Bifidobacterium breve reduces apoptotic epithelial cell shedding in an exopolysaccharide and MyD88-dependent manner

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Abstract

Certain members of the microbiota genus *Bifidobacterium*, are known to positively influence host well-being. Importantly, reduced bifidobacterial levels are associated with Inflammatory Bowel Disease (IBD) patients, who also have impaired epithelial barrier function, including elevated rates of apoptotic extrusion of small intestinal epithelial cells from villi; a process, termed 'cell shedding'. Using a mouse model of pathological cell shedding, we show that mice receiving *B. breve* UCC2003 exhibit significantly reduced rates of small intestinal epithelial cell shedding. Bifidobacterial-induced protection appears to be mediated by a specific bifidobacterial surface exopolysaccharide and interactions with host MyD88 resulting in downregulation of intrinsic and extrinsic apoptotic responses to protect epithelial cells under highly apoptotic conditions. Our results reveal an important and previously undescribed role for *B. breve*, in positively modulating epithelial cell shedding outcomes via bacterial- and host-dependent factors, supporting the notion that manipulation of the microbiota affects intestinal disease outcomes.

Key words

Bifidobacterium, Epithelial Cell shedding, Inflammatory Bowel Disease, Exopolysaccharide (EPS)

Introduction

Bifidobacteria represent one of the first colonisers of the infant gut and are prominent members of the adult gut microbiota [1, 2]. They have been linked to a number of health-promoting activities including the promotion of anti-tumour immunity [3], modulation of antimicrobial activities against pathogenic bacteria [4] and protection against relapse of Ulcerative Colitis [5, 6]. Despite these purported benefits, the molecular mechanisms underlying these protective effects by bifidobacteria remain largely unknown, although recently, components of their surface, including the exopolysaccharide (EPS) have been shown to play a significant role in modulating protective effects [7]. It is critical to obtain detailed insights into the mode of action by which microbiota members sustain and improve host health, as this will be central to future disease treatment/prevention strategies.

There is a growing body of evidence suggesting that the microbiota influences intestinal epithelial cell (IEC) function, including gene expression, cell division and energy balance [8-11]. These symbiotic bacterial/host relationships have co-evolved to the extent that the microbiota is indispensable for the maintenance of gut homeostasis [12]. Importantly, microbial dysbiosis, as indicated by a reduction in overall diversity, including specific reductions in *Bifidobacterium*, has been linked to Inflammatory Bowel Disease (IBD) [13-15], underlining the critical importance of host/microbe interactions in maintaining a steady state within the intestine.

The epithelium of the small intestine represents the first line of defence against entry of bacteria into host tissues. Cell division in the crypt, under physiological conditions, is counter-balanced by cell shedding from the villi to maintain homeostasis and integrity of the crypt/villus axis. When the epithelial cell is shed, a discontinuity in the villus epithelial monolayer is created, which potentially compromises the epithelial barrier. In health, epithelial barrier function is maintained [16], due to a dramatic redistribution of apical junction complex proteins including Zonula Occludin 1 (ZO-1), occludin 1 and E-cadherin, which form a funnel that surrounds the shedding cell and plugs the

resulting gap until the movement of neighbouring epithelial cells restores epithelial continuity [17-19].

TNF-α is a key cytokine in IBD. We and others, have shown that TNF-α induces apoptosis of villus tip epithelial cells causing excessive shedding, leading to breakdown of the epithelial barrier and micro-ulceration [16, 20]. Delayed repair of epithelial defects caused by excessive cell shedding contributes to the development of macroscopic ulceration [21]. Our studies with confocal endomicroscopy of patients with IBD in clinical remission have demonstrated that those patients with high rates of cell shedding are more likely to relapse than those with low shedding rates, demonstrating a causative link between barrier function and the inflammatory response [21].

Given reports of beneficial effects of certain members of the gut microbiota in IBD and potential roles of microbial dysbiosis in these diseases we hypothesized that certain health-promoting microbiota members, including *Bifidobacterium*, may play a role in protecting against the cell shedding response by modulating IEC function. To determine the contribution of bifidobacteria in cell shedding, we employed a well characterised *in vivo* mouse model in which pathological cell shedding is induced by intraperitoneal administration of Lipopolysaccharide (LPS), driving mononuclear cell expression of TNF-α and subsequent capase-3-positive shedding cells [22]. Our results suggest a particular bifidobacterial strain (i.e. human isolate *B. breve* UCC2003) positively modulates the small intestinal cell shedding response via host MyD88- and bacterial exopolysaccharide-dependent interactions which serve to significantly reduce apoptotic signalling in the epithelial compartment. This points at a previously unknown mechanism by which this *Bifidobacterium* microbiota member protects its host against pathological cell shedding. These findings may thus have important implications for the future design of therapeutic strategies in the context of intestinal diseases.

Materials and Methods

Animals

C57 BL/6 Jax mice (6-10 weeks) were obtained from Charles River. Vil-cre Myd88 transgenic mice (i.e. Cre recombinase expression causes truncation and resulting non-function of the MYD88 protein in IECs) were obtained from the Wellcome Trust Sanger Centre (kind gift from S. Clare).

Bacterial culture and inoculations

Bifidobacterium breve strains UCC2003, UCC2003del and UCC2003inv were used for animal inoculations. These strains and corresponding culturing conditions have been previously described in detail [7]. In brief, colonies were established from frozen glycerol stocks onto reinforced clostridial agar (RCA) plates before being subcultured to reinforced clostridial medium (RCM) and subsequently Man Rogosa Sharpe (MRS) medium (Oxoid, Hampshire) under anaerobic conditions. Bacteria were then purified by centrifugation and washing in PBS containing L-cysteine before being reconstituted in sterile PBS at a final concentration of approximately 1 x 10^{10} bacteria mL^{-1}. 0.1 mL of inoculum was then administered to mice by oral gavage in 3 x 24 h doses followed by plating of faecal pellets on RCA containing 50 mg L^{-1} mupirocin to confirm stable colonisation. Control mice received oral gavage of PBS only.

LPS injections and tissue collections

24 hours after the last doses of *B. breve* or PBS control, mice received an IP injection of 1.25 mg kg^{-1} LPS from Escherichia coli 0111:B4 (Sigma) or sterile saline (control) and mice were sacrificed 90 minutes post-challenge with LPS. Proximal small intestine was collected in 10% neutral buffered formalin saline (Sigma) and fixed for 24 h followed by paraffin embedding. Samples of proximal small intestine were also collected into RNA later (Manchester) for transcriptome analysis or frozen on dry ice for subsequent ELISA analysis. In some cases, proximal small intestine was also collected into Hanks buffered saline solution (HBSS) for isolation of intestinal epithelial cells.

Immunohistochemistry

5 µm sections of paraffin embedded small intestinal tissue were sectioned and used for immunohistochemistry. Following de-parafinisation and rehydration, tissue sections were treated with 1% hydrogen-peroxide in methanol to block endogenous peroxidases. Subsequently, slides were treated using heat-induced antigen retrieval in 0.01 M citrate acid buffer (pH 6) followed by incubation with a rabbit polyclonal anti-active Caspase-3 antibody (AF835:R&D systems). Visualisation of caspase-3 positivity was via a peroxidase-labelled anti-rabbit EnVision™ secondary antibody (Dako) and 3,3'-diaminobenzidine followed by counterstaining with haematoxylin. For macrophage staining, antibody against F4/80 antigen (ab6640:Abcam) was employed using biotinylated anti-rat (BA-9401) and Avidin-Biotin reagent (PK-6100) (Vector laboratories).

Quantification of caspase-3 positivity

IECs were counted on a cell positional basis from villus tip (Cell position (CP) 1) down towards the crypts under 400x magnification. 20 well orientated hemi-villi were counted per mouse and analysed using the Score, WinCrypts [23] and PRISM analysis software. IECs were defined as “normal” in cases where staining for active Caspase-3 was absent. Immuno-labelled cells with either unaltered or shedding morphology were treated as caspase-3 positive. Imaging was performed with an Olympus BX60 microscope and C10plus digital camera.

RNA isolation and real-time PCR

Samples fixed in RNAlater solution were processed through RNeasy plus mini spin columns to isolate total RNA (Qiagen). In brief, samples were homogenised using a rotor stator hand held homogeniser in buffer RLT before processing through a QIAshredder column and subsequently RNeasy mini-spin columns. Purified RNA was eluted into RNAase free water. Reverse transcription was performed using the Quantitect reverse transcription kit (Qiagen) and cDNA used for real-time PCR analysis. For real-time PCR, transcripts were amplified using Quantifast SYBR green mastermix (Qiagen) and Quantitect primer assays for TNF-α, TNF-R1, and F4/80 (EMR1). Expression of the housekeeping

gene Hypoxanthine-guanine phosphoribosyltransferase (HPRT; 5'-GACCAGTCAACAGGGGACAT-3' (sense) and 5'-AGGTTTCTACCAGTTCCAGC-3' (antisense) [24] was also determined. Cycling was performed on a Roche LightCycler 480 using the following conditions: 95^{o}C, 5 min then 40 cycles of 95^{o}C, 10 s; 60^{o}C, 35 s. Relative quantification of levels of transcript expression was calculated using the Pfaffl method [25] by comparing cycle threshold (Ct) value of each target gene to the CTvalue of housekeeper. Data are presented as a ''fold change'' in expression (normalized against control untreated mice/cells).

Isolation of IECs and FACS analysis

IECs were isolated using a modification of the Weiser methodology [26]. In brief, whole small intestine was collected in ice cold HBSS before being chopped into 0.5 cm^2 pieces and washed in a solution containing 0.154 M NaCl and 1 mM DTT and subsequently a solution containing 1.5 mM KCl, 96 mM NaCl, 27 mM Tri-sodium citrate, 8 mM NaH_2PO_4 and 5.6 mM Na_2HPO_4, pH 7.3. IECs were then isolated by incubation in PBS containing 1.5 mM EDTA and 0.5 mM DTT, shaking at 200 rpm and at 37^{o}C. Purity of epithelial preparations was confirmed by histological analysis of stripped intestinal mucosa and by FACS analysis of isolated cells. For FACS analysis $5x10^6$ cells were stained with anti-mouse CD45-A700 (Biolegend) on ice for 30 min. After two washes in HBSS containing 0.01 BSA, 2 mM EDTA, 20 mM HEPES, 0.01% NaN_3, propidium iodide was added (Biolegend) and samples analysed on a Sony FCS SH-800 flow cytometer. Data were analysed using FlowJo (TreeStar).

ELISA

Frozen proximal small intestinal samples were homogenised in extraction buffer containing protease inhibitors (Roche), cleared by centrifugation and analysed using a commercial ELISA kit TNF-α (eBioscience) as per manufacturer's protocol. Measurement of TNF-α immunoreactivity was at 450 nm using a Fluostar Optima plate reader (BMG Labtech).

Native PAGE, SDS-PAGE and Western blotting

Isolated intestinal epithelial cells were lysed in CelLytic MT reagent (Sigma) before centrifugation at 10,000 rpm for 10 minutes to pellet cellular debris. Supernatants were mixed with 2 x Laemmli sample buffer before being separated by sodium dodecyl sulfate (SDS)-PAGE with 3-14% acrylamide gel and transferred to Hybond-P PVDF membrane (GE Healthcare, Buckinghamshire, UK) and blocking with 5% marvel in with tris(hydroxymethyl)aminomethane. (Tris)-buffered saline containing Tween 20 (TTBS), immuno-staining was performed with 1/1000 anti-TNF-R1 antibody (Abcam) and 1/5000 Goat anti-Rabbit IgG HRP conjugate (Millipore) on reduced gel. Macrophage expression was analysed similarly using antibody against F4/80 antigen (Abcam) at 1:1000 and goat anti-rat IgG-HRP (SantaCruz, at 1:3000), on a non-reduced gel. Washes were in TTBS. For detection, Immobilon™ Western chemiluminescent HRP substrate (Millipore) was applied to the membrane as recommended by the manufacturer and signal was detected using a FluorChem E imaging system (Protein Simple). Band densities were quantified using Fiji [27].

PCR array analysis

Real Time ready Custom Panel 480 – 96+ PCR arrays were obtained (Roche) and quantitative PCR analysis performed. RNA was extracted from whole small intestinal tissue preserved in RNAlater reagent (Sigma) using RNeasy plus mini kits (Qiagen). Reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit followed by analysis of targets using LightCycler 480 Probes Master on a LightCycler 480 platform (all Roche). Standard protocols as per manufacturer recommendations were followed. CT values of target genes were normalised to expression of the housekeeping gene HPRT and fold change versus control samples calculated using the delta/delta CT method [25].

Statistical analysis

Experimental results were plotted and analysed for statistical significance with Prism5 software (GraphPad Software Inc). A p value of < 0.05 was used as significant in all cases.

Results

Lipopolysaccharide induces cell shedding from small intestinal villi in a dose dependent manner

Caspase-3 is activated in intestinal epithelial cells during their extrusion from the tips of small intestinal villi [18, 28]. Similar to previous reports, we found that control C57BL/6 mice receiving intra-peritoneal (IP) PBS injection showed low levels of cell shedding as evidenced by low level expression of cleaved caspase-3 (CC3) in the epithelial cell layer (Fig. 1A). Recent studies have demonstrated that following IP injection of mice with Lipopolysaccharide (LPS) isolated from *Escherichia coli* 0111:B4, a potent cell shedding response is induced, similar to that observed in relapsing IBD patients [22]. In agreement with these studies, we found a significant increase in CC3-mediated cell shedding at 90 minutes post-injection of 1.25 mg kg^{-1} LPS, not only at the villus tip, but also along the shoulders and sides of the villus (Fig. 1B). Effects of LPS on the cell shedding response were found to be dose dependent, in agreement with previous observations [22] (data not shown).

B. breve modulates LPS-induced cell shedding

Various members of the microbiota are known to promote a healthy gut [29], although the precise mechanisms behind this remain incompletely understood. We reasoned that because the integrity of the intestinal epithelium is intrinsically linked to the well-being of the host and because the microbiota is expected to impact on epithelial crosstalk, such health-promoting species might play a role in regulating cell shedding. To test this, groups of C57 BL/6 mice were initially dosed with vehicle control (PBS) or with 1 x 10^9 *B. breve* UCC2003 (isolated from a healthy infant) in 3 x 24 h doses orally to establish stable colonisation [7]. Colonisation was confirmed by faecal CFU counts on day 4 (Fig. S1). Mice were then administered LPS to induce pathologic cell shedding, followed by sacrifice at 1.5 h. Following dosing with *B. breve* UCC2003 and induction of cell shedding with LPS, mice showed a marked reduction in the levels of CC3-positive shedding cells compared to LPS treated control mice receiving PBS gavage (Fig. 2A-B). Cell count analysis confirmed significant

reduction in cell shedding at the majority of positions along the length of the villus in *B. breve* UCC2003 treated mice (Fig. 2C; $p < 0.001$, ANOVA). Thus, *B. breve* appears to modulate epithelial integrity/survival during periods of inflammatory insult.

Previous studies have indicated that bifidobacteria may modulate the composition of other microbiota members, and within the context of IBD, studies have linked microbiota disturbances with active disease. Thus, to determine if bifidobacterial colonisation impacts the gut microbiota, we analysed the community composition using a 16S rRNA-based sequencing approach. We found minor changes to the community structure in *B. breve* UCC2003 versus control treated mice (C57BL/6), but overall, no notable differences (but expected increase in Actinobacteria in the *B. breve* UCC2003 group) in microbiota class abundance between the treatment groups (Fig. S2). Bifidobacterial colonisation takes place along the gastrointestinal tract including the small/large intestine and caecum. RNAscope analysis showed that *B. breve* UCC2003 was found in intimate contact with the IECs of the small intestine in colonised C57BL/6 mice (Fig. S3). Together, these data suggest that colonisation with *B. breve* does not produce significant shifts in the overall gut microbiota community structure and that the observed protective effects after colonisation are more likely related to direct effects of *B. breve* , possibly through interactions with the intestinal epithelial cells.

<u>The mechanism of protection against LPS-induced cell shedding is TNF-α independent</u>

LPS-induced cell shedding is caused by the release of TNF-α from lamina propria tissue-resident macrophages, which binds to TNF-receptor 1 (TNF-R1), on intestinal epithelial cells [22], thereby driving the apoptotic response. Conditioning of macrophage responses by the microbiota has been reported previously [30] and, consistent with this data, bacteria such as *B. breve* have been described to possess immune-modulatory properties [31]. Thus, to determine whether the cell shedding outcome, as modulated by *B. breve*, was caused by reduced expression of TNF-α from macrophages, we isolated RNA and protein from whole small intestine of control and *B. breve*

UCC2003-treated C57BL/6 mice following LPS-mediated induction of cell shedding. As shown in Fig. 3A no significant difference in levels of TNF-α protein was observed between groups and this was confirmed at the transcriptional level (data not shown). We also found no changes in expression of TNF-α in the plasma of *B.* breve UCC2003 -treated versus control mice following LPS-induced cell shedding (Fig. 3B) or any significant difference in the numbers/levels of $F4/80^{+}$ macrophages infiltrating the small intestine (Fig. 3C-F). Together, these data suggest that modulation of the reduced cell shedding response is independent of TNF-α induction. Since the microbiota may be able to interact directly with IECs we postulated that *B. breve* modulates a signalling pathway downstream of the TNF-α ligand. To test whether expression of TNF-R1 was altered in the epithelium following dosing with *B. breve* UCC2003, IECs were isolated from whole small intestinal tissue using a modified Weiser methodology [32], after which purity of the IEC population was confirmed by histological analysis of stripped intestinal tissue and FACS analysis (Fig. 3G&H). Subsequent quantitative RT-PCR and Western blot analysis of isolated IEC populations showed no changes to expression of the TNF-R1 transcript or protein following exposure to *B. breve* UCC2003 (Fig. 3I-K), suggesting that there is no impairment of signalling at the level of the receptor.

Functional epithelial MyD88 signalling is required for *B. breve*-mediated protection against cell shedding

Intestinal epithelial cells sample microbe-associated molecular patterns (MAMPS) of the intestinal luminal contents using a variety of receptors including members of Nucleotide-binding Oligomerization Domain (NOD) family, C-type lectin receptors (CLR) family and the Toll-like receptor (TLR) superfamily. MyD88 is a critical adaptor protein in signalling downstream of the majority of the TLR family members [33]. We thus used epithelial-specific (Vil-Cre) MyD88 knockout mice to determine whether *B. breve* elicits its protective effects via epithelial TLR signalling pathways.

C57BL/6 $MyD88^{-/-}$ villin-cre mice (i.e. IEC MyD88 KO mice) colonised with *B. breve* UCC2003, showed similar rates of LPS-induced cell shedding to PBS gavaged IEC $MyD88^{-/-}$ mice. In comparison, control

mice (i.e. C57BL/6 MyD88$^{+/+}$ villin-cre), showed the expected protection against cell shedding in the presence of *B. breve* UCC2003 (Fig. 4A-D). Furthermore RT-PCR analysis of IEC homogenates showed increased expression of TLR2 in *B. breve* UCC2003 colonised mice when compared to control mice (i.e. PBS, Fig. 4E). Taken together, these data indicate that functional MyD88 signalling, potentially via TLR2is required for modulating the protective effect of *B. breve* against cell shedding outcomes.

B. breve EPS plays a role in modulating protection against LPS-induced cell shedding

Recently, a number of functions modulated by bifidobacteria have been shown to be mediated through surface-associated EPS including resistance to gut infection [7]. Interestingly, the *eps* gene clusters represent a relatively conserved feature of bifidobacterial genomes, including those of the species *B. breve* [34]. In order to investigate the role of EPS in modulating the response against cell shedding, we used a deletion mutant (*B. breve* UCC2003-EPSdel) that expresses neither EPS1 nor EPS2 [7]. Mice were stably colonised by dosing with *B. breve* EPS-positive or EPS-negative strains followed by challenge with LPS (Fig.S1). Strikingly, when colonised with the *B. breve* UCC2003-EPSdel, no protection against cell shedding was observed in control (i.e. PBS) versus colonised mice (Fig. 5A&B).

B. breve UCC2003 controls EPS biosynthesis via a bidirectional gene cluster which results in expression of either EPS1 (*B. breve* UCC2003) or EPS2 (*B. breve* UCC2003-EPSInv) [7]. Thus, to gain further insights into the role of a different EPS in the protective cell shedding response, we undertook studies using *B. breve* UCC2003-EPSInv. Colonisation with EPS2 expressing *B. breve* (i.e. *B. breve* UCC2003-EPSInv) also failed to show any protection against LPS-induced cell shedding, suggesting considerable variation in the protective response dependent upon EPS genetic and chemical structure and organisation (Fig. 5C&D). All strains are directly compared in Fig.S4.

Together, these studies emphasize the striking strain variant specificity that is observed with regard to the individual protective effects of these bacteria following LPS-induced cell shedding. This is

likely regulated by the specific molecules produced by each strain, including the EPS. This highlights the critical need to fully genetically characterise 'probiotic' strains of bacteria to enable a detailed dissection of their functional effects *in vivo* for optimal translation to human patients.

B. breve EPS attenuates inflammatory and apoptosis signalling

In order to gain further insight into the changes taking place in the small intestine following colonisation with *B. breve* UCC2003 and the influence of EPS, whole small intestinal samples from control (i.e. PBS) and colonised (EPS-positive, *B. breve* UCC2003 and EPS-negative, *B. breve* UCC2003-del) mice following challenge with LPS were analysed using a custom RT-PCR array (Fig. 6: 49/84 targets are shown, full set of data is displayed in Fig. S5A&B) to look for transcriptional changes to key inflammatory transcripts and those involved in the apoptotic cascade. Interestingly, small intestinal samples from *B. breve* UCC2003-EPSdel colonised mice (Fig. 6A and Fig. S3A&B) showed significant increases (> 2-fold and $p < 0.01$) in IL-6 and Tnfrs15 when compared to control and LPS challenged mice. Moreover numerous other apoptotic and inflammatory genes were significantly upregulated (> 2-fold, $p<0.01$) including Bad, Cycs (Cytochrome C, Somatic), casp4, Fas, Traf5 and Tnfrs9. In contrast in EPS-positive colonised mice (i.e. *B. breve* UCC2003) our analysis showed only subtle changes to the expression of the majority of the targets, when compared to control LPS animals. In addition, whilst significant elevation (> 2-fold and $p < 0.05$) in IL-6 and Tnfrs15 was observed following colonisation with *B. breve* UCC2003, Tnfrs15 expression was markedly decreased versus *B. breve* UCC2003-EPSdel colonised mice (3-fold vs 16-fold increase). These data suggest that signalling via EPS may downregulate inflammatory and apoptotic networks which would otherwise lead to elevated cell shedding.

Discussion

We report that colonisation of mice with *B. breve* significantly reduces pathological/apoptotic epithelial cell shedding, through a previously unknown mechanism involving bifidobacterial EPS-MyD88 signalling.

The gut microbiota appears central to maintaining epithelial barrier integrity and importantly disturbances in the microbiota appear pivotal in IBD pathogenesis. Indeed, IBD patients (paediatric and adult cohorts) have been shown to possess a reduced overall microbiota diversity and reductions in specific genera including *Clostridium*, *Bacteroides, Faecalibacterium* and, of particular interest here *Bifidobacterium* [6, 15, 35]. Previous clinical trials have shown that administration of bifidobacterial strains can reduce the incidence of relapse in patients suffering from IBD [36]. Following LPS-induced cell shedding, we observed that *a priori* administration of *B. breve* UCC2003 (which is a human-isolated strain, thus more translationally relevant) conferred a significant level of protection which manifested as significantly reduced caspase-3 positivity within the villus epithelium (Fig. 2A-C). Previous studies have highlighted that bifidobacterial supplementation may also modulate the wider microbiota in mouse models [37]. However, our data indicates that whilst there are modest differences between PBS and *B. breve* colonised mice (as indicated by taxa abundance), there are no notable differences (with high variability between animals) suggesting limited effects on overall microbiota profiles (Fig. S1). These data therefore suggests a more direct link between bifidobacteria and maintenance of epithelial integrity in the prevention of intestinal inflammation.

Previous studies have indicated that *Bifidobacterium* predominantly colonises the colon of infants and adults, as determined from faecal, mucosal scrapings or biopsy samples [38, 39], however, in this work (using a murine model), we have described SI-specific responses. From a translational perspective, in humans these protective cell shedding responses may result from bifidobacteria

cross-talk in the lower SI. Although difficult to measure in humans, previous studies have indicated *Bifidobacterium* colonisation in the lower SI (i.e. the ileum, as we observe in our model). Notably, select studies using Ileostomy effluents and illeum biopsies have indicated Bifidobacteria (specifically B. animalis subsp. lactis and *B. breve* respectively) are present in this area of the infant and adult gastrointestinal (GI) tract [40, 41]. Therefore, in the human context, we may observe direct SI signalling via resident bifidobacteria and/or remote SI feedback signalling from colonic bifidobacteria epithelium cross-talk, which could be tested in future clinical intervention studies.

As previously mentioned, studies have shown that this experimental model of LPS-induced cell shedding is driven by an induction in expression of TNF-α from the intestinal mucosa [22, 42]. One of the key functions of the gut microbiota is induction of tolerogenic or anti-inflammatory immune responses and thus we hypothesised that bifidobacteria may reduce cell shedding as a direct result of inhibiting TNF-α and macrophages – a potential source of TNF. However, we were unable to detect any changes in levels of TNF-α expression or macrophage infiltration from *B. breve* UCC2003-treated or control (i.e. PBS) mice (Fig. 3C), suggesting that the protection conferred by *Bifidobacterium* strains is TNF-α independent. Previous studies have indicated that colonisation of *B. breve* UCC2003 during homeostatic conditions does not induce differences in splenic TNF-α-positive macrophage numbers when compared to non-colonised controls [7]. Coupled with the lack of change in expression in TNF-R1 following colonisation (Fig. 3H&I), it appears that macrophages, TNF-α production and TNF-R1 signalling are not involved in modulating this protective response and suggests that *B. breve* UCC2003 acts preferentially from the luminal side through interactions with the intestinal epithelial cells. However, we cannot exclude the potential for EPS to block signalling via TNF-R1, however TNF-R1 expression appears to be restricted to the basolateral surface of epithelial cells and thus it would not be expected that *B. breve* (and thus the EPS) would have direct access to this cellular compartment for direct inhibition via binding [43]. Furthermore, quantification of downstream effectors (Fig. S3) including FADD, TRAF2 and caspase 2 and 8 does not significantly

differ between *B. breve* UCC2003 and *B. breve* UCC2003-del colonised mice, which suggests EPS does not play a key role via TNF-R1.

To delineate these protective luminal bifidobacterial-epithelial interactions, we utilised epithelial-specific MyD88 KO mice which is a key adaptor protein downstream of microbe-TLR signalling. Notably, mice carrying truncated epithelial MyD88 (i.e. C57BL/6 MyD88$^{-/-}$ villin-cre) showed no protection against cell shedding after colonisation of *B. breve* UCC2003 (Fig. 4B&D), which was in stark contrast to MyD88-positive control animals that again showed significant protection against LPS-induced cell shedding (Fig. 4A&C). Furthermore, we observed significant increases in IEC TLR2 expression in *B. breve* UCC2003 colonised mice. Interestingly, previous work has indicated that TLR2 may enhance ZO-1 associated intestinal epithelial barrier integrity [44], and other studies indicate that mice deficient in MyD88 signalling have increased susceptibility to intestinal inflammation [12]. In a UV model of apoptosis, MyD88 signalling appears to reduce caspase-3 and in turn increase cell survival and more recently *B. bifidum* has been shown to reduce apoptosis *in vitro* (necrotising enterocolitis IEC-6 cell model) also indicated by reduced CC3-positive cells [45]. Thus our data, in tandem with these studies, indicate that *B. breve* UCC2003 may regulate epithelial integrity in response to LPS-induced cell shedding (as marked by caspase-3) via these central MyD88 signalling mechanisms, potentially downstream of TLR2.

Having determined the importance of host molecule MyD88, we next sought to determine if there was a specific bifidobacterial molecule central to the observed protective response. Since we have previously shown that the surface EPS of *B. breve* UCC2003 can regulate the host response [7], we investigated the ability of an EPS mutant, *B. breve* UCC2003-EPSdel (complete deletion of *eps* biosynthetic cluster) to modulate LPS-induced cell shedding. Notably, mice receiving *B. breve* UCC2003-EPSdel showed no significant protection against cell shedding when compared to EPS-positive (i.e. *B. breve* UCC2003) colonised mice (Fig. 5A&C), suggesting an important role for this EPS

in microbe-host crosstalk. Importantly, EPS structures can be recognised via TLR2 (and signal via MyD88) and previous work with the Polysaccharide A (PSA) capsule of *Bacteroides fragilis* highlights that PSA can modulate dendritic cell and T regulatory cell function via TLR2 signalling [46, 47]. Additionally, previous work has highlighted that a strain of *B. breve* (Yakult strain) can also induce IL-10 producing T regulatory cells via TLR2, however they did not determine if this was via an EPS-specific mechanisms [48]. Furthermore, recent studies using *Bacillus subtilis* have demonstrated that the EPS capsule of this bacterium is able to protect against intestinal inflammation in a murine model of colitis (in this instance via TLR4), providing further support for the likely role of bifidobacterial EPS in the effects observed in these studies [49]. Notably, the probiotic genus *Lactobacillus* also produces distinct EPSs, which are structurally similar to those observed in bifidobacteria [50]. Recently, within an *in vitro* system (HT29-19A epithelial cell line), the EPS from *Lactobacillus acidophilus* 5e2 was shown to increase IL-8 expression and also TLR2 expression (we also observe that *B. breve* UCC2003 induces IEC TLR2 expression), and additionally up-regulation of TLR2 was found to potentially 'sensitise' epithelial cells to subsequent stimulation with peptidoglycan (a TLR2 agonist) [51]. Furthermore, the authors also observed a modest increase in TLR4 expression after addition of EPS, but did not detect any significant modulation of IL-8 responses after priming with EPS and subsequent addition of LPS, which may indicate less of a role for EPS-TLR4 interactions [51]. From a more systemic perspective, in the instance that *Lactobacillus* or indeed *B. breve* UCC2003 potentially translocate across the epithelial barrier, it maybe be expected they could directly influence macrophage function and previous studies have shown that *L. casei* Shirota can dampen down inflammatory macrophages responses and *L. rhamnosus* EPS has also been shown to modulate macrophage function *in vitro*, but on this occasion induce pro-inflammatory responses [52, 53]. Ideally we would test our *B. breve* strains in TLR2 and/or TLR4 KO animals; unfortunately, previous work has shown that these mice do not respond to LPS and thus would not have a cell shedding response making these further studies not possible. However, in studies using RNAscope, we found significant numbers of *B. breve* UCC2003 associated with the villi

in colonised mice (Fig. S2), suggesting that direct signalling interactions between the bacteria (possibly via EPS and TLRs, and *B. breve* UCC2003 colonisation increases TLR2 expression) and IECs may play an important role in modulating this response. These data alongside our findings suggest that *B. breve* EPS may regulate cell shedding by acting as TLR ligands via MyD88, leading to protective epithelial responses.

To probe these EPS-epithelial interactions further we took advantage of the bi-directional *eps* gene cluster in *B. breve* UCC2003 which can express two genetically and importantly chemically distinct surfaces EPSs [7]. All previous studies utilised EPS1 (i.e. with *B. breve* UCC2003), but we also determined responses following EPS2 (i.e. *B. breve* UCC2003-EPSInv) colonisation. Strikingly and contrary to our expectations we found that this isogenic strain was unable to confer protection against LPS-induced cell shedding (Fig. 5B&D). Importantly, EPSs are comprised of repeating mono- or oligosaccharides linked by various glycosidic linkages, and the three dimensional structures and other physiochemical features of EPSs can vary widely [54]. The variability in chemical composition of these two *B. breve* EPSs (previous work suggests the EPSs may include glucose, galactose and/or the N-acetylated versions of these two sugars in different ratios or composition [7]) could in part explain the different modulatory properties of this beneficial microbe in relation to receptor-ligand binding and further highlights the issues with significant strain (or in this case isogenic), variation in effects on host responses. Importantly, these different EPS-epithelium protective responses do not appear to be linked to colonisation ability as all strains colonised mice at similar levels (Fig. S1). Previous limited studies have indicated that specific chemical structures of EPSs such as PSA of *B. fragilis* (comprised of an unusual repeating tetrasaccharide moiety, free carboxyl, phosphate, and amino groups, that contribute to its zwitterionic nature) is important for function [46]. Additionally, *in vitro* studies on *L. reuteri* strains (DSM 17938 and L26 Biocenol™) indicates both EPSs are high molecular weight d-glucan polysaccharides with differing spatial conformations, which may relate to

induction of different cytokine responses, however the direct chemical structures involves in this modulation have yet to be defined [55]. Future challenges will include studies to fully chemically characterise the different strains of 'probiotic' bacteria, as evidently, significant differences in response to small strain variations (including variations in EPS expression and structure and also other MAMPS) may impact beneficial host responses [56, 57].

We have previously shown that EPS-positive *B. breve* UCC2003 does not induce inflammatory host responses after colonisation, which we hypothesise is to the advantage of the bacterium and host for maintaining efficient symbiosis and homeostasis [7]. Interestingly, when we probed the downstream signalling transcriptional events after colonisation and LPS challenge we determined that presence of EPS1 (i.e. *B. breve* UCC2003) appeared to attenuate apoptosis-induced signalling activation, which was in stark contrast to mice colonised with the *B. breve* UCC2003-EPSdel strain which had significantly elevated apoptotic gene expression (Fig. 6). Importantly, previous work has demonstrated that activation of MyD88 can downregulate several of these genes including Fas (CD95) [58]. Fas is a cell surface receptor and member of the TNF superfamily and when bound by its ligand it induces apoptosis through the assembly of a multiprotein complex called the DISC which in turn activates caspase 8 (i.e. extrinsic apoptosis pathway) [59]. Further evidence of an EPS-specific mechanism attenuating epithelial apoptosis comes from observation that Bad, Cycs, casp4, Traf5 and Tnfr9 are upregulated in the intestinal mucosa of mice colonised by *B. breve* UCC2003-del compared to *B. breve* UCC2003 colonised mice. Bad is a pro-apoptotic (BH3-only) member of the bcl-2 family that antagonises the anti-apoptosis proteins bcl-2, bcl-xl and bcl-2 allowing the activation of bax/bak oligomers and the release of cytochrome c from the mitochondria. Within the same pathway, Cycs encodes the heme protein cytochrome c, which forms a multiprotein complex called the apoptosome which activates a cascade of proteases called caspases which cause apoptotic cell death [60]. Traf5 is a scaffold protein that forms a multiprotein complex with TRAF2, RIP1 and the

TNF receptor and potentially can mediate the activation of apoptosis and NF-κB [61]. We have previously shown that NF-κB1 inhibits LPS-induced apoptotic cell shedding whereas NF-κB2 stimulates apoptotic cell shedding [22]. TNFRF9 (CD137) is expressed on T cells and has been reported to enhance their cytolytic activity [62]. These data strongly suggest that mechanistically *B. breve* UCC2003, via EPS, may block intrinsic and extrinsic apoptosis signalling (via activation of MyD88) during inflammation to protect epithelial cells under highly apoptotic conditions.

In summary, we have demonstrated that certain bifidobacteria, i.e. *B. breve* UCC2003 are able to protect against pathologic cell shedding induced by IP injection of LPS and that this protection appears to be independent of TNF-α production by resident tissue macrophages. Using wild type and mutant *B. breve*, we have demonstrated that a specific EPS is able to confer this protection, and using knockout mice, have shown that this protection appears contingent on functional signalling downstream of the epithelial TLR family members and modulation of pro-apoptotic gene pathways. Understanding how health promoting species of bacteria such as the *Bifidobacterium* genus interact with the intestinal epithelium and how these species confer their protective effects may drive progress toward understanding how pathologic cell shedding in IBD patients is linked to changes in the intestinal microbiota. Future human studies could be considered to address issues of microbial dysbiosis and relation to the cell shedding response and to what extent microbial dysbiosis is linked to periods of remission and relapse in such patients.

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Ethics statement

All experiments were performed under the UK Regulation of Animals (Scientific Procedures) Act of 1986. The project licence (PPL 80/2545) under which these studies were carried out was approved by the UK Home Office and the UEA Ethical Review Committee. Mice were sacrificed by CO_2 and cervical dislocation.

Author Contributions

K.R.H, A.J.M.W, and L.J.H., designed research; K.R.H, C.A.G, L.C.H, S.M, C.J.W and J.K .F. performed research; D.v.S contributed new reagents/ analytic tools; K.R.H, D.v.S, A.J.M.W, and L.J.H., analysed data; and K.R.H, D.v.S, A.J.M.W and L.J.H. wrote the paper.

Competing Interests

We declare we have no competing interests.

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Figures and Figure legends

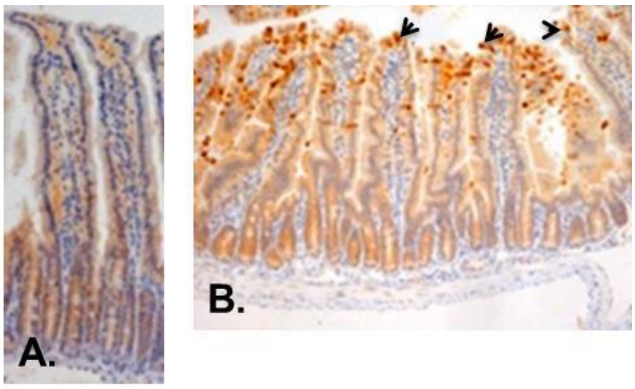
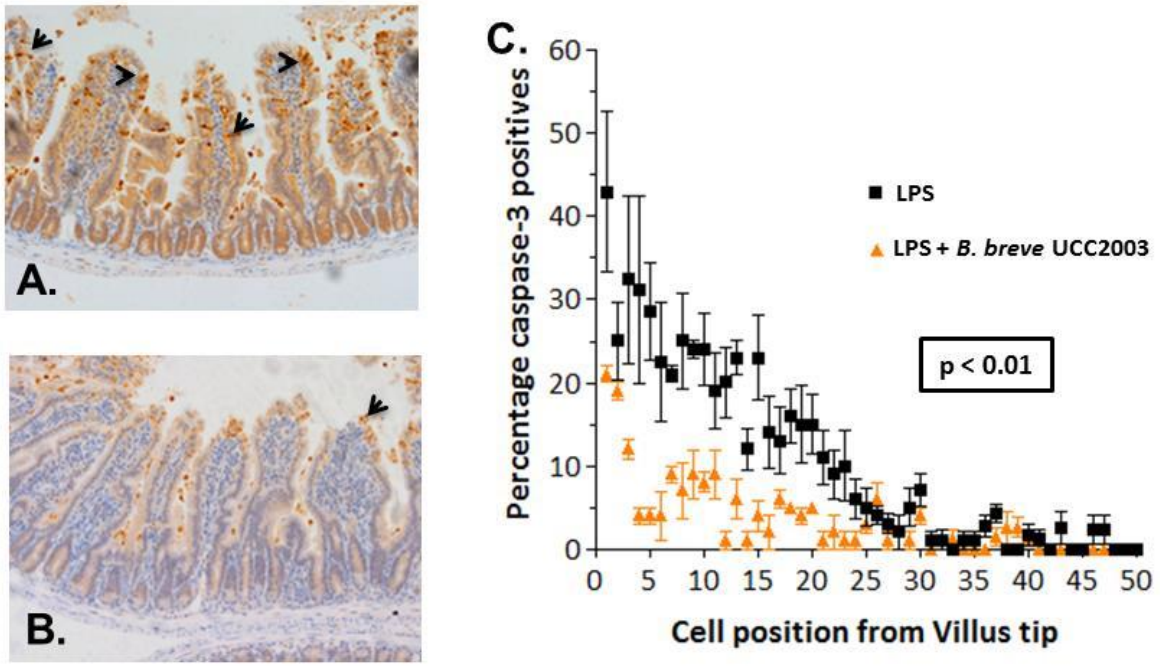


Figure 1: LPS challenge induces cell shedding from the small intestinal villi

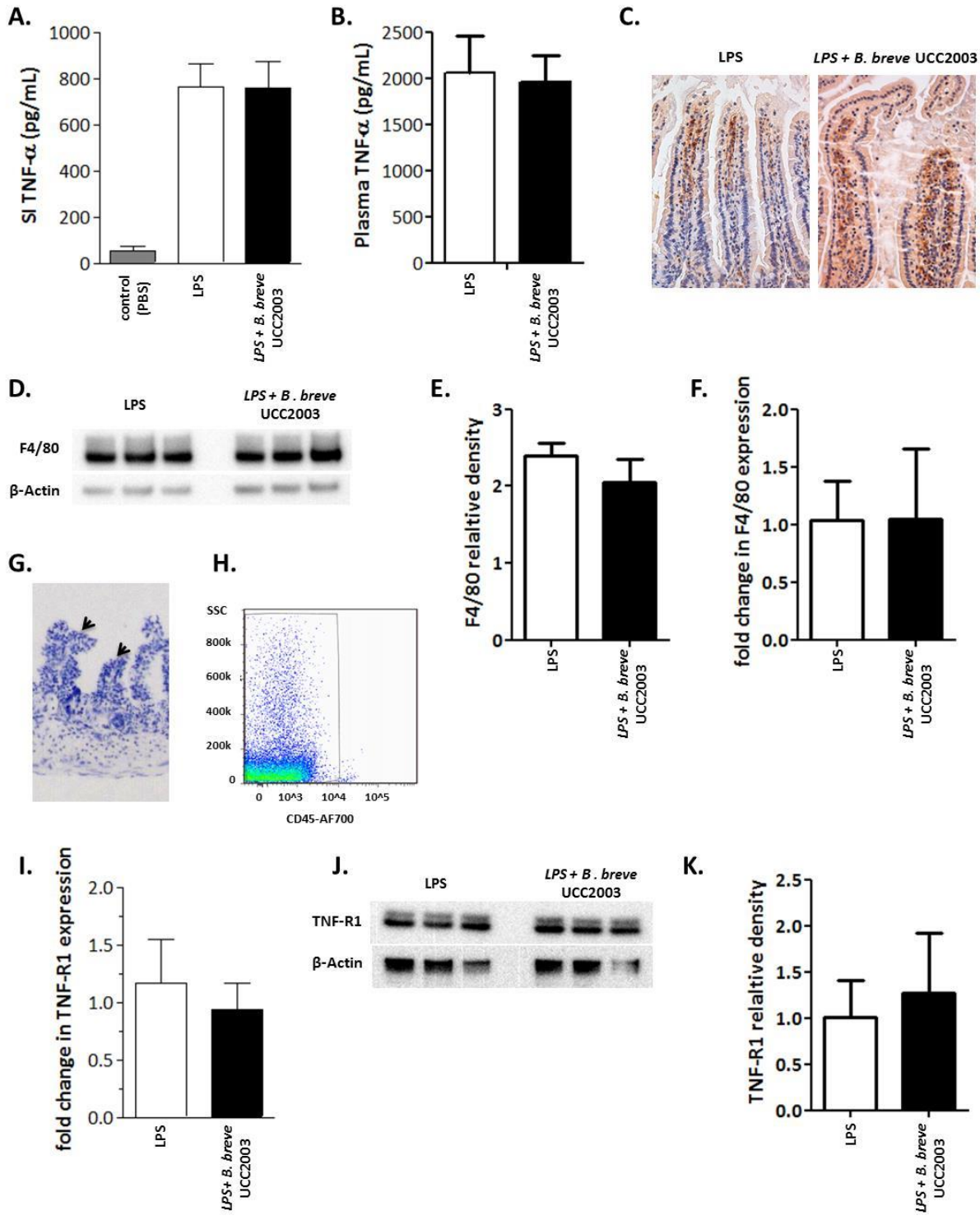
C57BL/6 mice were administered either **(A)** PBS (control) or **(B)** LPS by IP injection and proximal small intestines removed after 1.5 h for immunohistochemistry and stained with anti-CC3 (i.e. brown cells indicate shedding event), also highlighted by arrows. A representative picture for each group is shown (12 mice per group, two independent experiments).



693

694 Figure 2: *B. breve* UCC2003 protect against LPS-induced cell shedding

695 C57 BL/6 mice received three daily oral gavage doses of **(A)** PBS or **(B)** ~1 x 10^9 *B. breve* UCC2003
696 followed by IP challenge with LPS 24 h later, representative picture shown. Formalin fixed, paraffin-
697 embedded intestinal sections were stained with anti-CC3 and **(C)** quantified using the WinCrypts and
698 Score programs, 20 well orientated hemi-villi were counted/mouse. Data are mean ± SD, *n* = 12 (2
699 independent experiments) analysed with Mann–Whitney U test.

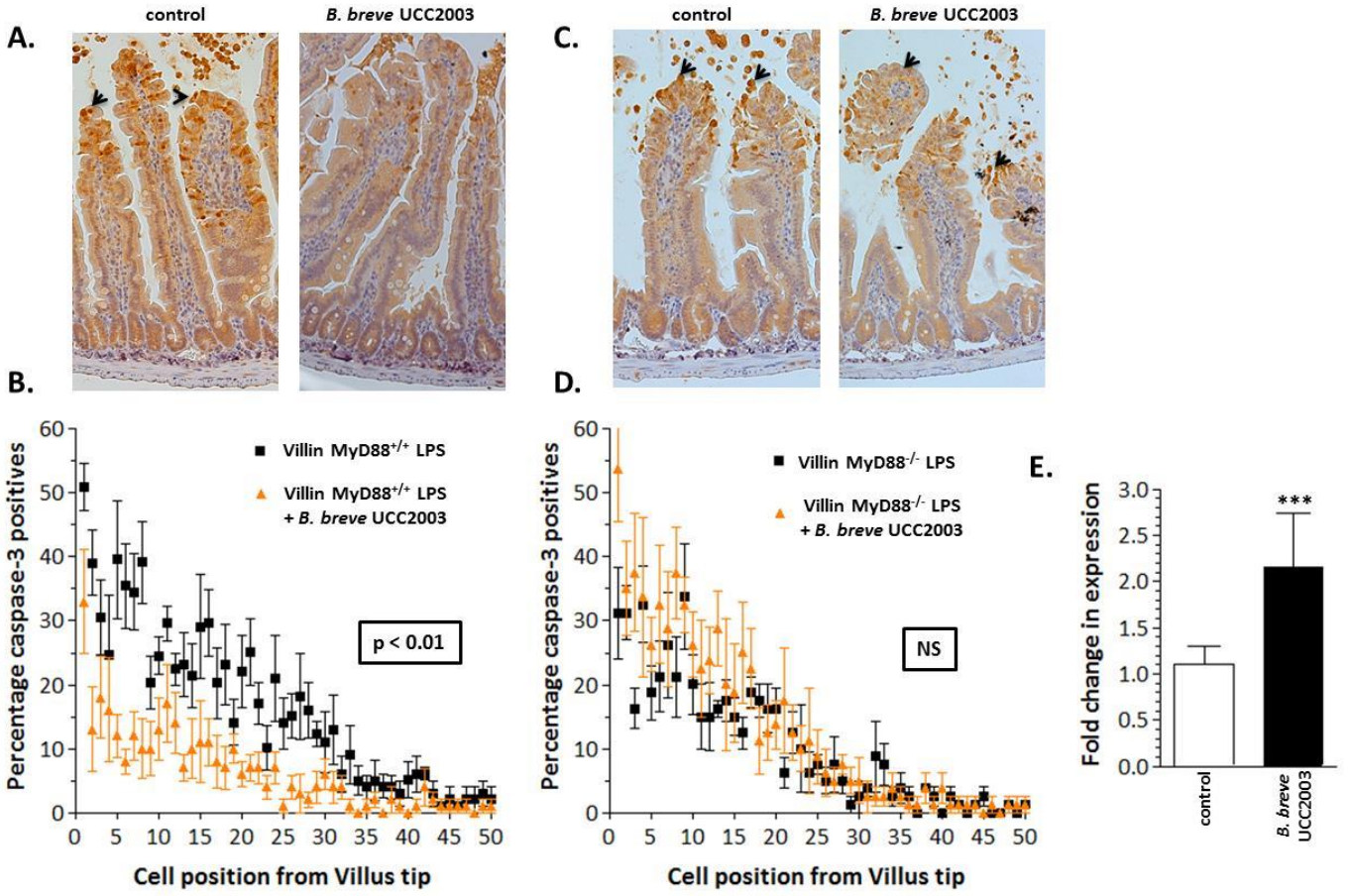


700

701 Figure 3: The cytoprotective effect of *B. breve* is not mediated by the TNF-α signalling pathway

702 C57BL/6 mice were gavaged with PBS or *B. breve* and challenged with PBS or LPS for 1.5 h. Columns
703 show TNF-α levels (via ELISA) in **(A)** whole small intestine intestinal homogenates or **(B)** plasma ± SD.
704 **(C)** Representative immuno-histochemical staining for F4/80^{+} macrophages (brown cells) in control

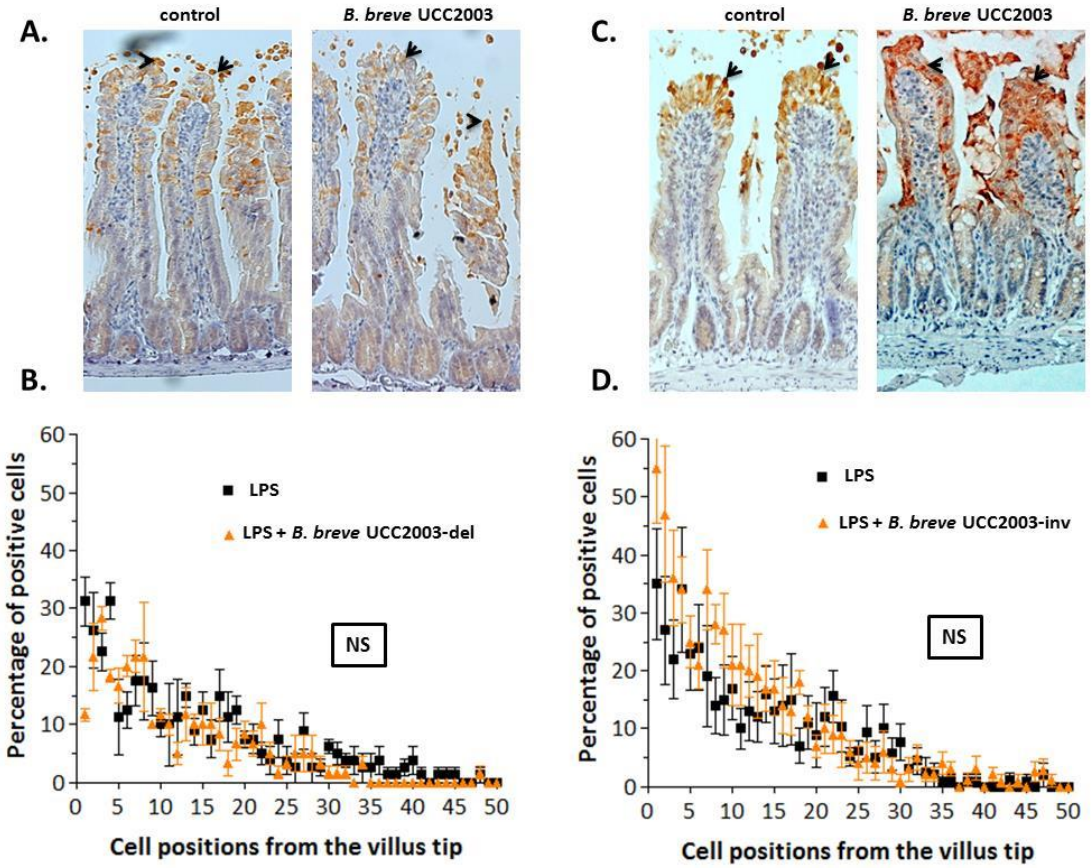
or *B. breve* colonised mice. **(D)** Western blot analysis (F4/80 or house-keeping β-actin) of whole small intestinal homogenates, with **(E)** columns showing relative density of F4/80 from (from D) whole intestinal homogenates. **(F)** Columns show F4/80 expression via RT-PCR ± SD. **(G)** Representative histology image of epithelial cell stripping protocol (modified Weiser method) leaving LP intact (as indicated by arrows) and **(H)** FACS analysis for purity (anti-CD45). **(I)** Columns shown TNF-R1 expression via RT-PCR ± SD and **(J)** Western blotting for protein expression in isolated intestinal epithelial cells, with **(K)** columns showing relative density of TNF-R1 (from J). . *n* = 9 mice per group are representative of three experiments analysed with ANOVA Kruskal–Wallis test with Dunn's multiple comparison test (Fig. 3A), and with Mann–Whitney U test (Fig. 3B, E, F, I and K).



718

719 Figure 4: The cytoprotective effect of *B. breve* is MyD88 dependent

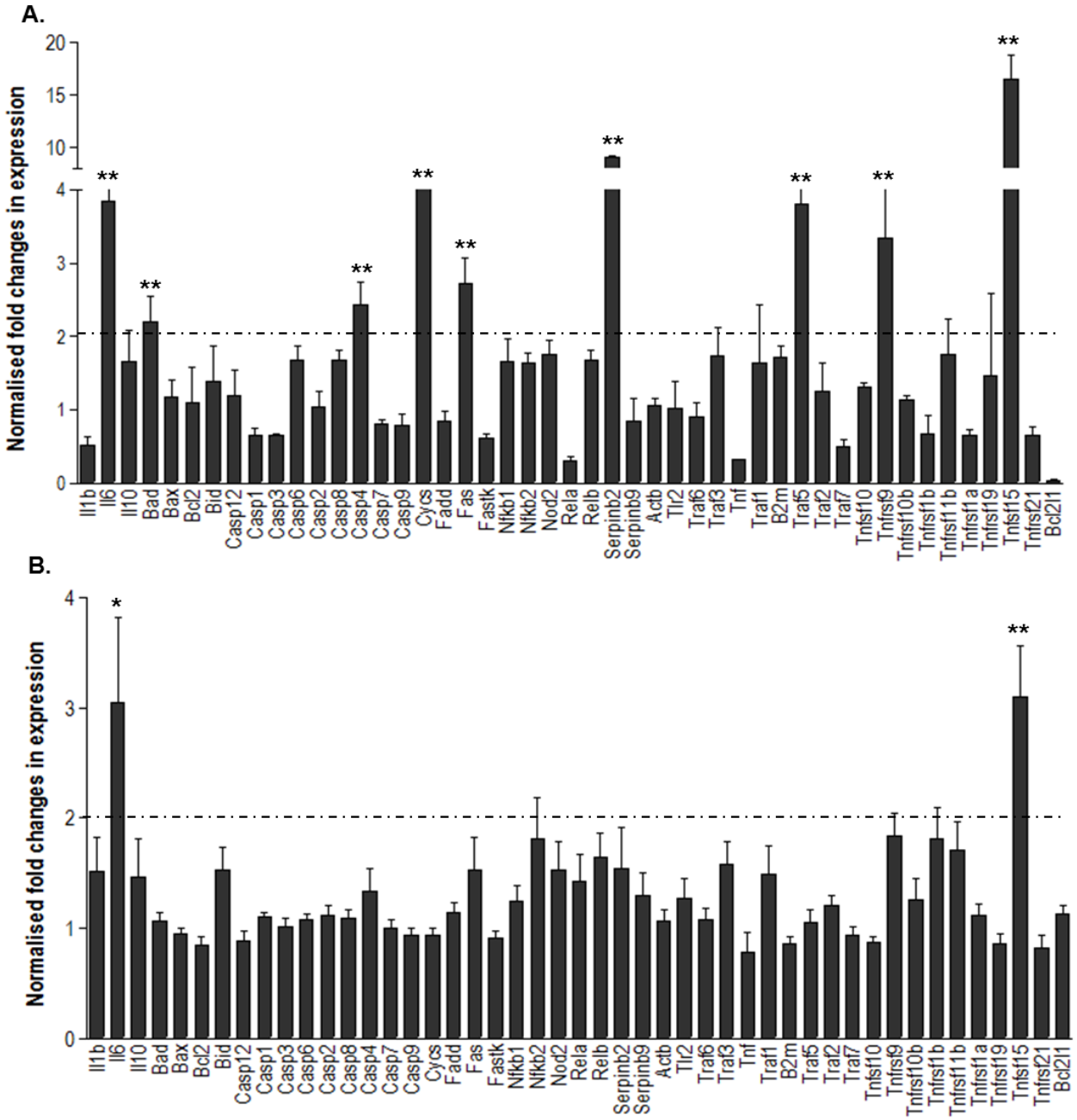
720 **(A, B)** IEC MyD88$^{+/+}$ mice and **(C, D)**, IEC MYD88$^{-/-}$ mice were gavaged with PBS (control) or *B. breve*
721 and challenged with LPS. Paraffin-embedded intestinal sections were stained with anti-CC3 and
722 quantified using the WinCrypts and Score programs. **(E)** Columns shown TLR2 expression via RT-PCR.
723 Data are mean ± SD, *n* = 12 (two independent experiments) analysed with Mann–Whitney U test.



724

725 Figure 5: *B. breve* EPS plays a role in modulating the cytoprotective effect

726 C57BL/6 mice were gavaged with either *B. breve* UCC2003 or **(A, B)** *B. breve* UCC2003del (i.e. EPS-
727 negative) or **(C, D)** *B. breve* UCC2003inv (i.e. EPS2). Formalin fixed, paraffin-embedded intestinal
728 sections were stained with anti-CC3 and quantified using the WinCrypts and Score programs. Data
729 are mean ± SD, *n* = 12 (two independent experiments) analysed with Mann–Whitney U test.



730

731 Figure 6: *B. breve* EPS attenuates inflammatory and apoptosis signalling

732 Whole small intestinal homogenates from LPS challenged **(A)** *B. breve* UCC2003-EPSdel and **(B)** *B.*
733 *breve* colonised mice compared to control (i.e. PBS) were analysed using a custom RT-PCR array.
734 Data are mean ± SD, n = 6 (two independent experiments), *P < 0.05 and **P < 0.01 and analysed
735 with Mann–Whitney U test.

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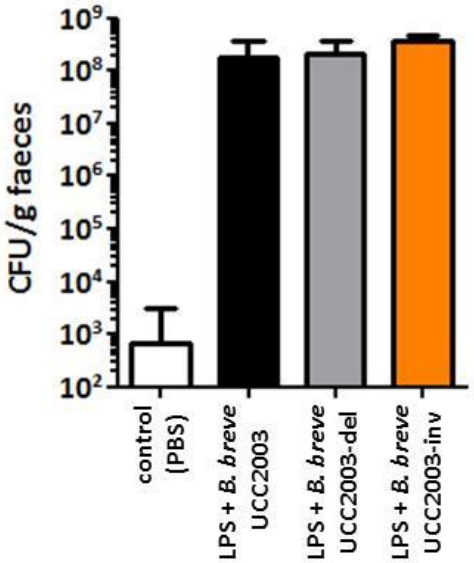
737 **Supplementary Information**



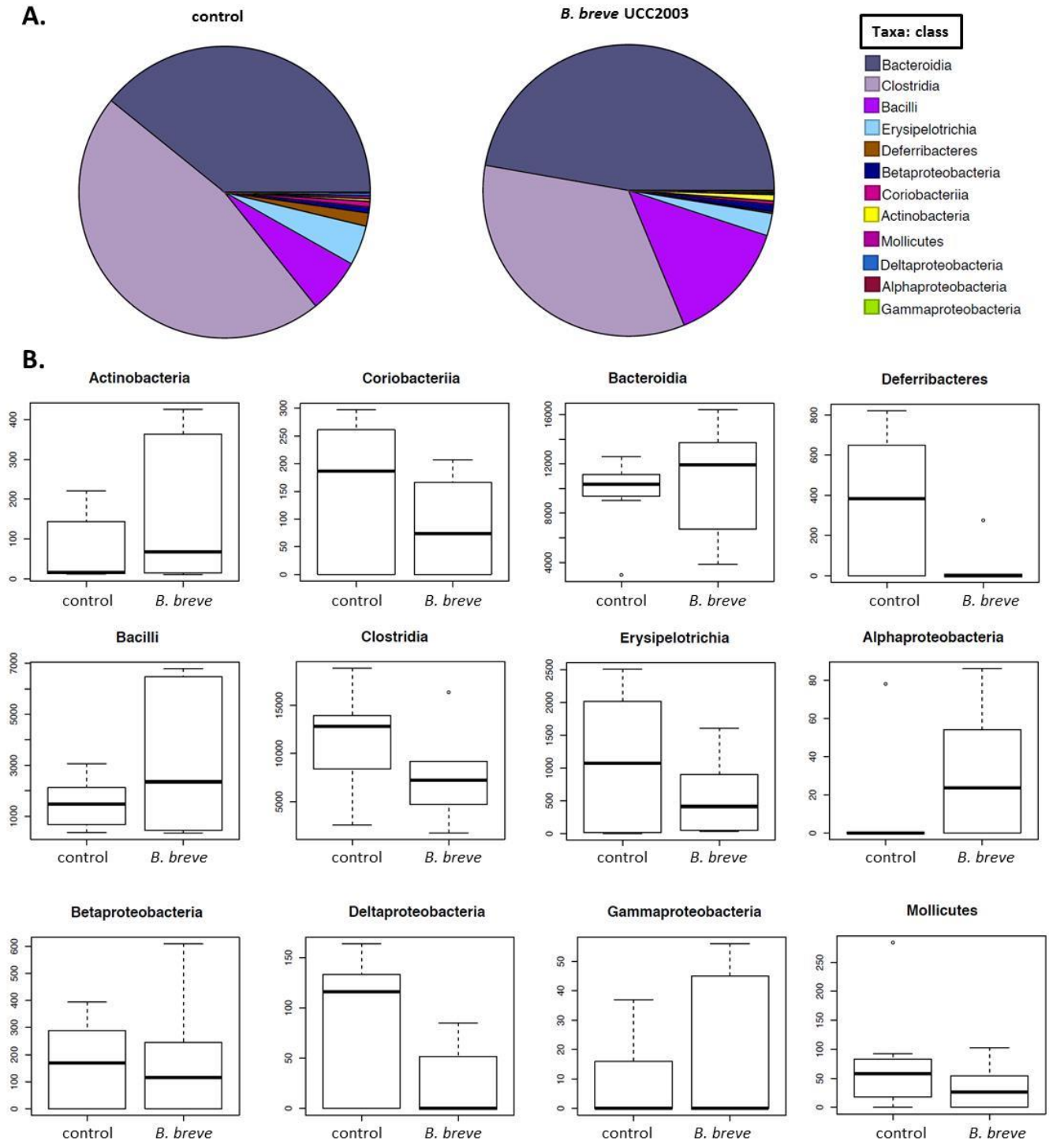
738

739 Supplementary Figure 1: *B. breve* strains stably colonise murine GI tract.

740 Faecal samples were collected from control (i.e. PBS), *B. breve* UCC2003, *B. breve* UCC2003-del and

741 *B. breve* UCC2003-inv colonised mice on day 4 (after 3 x 24 h doses at ~1 x 10^9) and plated on RCA (+

742 mupirocin) and CFU enumerated at 24 hours.



743

744 Supplementary Figure 2: *B. breve* UCC2003 does not notably impact faecal microbiota profiles.

745 Faecal samples from control (i.e. PBS) and *B. breve* UCC203 (after 3 x 24 h doses at ~1 x 10^9) were

746 collected and processed for 16s Illumina sequencing. **(A)** Pie chart depicting comparison of average

747 taxonomic content for two groups at class level taxonomic profile. **(B)** Individual boxplot of each taxa

748 at class level taxonomic profile for two groups (i.e. control and *B. breve* UCC2003), where read
749 abundance data was normalised for both the groups.

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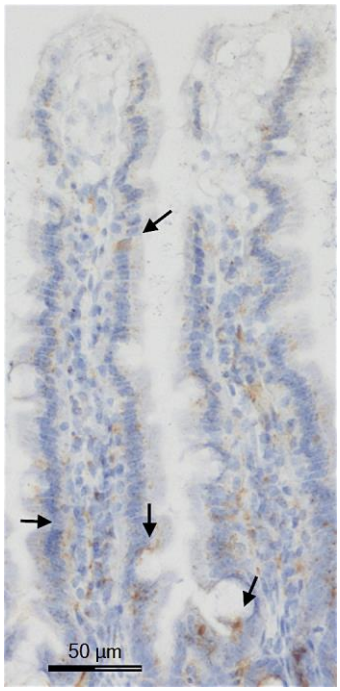
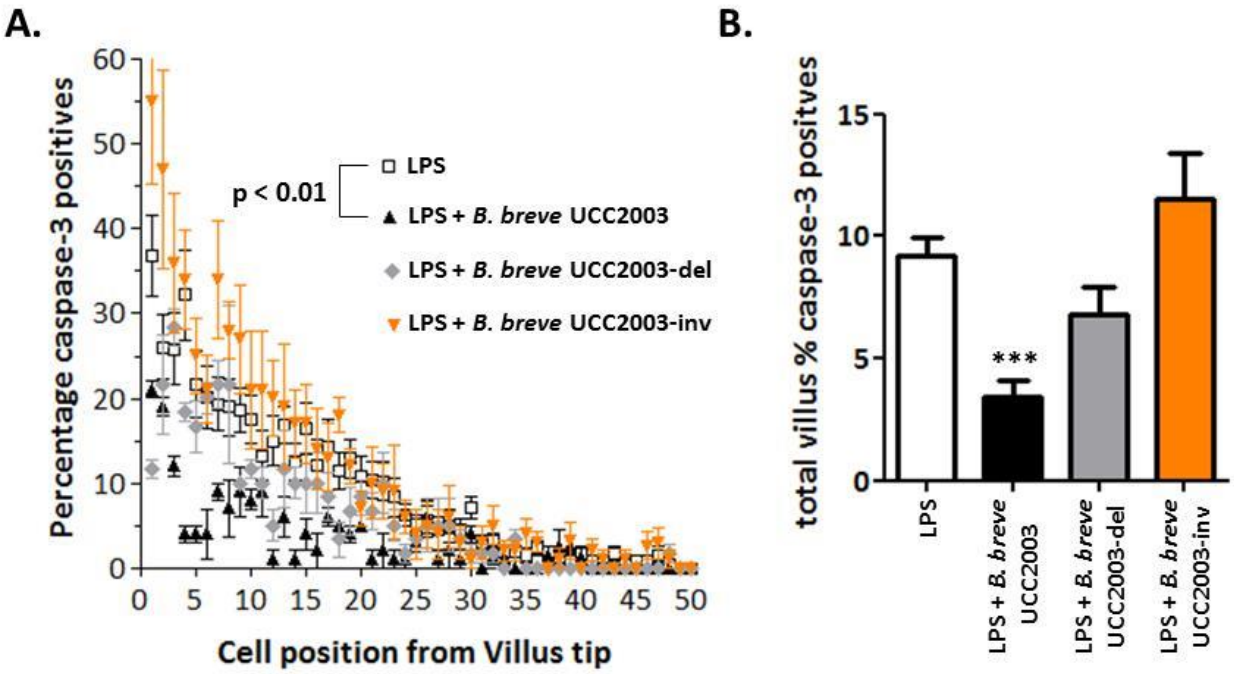


Supplementary Figure 3: *B. breve* UCC2003 resides in close contact with the small intestinal epithelium. Representative RNAscope staining for *B. breve* (*B. breve* specific probe) brown cells and arrows) in the small intestine of *B. breve* colonised mice (after 3 x 24 h doses at ~1 x 10^9).



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772 Supplementary Figure 4: Bifidobacterial EPS modulates cell shedding.

773 Combination of counts from Figures 2, 4 and 5 to provide representative overview of LPS-induced
774 cell shedding responses between *B. breve* strains. C57BL/6 mice were gavaged (3 x 24 h doses at ~1
775 x 10^9) with either *B. breve* UCC2003 or *B. breve* UCC2003del (i.e. EPS-negative) or *B. breve*
776 UCC2003inv (i.e. EPS2) and challenged with LPS. **(A)** Formalin fixed, paraffin-embedded intestinal
777 sections were stained with anti-CC3 and quantified using the WinCrypts and Score programs along
778 villus length, **(B)** average percentage of total caspase-3 positive events. Data are mean ± SD, *n* =
779 12/group (two independent experiments) analysed with Mann–Whitney U test.

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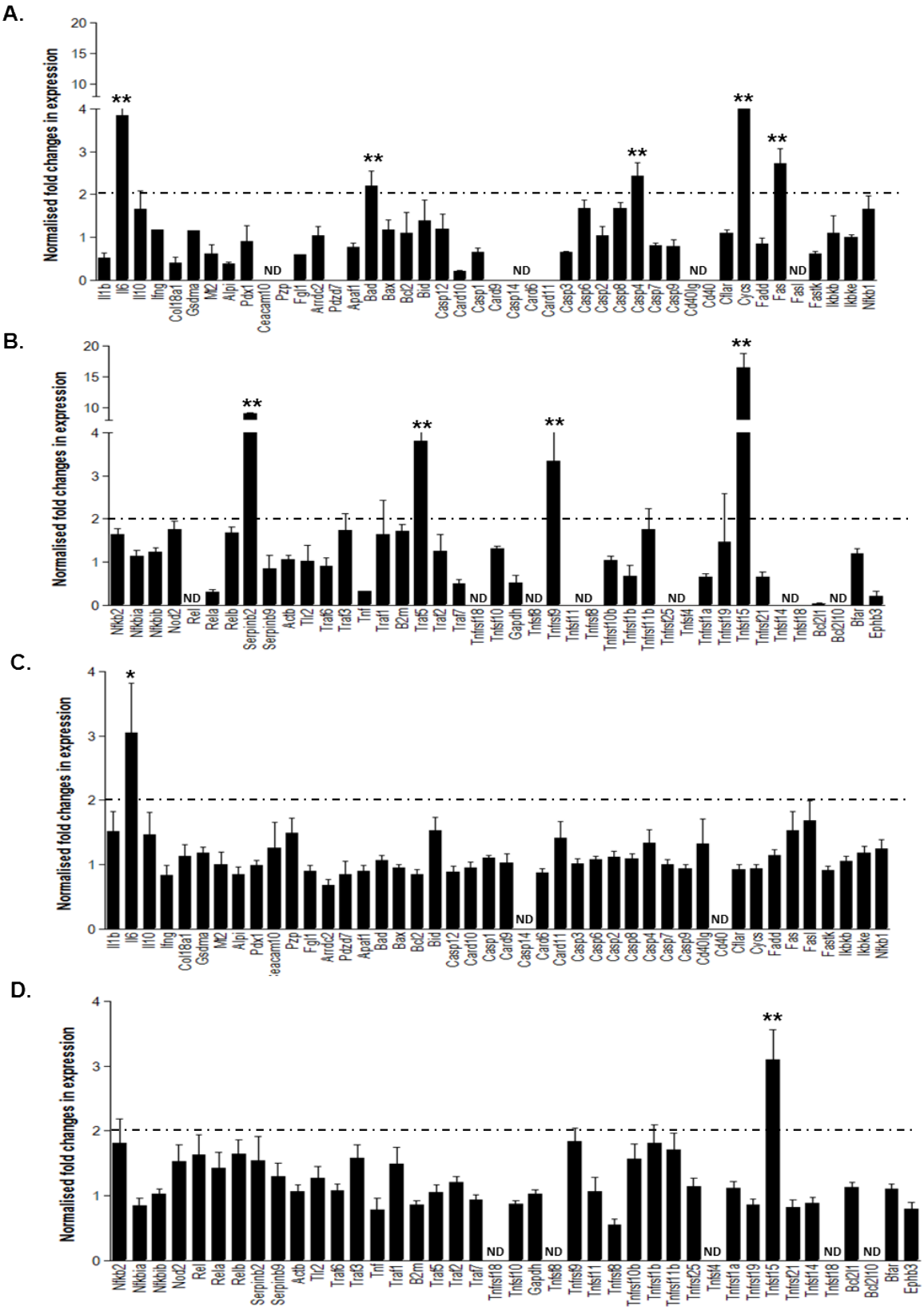

781

782 **Supplementary Figure 5:** Whole small intestinal homogenates from LPS challenged **(A, B)** *B. breve*

783 UCC2003-EPSdel and **(C, D)** *B. breve* colonised mice compared to control (i.e. PBS) were subjected to

784 custom array RT-PCR. Data are mean ± SD, n = 6 (two independent experiments), *P < 0.05 and **P <

785 0.01, non-detectable (ND), and analysed with Mann–Whitney U test

RNAscope

RNAscope was performed using a commercial kit from Advanced Cell Diagnostics (California, USA) as per the manufacturer's instructions. Briefly, 5 µm formalin fixed paraffin embedded small intestinal tissue was mounted on Superfrost plus slides (ThermoFisher) before baking in a dry oven at 60^{o}C for 1 h. Slides were then deparaffinised with Xylene and 100% ethanol before applying Pre-treat solution 1 for 10 minutes at room temperature. Slides were then washed in distilled water before incubating in boiling Pre-treat 2 solution for 15 minutes. Following further washes, Pre-treat solution 3 was applied in a humidified chamber at 40^{o}C for 30 minutes. After further washes, *B. breve* UCC2003 specific probe or Cyclophylin B control probe was hybridised to the slides for 2 h at 40^{o}C. Following washing in wash buffer, a series of amplification probes (AMP1 to AMP6) were sequentially bound and washed to/from the slides before signal detection using DAB substrate as per the manufacturer's recommendations. Slides were then counterstained with haematoxylin, dehydrated and mounted for visualisation.

Faecal DNA extractions, quantification and sequencing

DNA was extracted from murine faecal samples using the FastDNA^{TM} SPIN Kit for Soil (MP Biomedicals) following the manufacturer's instructions but incorporating an extension of the initial bead-beading time to three minutes.

The concentration of bacterial DNA was quantified using Qubit and normalised to 5 ng ml^{-1} for all samples. Extracted DNA was used as a template for PCR amplification of the V4 region of the 16S rRNA gene, 5' AAT GAT ACG GCG ACC ACC GAG ATC TAC A and, 5' CAA GCA GAA GAC GGC ATA CGA GAT AAC T. Amplification conditions of the PCR were: 1 cycle of 94°C 3 min and 25 cycles of 94°C for 45 s, 55°C for 15 s and 72°C for 30 s using a 96 well Thermal Cycler PCR machine. 16S RNA gene libraries were sequenced on the Illumina MiSeq platform with 250 bp paired end reads.

Sequence processing

All raw sequence reads were processed through quality control using FASTX-Toolkit [63] keeping a minimum quality threshold of 33 for at least 50% of the bases. Reads that passed the threshold were aligned against SILVA database (version: SILVA_119_SSURef_tax_silva) [64] using BLASTN (ncbi-blast-2.2.25+; Max e-value 10e-3) [65] separately for both pairs. After performing the BLASTN alignment, all output files were imported and annotated using the paired-end protocol of MEGAN [66].

Taxonomic annotation

For processing the BLAST files by MEGAN6, we used parameter settings of "Min Score = 50", "Top Percent = 10". Some reads which did not have any match to the respective database were placed under a "No hit" node, and some reads that were originally assigned to a taxon that did not meet our selected threshold criterion were pushed back using the lowest common ancestor (LCA) algorithm to higher nodes where the threshold was met. After importing datasets in MEGAN, we obtained MEGAN-own "rma files" for each data mapped onto NCBI taxonomy based on our selected threshold. Further, all the files were compared and analysed within MEGAN.

Statistical analysis

For microbiota analysis R software was used [67]. Average community profile comparison of two groups are displayed using pie charts in MEGAN. Abundance matrices were depicted using boxplots in R for each taxa showing comparison of two groups.