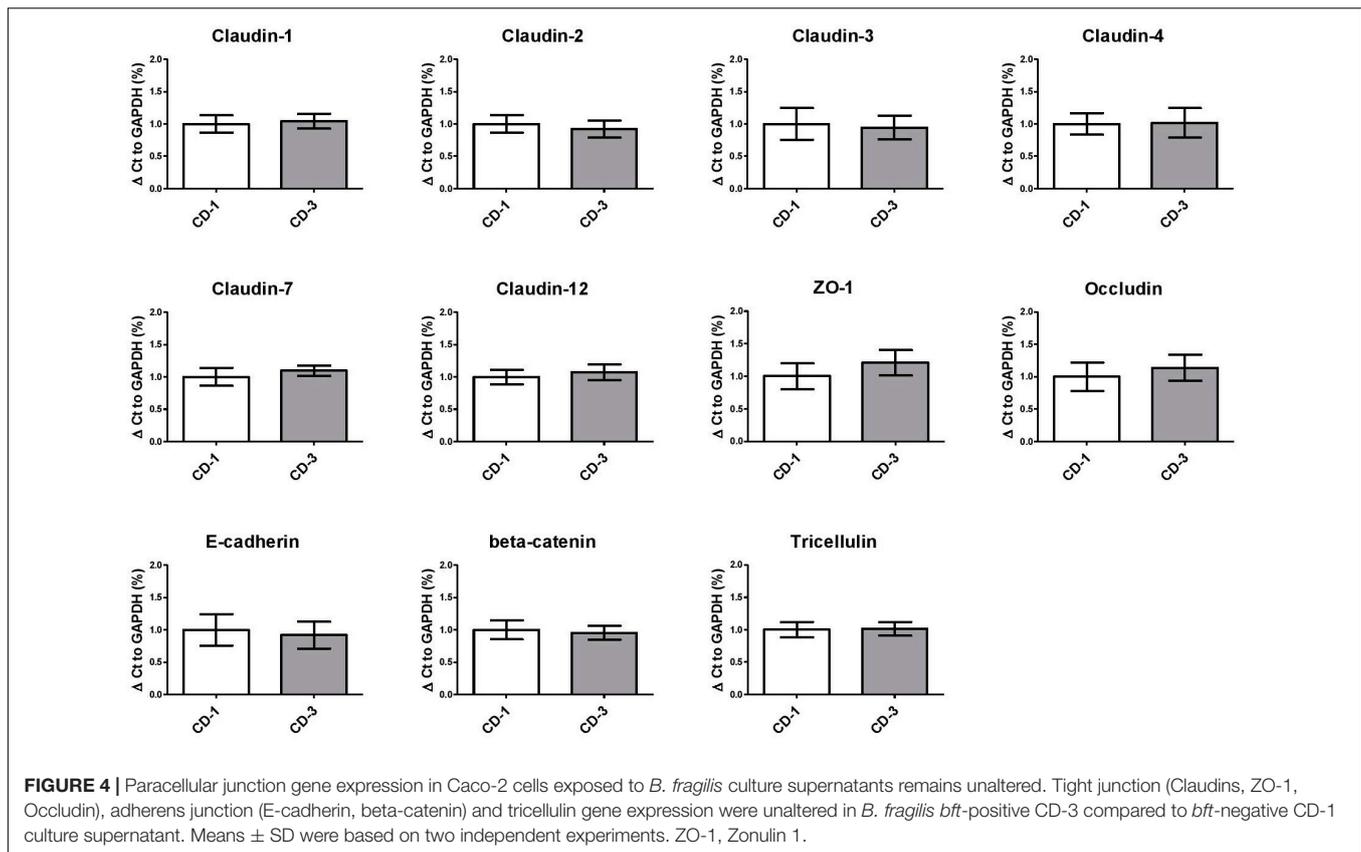
## DISCUSSION

Previously, *B. fragilis* colonization has been investigated in CD exacerbations, together providing inconclusive results (Prindiville et al., 2000; Wills et al., 2014; Schäffler et al., 2016; Tedjo et al., 2016). Based on previous research, we hypothesized that *B. fragilis* and its virulence factors *bft* and *ubb* are associated with exacerbations and may contribute to exacerbations by

disruption of the intestinal epithelial barrier (Wells et al., 1996; Chambers et al., 1997; Obiso et al., 1997; Wu et al., 1998; Riegler et al., 1999).

In the present study, we indeed observed a higher *B. fragilis* prevalence among active CD patients when compared to patients in remission, but not in virulence factor-positive strains. Furthermore, disruption of the intestinal epithelial barrier by supernatant of various (non)virulent *B. fragilis* strains could not be observed. Intriguingly, Bft-positive culture supernatant even led to a significant increase of intestinal epithelial barrier resistance in Caco-2 monolayers.

The cross-sectional investigation of *B. fragilis* in our large CD cohort demonstrated a higher prevalence in active CD patients. However, no differences in relative abundance were detected when analyzing *B. fragilis* positive samples. Logistic regression analysis confirmed the association between *B. fragilis* colonization and disease activity and additionally highlighted an increased likelihood of *B. fragilis* carriage among patients with a stricturing disease behavior. To our knowledge, an association with disease behavior has previously not been reported. Previous research has shown that *B. fragilis* lipopolysaccharide can activate Toll-like receptor 4, which in



turn has been associated with liver fibrosis and was suggested as a potential mechanism in stricturing CD (Mancuso et al., 2005; Li et al., 2019). This potential mechanism needs further investigation to clarify whether *B. fragilis* can induce fibrosis and may thereby directly contribute to the development or persistence of strictures.

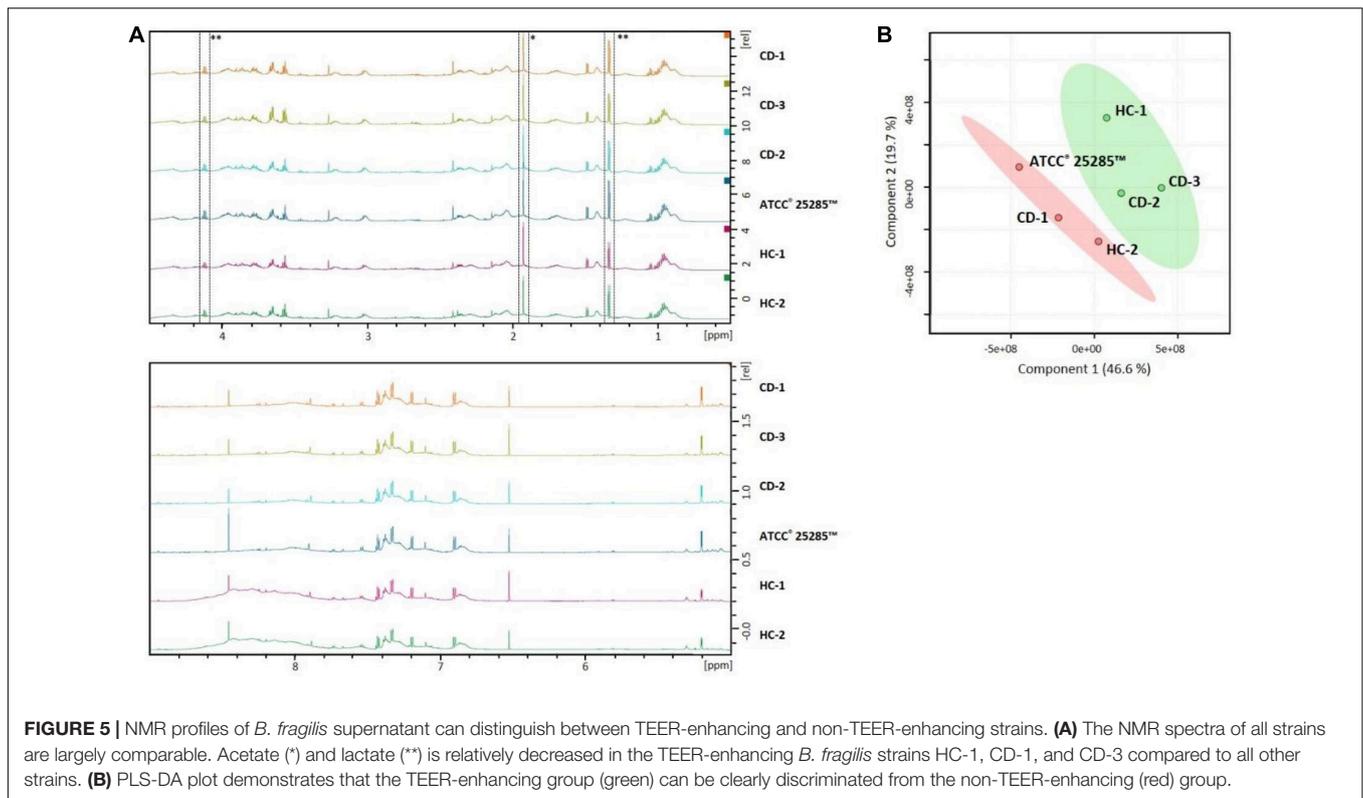
To study the relevance of *B. fragilis* virulence factors, we next compared the prevalence and relative abundance of *bft* and *ubb* positive strains. Bft and Ubb are two *B. fragilis* proteins, which have previously been shown to affect the host via direct and indirect interactions, respectively (Wu et al., 1998; Stewart et al., 2018). The metalloprotease Bft has been shown to disrupt the epithelial barrier by cleaving

the adherens junctions protein E-cadherin *in vitro* (Wu et al., 1998), whereas Ubb was found to mimic human Ubb and led to antibody formation *in vivo* (Stewart et al., 2018). These two compounds were therefore considered of interest and detected in the fecal metagenomes of CD patients.

In our cohort, no difference was found in virulence factor positive *B. fragilis* strains when comparing active and remissive CD. Further analyses on associations between virulence factor carriage and CD related parameters were not conclusive due to the overall low prevalence. Although previous rather small scale studies on *bft* in CD patients could also not demonstrate a link with disease exacerbations (Prindiville et al., 2000; Zitomersky et al., 2013) and studies on *ubb* among CD patients are lacking, their gene products might still play a role in intestinal barrier dysfunction and thereby contribute to intestinal inflammation. We therefore subsequently investigated representative bacterial strains for their effect on intestinal epithelial barrier function using a validated Caco-2 barrier function model (Srinivasan et al., 2015). To investigate the functional impact, we chose to apply bacterial culture supernatant as it contains all secreted products, including proteins, metabolites and MVs (Kaparakis-Liaskos and Ferrero, 2015; Sicard et al., 2017; Parker et al., 2018). *In vivo*, these products can interact with the host by directly targeting the epithelial lining or indirectly via the immune system (Kaparakis-Liaskos and Ferrero, 2015; Parker et al., 2018; Badi et al., 2019).

**TABLE 2 |** Distinctive *B. fragilis* coding sequences.

Predicted/hypothetical protein	Length (aminoacids)
Fragilysin precursor (bftP_1)	406
Fragilysin precursor (bftP_2)	397
Transposase/IS protein	281
Transposase	507
Putative amidoligase enzyme	315
Hypothetical protein	436
Hypothetical protein	84
Hypothetical protein	76
Hypothetical protein	181



In contrast to our hypothesis, none of the investigated supernatants led to a disrupted epithelial barrier when examined with TEER analysis. On the contrary, *bft*-positive culture supernatant significantly increased epithelial barrier resistance. The observed TEER increase was comparable for both, *bft-1*- and *bft-2*-positive isotypes, which include the most prevalent isotypes in the human gut (Sears, 2009). Even in the presence of the inflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$ , which are known to disrupt tight junctions (Eeckhaut et al., 2013; Sturgeon and Fasano, 2016), *bft*-positive culture supernatant evoked an equivalent TEER increase. As previous studies have described a barrier disrupting effect induced by Bft in some other colonic (adeno)carcinoma cell lines, such as HT-29 or T84 (Chambers et al., 1997; Obiso et al., 1997), but not in others, for instance Caco-2, NCI-H508 and LS174T (Van Tassell et al., 1992), we also tested the effect of *bft*-positive culture supernatant on a more physiological CD patient-derived colonic organoid model of epithelial barrier function (Xu et al., 2018). This likely confirmed that exposure to *bft*-positive and *bft*-negative *B. fragilis* culture supernatant did not lead to barrier disruption or any conspicuous morphological changes, such as cell rounding, although it remains possible that organoids from different hosts might respond differently.

To further elucidate mechanisms underlying the pronounced TEER elevation, junctional gene expression was examined. The qPCR-based approach showed that altered gene expression levels of junctional proteins are unlikely to contribute to the TEER elevation evoked by Bft-positive culture supernatant.

Furthermore, induction of proliferation via the previously described Bft-induced upregulation of the proto-oncogene *c-myc* (Wu et al., 2003) was found to be unlikely. Other cellular mechanisms might rather be involved in the detected TEER increase, such as alterations in ion flux or post-translational modifications of junctional proteins (Srinivasan et al., 2015; Shigetomi and Ikenouchi, 2018). We further excluded a medium-based effect, since the bacterial culture medium was the same for all strains and was also used in the negative control.

Altogether, the above findings are not in line with previous research on the mechanisms of Bft. Several studies showed that isolated Bft could significantly decrease TEER via the cleavage of the adherens junction protein E-cadherin (Wells et al., 1996; Chambers et al., 1997; Obiso et al., 1997; Wu et al., 1998). In those studies, Bft was purified and titrated to approximate physiological concentrations (Van Tassell et al., 1992; Wells et al., 1996; Chambers et al., 1997; Obiso et al., 1997; Wu et al., 1998). In the present study, we chose to apply the complete culture supernatant of 24 h bacterial cultures instead of purified Bft, to include other secreted molecules and mimic a more physiological situation. Although we succeeded to detect secreted Bft in the supernatant, using a previously established HT-29 cell rounding assay (Weikel et al., 1992), Bft concentrations were below the detection limit of western blot. Furthermore, for barrier function analysis Caco-2 cells were used instead of HT-29 or T84 cells, which might vary in Bft susceptibility (Van Tassell et al., 1992; Chambers et al., 1997; Obiso et al., 1997).

Next to a lack of epithelial barrier disruption in our Caco-2 model, we showed that *bft*-positive culture supernatant also

did not disrupt the epithelial barrier in more physiological, human colonic organoids as mentioned above. This suggests that our findings are not merely the result of a potentially more resistant Caco-2 phenotype. However, as it has been previously described that the response to Bft differs between cell lines, we cannot completely rule out that the lack of response in both, the Caco-2 model and human colonic organoids from a CD patient, might be because of a more resistant phenotype. Based on the previously reported E-cadherin cleavage and subsequent barrier disruption (Obiso et al., 1997; Wu et al., 1998), it seemed rather unlikely that Bft is contributing to the observed increased TEER values in Caco-2 monolayers. We could exclude Bft as causing factor, using additional *bft-1*- and *bft-2*-positive reference strains with their respective *bft*-deletion mutants. Both, wild type and mutants-derived supernatants showed similar TEER elevation as observed in the original *bft*-positive strains. Therefore, further research was conducted on identifying the potential factor that might induce the observed TEER elevation. As part of the bacterial secretome, *B. fragilis* MVs were isolated from the supernatant and applied on the Caco-2 monolayers. Since TEER values did not change after 24 h incubation, MVs were excluded as TEER elevating factors. Next, whole genome sequencing was applied to identify strain specific coding sequences, indicating differences in potentially secreted proteins. The predicted proteins were related to the pathogenicity island, including *bft* and *metalloprotease II (mpII)*, the transposon machinery, including transposase like protein and putative transposase/insertion sequence protein, and a putative amidoligase was predicted. So far, there is no reported evidence on interaction with amidoligases and tight or adherens junctions. Considering the related functions of the other predicted proteins, they seem rather unlikely to induce TEER elevation, except for MPII, which has earlier been shown to bind to E-cadherin (Remacle et al., 2014). Since the close phylogeny of one TEER-elevating and one non-TEER-elevating strain combined with our observed TEER elevation in the *bft* deletion mutants offer limited possibilities for the involvement of other genes, we hypothesize that MPII likely contributed to the TEER elevation. Finally, relative concentrations of acetate and lactate were lower in TEER-increasing strains. However, short chain fatty acids, including acetate, and lactate are reported to rather strengthen the epithelial barrier (Bruininx et al., 2010; Zheng et al., 2017). The lower acetate and lactate concentrations found in the supernatant of TEER-enhancing strains are therefore unlikely to explain the TEER-enhancing effect. Altogether, we could not elucidate the mechanism underlying the significant increase in TEER by *bft*-positive *B. fragilis* strains.

Besides the effect of Bft-containing supernatant, we further evaluated the impact of *ubb*-positive strains. As mentioned above, we could not observe epithelial disruption in this condition. So far, little is known about the pathophysiological impact of Ubb, but it has been associated with chronic auto-immune diseases, including ulcerative colitis (Stewart et al., 2018). Since we could not find an impact on barrier function, future studies should focus on the interaction of Ubb with other pathophysiological factors, especially pro-inflammatory immune mediators, in Crohn's disease. A combined clinical and *in vitro*

study could explore the presence of anti-Ubb antibodies in CD patients and the subsequent immune response.

In summary, this study shows a higher *B. fragilis*, but not *bft* or *ubb*, prevalence in CD exacerbations and an association with a stricturing disease course. Surprisingly, the direct effect of *B. fragilis* products on colonic epithelial cells led to a significant TEER increase in *bft*-positive strains, which might indicate a barrier stabilizing effect. This was, however, not due to Bft. More detailed pathophysiological mechanisms and a potential clinical relevance need further investigation. Additionally, this study clearly stresses the need to investigate functional host-microbe interactions to pursue on taxonomic and functional associations based on microbiome research.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: <https://www.ncbi.nlm.nih.gov/PRJEB41450>.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of the Maastricht University Medical Centre+. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

PS, HB, DJ, and JP conceived this study. HB, FS, LB, AB, DJ, and JP designed the experiments. HB and MP carried out the patient inclusion and sampling. HB, CJ, LB, and PX conducted the experiments (HB: qPCR, TEER, WB, cell rounding, organoids; CJ: whole genome sequencing; LB: NMR spectroscopy; PX: organoids). HB, DJ, and JP drafted the manuscript. All authors revised the article and approved the final version for submission.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2021.598232/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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