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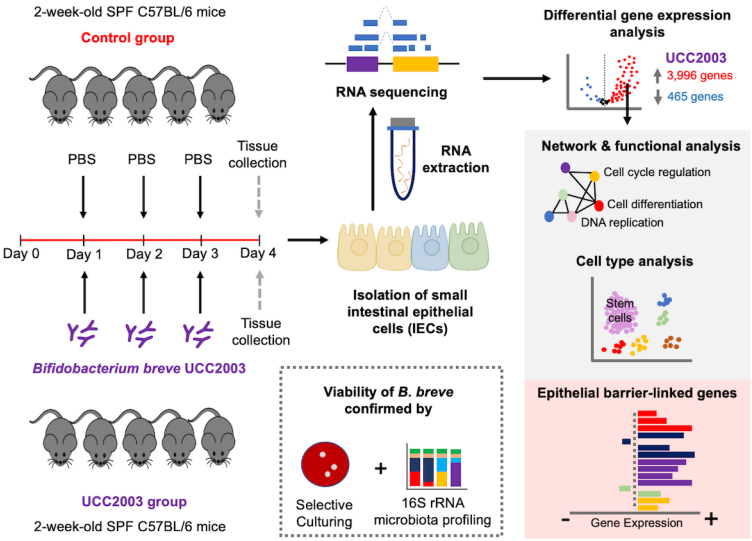
2-week-old SPF C57BL/6 mice
Control group
PBS
PBS
PBS
Tissue collection
Day 0
Day 1
Day 2
Day 3
Day 4
Tissue collection
Bifidobacterium breve UCC2003
UCC2003 group
2-week-old SPF C57BL/6 mice
RNA sequencing
RNA extraction
Isolation of small intestinal epithelial cells (IECs)
Viability of B. breve confirmed by
Selective Culturing
16S rRNA microbiota profiling
Differential gene expression analysis
UCC2003
3,996 genes
465 genes
Network & functional analysis
Cell cycle regulation
Cell differentiation
DNA replication
Cell type analysis
Stem cells
Epithelial barrier-linked genes
Gene Expression

Title

***Bifidobacterium breve* UCC2003 induces a distinct global transcriptomic programme in neonatal murine intestinal epithelial cells**

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Summary

The underlying health-driving mechanisms of *Bifidobacterium* during early life are not well understood, particularly how this microbiota member may modulate the intestinal barrier via programming of intestinal epithelial cells (IECs). We investigated the impact of *Bifidobacterium breve* UCC2003 on the transcriptome of neonatal murine IECs. Small IECs from two-week-old neonatal mice administered *B. breve* UCC2003 or PBS (control) were subjected to global RNA-Seq, and differentially expressed genes, pathways and affected cell types determined. We observed extensive regulation of the IEC transcriptome with ~4,000 genes significantly up-regulated, including key genes linked with epithelial barrier function. Enrichment of cell differentiation pathways were observed, along with an overrepresentation of stem cell marker genes, indicating an increase in the regenerative potential of the epithelial layer. In conclusion, *B. breve* UCC2003 plays a central role in driving intestinal epithelium homeostatic development during early life and suggests future avenues for next-stage clinical studies.

Key words: RNA-Seq, *in vivo*, *Bifidobacterium breve*, intestinal epithelial cells, differential gene expression, early life

Introduction

Bifidobacterium represents a keystone member of the early life gut microbiota (Arrieta et al., 2014, O'Neill et al., 2017, Derrien et al., 2019). Certain species and strains are found at high levels in vaginally delivered breast-fed infants including; *Bifidobacterium longum* subsp. *infantis*, *B. longum* subsp. *longum*, *B. bifidum*, *B. pseudocatenulatum* and *B. breve* (Dominguez-Bello et al., 2010, Mikami et al., 2012, Nagpal et al., 2017, Stewart et al., 2018). As a dominant member of the neonatal gut microbiota, *Bifidobacterium* is associated with metabolism of breast milk, modulation of host immune responses, and protection against infectious diseases (Fukuda et al., 2012, Ling et al., 2016, Robertson et al., 2019, Lawson et al., 2020, Patole et al., 2016, Baucells et al., 2016, Jacobs et al., 2013, Plummer et al., 2018). However, the mechanisms driving improved health outcomes during early life are largely underexplored and are likely strain dependent.

A key interface between *Bifidobacterium* and the host is the intestinal epithelial cell (IEC) barrier (Thoo et al., 2019, Groschwitz and Hogan, 2009). Previous studies have indicated that certain strains of *Bifidobacterium* specifically modulate IEC responses during inflammatory

insults, which may help protect from certain gut disorders (Hsieh et al., 2015, Srutkova et al., 2015, Grimm et al., 2015). In murine experimental models, previous work by our group has shown that infant-associated *B. breve* UCC2003 modulates cell death-related signalling molecules, which in turn reduces the number of apoptotic IECs (Hughes et al., 2017). This protection from pathological IEC shedding appeared to be via the *B. breve* exopolysaccharide (EPS) capsule and the host-immune adaptor protein MyD88. Another strain of *B. breve*, NumRes 204 (commercial strain) has also been shown to up-regulate the tight junction proteins Claudin 4 and Occludin in a mouse colitis model (Zheng et al., 2014, Plantinga et al., 2011).

Many of the studies to date have focused on the role of *Bifidobacterium* and modulation of IECs in the context of acute or chronic gut inflammation, with expression profiling limited to specific immune or apoptosis signalling targets (Plaza-Diaz et al., 2014, Riedel et al., 2006, Liu et al., 2010, Hsieh et al., 2015). As many of these studies have involved pre-colonisation of the gut with *Bifidobacterium* strains, followed by inflammatory insult, this suggests that initial priming during normal 'healthy' conditions may modulate subsequent protective responses. Furthermore, these studies have often been performed in adult mice rather than exploring effects during the early life developmental window, where *Bifidobacterium* effects are expected to be most pronounced. Previous work has indicated that there is significant modulation of the neonatal IEC transcriptome in response to gut microbiota colonisation, but to date no studies have probed how particular early life associated microbiota members, like *Bifidobacterium* may modulate neonatal IEC responses (Pan et al., 2018). Thus, to understand if and how *Bifidobacterium* may modulate IEC homeostasis during the early life developmental window, we administered *B. breve* UCC2003 to neonatal mice and profiled transcriptional responses in isolated small intestine IECs using global RNA-Seq. Our analysis indicated whole-scale changes in the transcriptional programme of IECs (~4,000 significantly up-regulated genes) that appear to be linked to cell differentiation/proliferation and immune development. Notably the stem cell compartment of IECs seemed to elicit the strongest gene signature. These data highlight the role of *B. breve* UCC2003 in driving early life epithelial cell differentiation and maturation; impacting intestinal integrity and immune functions, which provides a mechanistic basis for understanding associated health-promoting effects.

Results

To examine the effects of *B. breve* UCC2003 on the transcriptional profiles of host IECs under homeostatic conditions, we extracted RNA from isolated IECs of healthy two-week old neonatal mice (control group) and mice gavaged with *B. breve* UCC2003 for three consecutive days (n=5 per group). Isolated RNAs from IECs were subjected to RNA-Seq to determine global mRNA expression (Figure 1). Subsequently, Differential Gene Expression (DGE) analysis was performed to understand *B. breve*-associated gene regulation.

Minimal impact of *B. breve* UCC2003 on the wider neonatal gut microbiota

Initially, we examined for the presence of *B. breve* UCC2003 in the gut microbiome and impact on the wider microbiota using culture and 16S rRNA microbiota profiling approaches (Figures 2a-b). We observed high levels of *B. breve* UCC2003 across the four days in faecal samples, with higher levels of viable *B. breve* UCC2003 within the colon (~10^8 CFU/g), when compared to the small intestine (~10^5 CFU/g; Figure 2b). Based on 16S rRNA analysis, relative abundance of *Bifidobacterium* increased significantly in the UCC2003 group (P=0.012) following bacterial administration, while the control group displayed very low relative *Bifidobacterium* abundance (~0.01%; Figure 2c). Principal component analysis (PCA) on gut microbiota profiles (control vs UCC2003) showed a distinct change in microbial community composition in the UCC2003 group primarily driven by increased relative abundance of *Bifidobacterium*, which may also correlate with increased overall microbial diversity in the UCC2003 group (Figure 2d-e). Linear Discriminant Analysis (LDA) also indicated that *Bifidobacterium* was uniquely enriched in UCC2003 group, and low relative abundance (<2%) microbiota members such as *Streptococcus*, *Ruminococcus*, *Prevotella* and *Coprococcus* were significantly lower (Figure 2f-g). Overall, administration of *B. breve* UCC2003 appeared to minimally impact the wider gut microbiota, without significantly altering relative abundance of other major resident taxa including *Lactobacillus*, *Bacteroides* and *Blautia* compared to the control group.

Impact of *B. breve* UCC2003 on the neonatal intestinal epithelial transcriptome

To understand the distribution of samples based on IEC gene expression profiles we performed PCA analysis (Figure 3a; Table S1). All samples clustered according to group (control vs UCC2003), suggesting a significant impact of *B. breve* UCC2003 on gene expression profiles, with distance-wise clustering (Jensen-Shannon) also supporting separation of experimental groups (Figure 3b). To define Differentially Expressed Genes

(DEG), we employed a filter of absolute log2(fold change) > 1.0 (with adjusted $p < 0.05$), which equates to a minimum two-fold change in gene expression (Figure 3c-e; Table S2). After analysis, a total of 3,996 DEGs were significantly up-regulated, while 465 genes were significantly down-regulated in *B. breve* UCC2003 supplemented animals when compared to controls (Figure 3c and 4a). Notably, we also performed the same experimental protocol on healthy mice aged 10-12 weeks, and we did not observe any significant DEGs, suggesting *B. breve* UCC2003 modulation of IECs is strongest within the early life window under homeostatic conditions.

To determine the functional role of the DEGs, we examined the most significantly regulated genes ranked by False Discovery Rate (FDR) adjusted p values (or, *q* values). We first looked at the top 20 up-regulated DEGs in the *B. breve* UCC2003 experimental group (Figure 4b). Most genes annotated with known biological processes were cell differentiation and cell component organisation functions including *Ccnb1ip1*, *Hist1h4b*, *Vps13b* and *Fgd4* (annotated in the PANTHER Gene Ontology [GO] Slim resource). Two genes were involved in cell death and immune system processes, namely *Naip6* and *Gm20594* (Table S3). When we ranked the top-regulated genes using log2-fold change, we observed increased expression of *Creb5*, which is involved in the regulation of neuropeptide transcription (cAMP response element binding protein; CREB) (Figure 4c). CREB is also known to regulate circadian rhythm, and we also identified additional circadian-clock-related genes that were significantly up-regulated including *Per2* and *Per3*. We noted that several top down-regulated DEGs were annotated as genes involved in metal binding, or metal-related genes including *Mt1*, *Mt2*, *Hba-a1*, *Hbb-bt* and *Ftl1-ps1* (Figure 4d; Table S4).

Regulation of intestinal epithelial barrier-associated genes

As *B. breve* strains have been previously shown to modulate certain tight junction/barrier-related proteins, we next investigated DEGs associated with intestinal epithelial barrier development/intestinal structural organisation (Figure 4e). Several tight-junction (TJ) structural-associated DEGs were observed, including Claudin-encoding gene *Cldn34c1* (Log2 fold-change [LFC] 3.14), Junction Adhesion Molecules-encoding genes *Jam2* (LFC 2.9), and Tight Junction protein (also called Zonula Occludens protein; ZO) -encoding gene *Tjp1* (LFC 1.49). Genes that encode integrins (involved in regulation of intracellular cytoskeleton) also exhibited a trend of increased expression (13/14; 92.8%). Both Piezo genes, which assist in tight junction organisation, *Piezo1* (LFC 1.25) and *Piezo2* (LFC 1.9), were significantly up-regulated in the *B. breve* UCC2003 treated group.

Over 90% of cadherins, proteins associated with the assembly of adherens junctions (Figure 4e) were up-regulated; including *Pcdhb14* (LFC 2.8), *Pcdhgb4* (LFC 2.7), *Pcdh8* (LFC 1.3), *Fat1* (LFC 1.5) and *Dsg2* (LFC 1.1). Interestingly, several genes (4/7; 57.1%) involved in mucus layer generation were significantly up-regulated in the UCC2003 experimental group including *Muc2* (LFC 2.2), *Muc6* (LFC 3.7), *Muc5b* (LFC 2.9), and *Muc4* (LFC 1.24). Genes *Gja1* (LFC 3.59) and *Gjb8* (LFC 2.63) that encode gap junction proteins were also up-regulated. In addition, we also investigated differential expression of genes associated with integrin assembly and downstream integrin signalling pathways (Figure 4f). Over 70% (16/21) of these genes were up-regulated, with 52.3% (11/21) significantly increased in gene expression in the UCC2003 group (LFC >1.0).

We observed increased expression of genes associated with IEC barrier development including cadherins, gap junctions, integrins, mucus layer-associated genes, and several key tight junction proteins. These strongly induced gene expression profiles suggest that *B. breve* UCC2003 is involved in enhancing epithelial barrier development in neonates.

Modulation of cell maturation processes

We next sought to understand the biological functions of up-regulated DEGs by employing PANTHER GO-Slim functional assignment, and process/pathway enrichment analysis (see Figure S1; Table S5 and S6). DEGs were predominantly involved in general biological processes including cellular process (901 genes) and metabolic process (597 genes; Table S7). At the molecular function level, DEGs were primarily assigned to binding (868 genes) and catalytic activity (671 genes; Table S8), with Olfactory Signalling Pathway and Cell Cycle (biological) pathways also found to be enriched (Table S9).

To delve further into the data, we constructed a signaling network based on up-regulated DEGs (*n*=3,996) with the aim of identifying specific gene networks involved in important signalling pathways (Figure 5a). Overall, 1,491 DEGs were successfully mapped (37.3%) to a signalling network that comprised 8,180 genes. Four individual clusters of genes were detected, with functional assignment and pathway analysis implemented on these clusters (Figure 5a). All gene clusters were associated with cell differentiation and maturation, with cluster 1 (68 genes) linked specifically with DNA replication and transcription, cluster 2 (26 genes) with cell growth and immunity, cluster 3 (11 genes) with cell replication, and cluster 4 (72 genes) related to cell cycle and cell division (Table S10).

Intestinal cell type analysis on DEGs identifies significant enrichment of epithelial stem cells

IECs include several absorptive and secretory cell types, namely enterocytes, Paneth cells, goblet cells, enteroendocrine cells, tuft cells and stem cells. As these cells perform different functions in the gut, it was important to understand whether *B. breve* UCC2003 had a cell type specific effect on the intestinal epithelium. Using known cell type specific gene markers (Haber et al., 2017), we identified cell type gene signatures modulated within the UCC2003 group (Figure 5b-c). Importantly, all cell type markers were well represented in the expressed genes of the whole IEC transcriptomics data from both groups (control + UCC2003), thus validating the presence of all IEC types in our study data (Figure 5b). Cell type analysis of genes differentially expressed after *B. breve* UCC2003 supplementation, revealed that stem cell marker genes were significantly enriched (30%; $P < 0.05$) among the six IEC types (Table S11). Signatures of other cell types were also present (linking to marker genes in the DEG list) but not significantly overrepresented: Tuft cells (22%), enteroendocrine cells (18%), goblet cells (15%), Paneth cells (15%) and enterocytes (13%; Figure 5c). These data indicated that intestinal epithelial stem cells, cells primarily involved in cell differentiation, were the primary cell type whose numbers and transcriptomic programme were regulated by *B. breve* UCC2003.

Further investigation of this stem cell signature revealed that of the 37 differentially expressed marker genes, 35 are up-regulated in the presence of *B. breve* UCC2003. This indicates an increase in the quantity of stem cells or semi-differentiated cells in the epithelium, consistent with the overrepresentation of cell cycle and DNA replication associated genes observed in the whole differential expression dataset. Functional analysis of the 37 stem cell signature genes revealed only one overrepresented process – Regulation of Frizzled by ubiquitination ($P < 0.05$), which is a subprocess of WNT signalling. WNT signalling is important in maintaining the undifferentiated state of stem cells (Nusse, 2008).

Finally, we employed a network approach to predict key transcription factor (TF) regulators of the differentially expressed stem cell marker genes, through which *B. breve* UCC2003 may be acting (Figure 5d). Using the TF-target gene database, DoRothEA, we identified expressed TFs known to regulate these genes (Garcia-Alonso et al., 2019, Holland et al., 2019). Five genes had no known and expressed regulator, thus were excluded. Hypergeometric significance testing was used to identify which of these TFs are the most influential (see Methods for details). This analysis identified 32 TF regulators (Figure 5d). Of these

regulators, 12 were differentially expressed in the IEC dataset (all up-regulated): *Fos, Gabpa, Rcor1, Arid2, Tead1, Mybl2, Mef2a, Ahr, Pgr, Kmt2a, Ncoa2* and *Tcf12.* Functional analysis of all the TF regulators and their targeted genes together, revealed overrepresented functions relating to WNT signalling, histone methylation for self-renewal and proliferation of hematopoietic stem cells and nuclear receptor (incl. estrogen) signalling (Table S12). These data provide evidence that *B. breve* UCC2003 directly affects key transcriptomic programmes regulating drives specific signalling processes, particularly within stem cells.

Discussion

The early life developmental window represents a crucial time for microbe-host interactions that impacts health both in the short- and longer-term. Understanding how specific microbiota members modulate host responses in pre-clinical models may help the design and development of next-stage targeted microbiota therapies in humans. Here we investigated how *B. breve* UCC2003 induces genome-wide transcriptomic changes in small intestine IECs of neonatal mice. We observed that *B. breve* had a global impact on the IEC transcriptome, evidenced by the large number of significantly up-regulated genes and pathways related to cell differentiation and cell proliferation, including genes associated with epithelial barrier function. We propose that *B. breve* may act as a key early life microbiota member driving fundamental cellular responses in murine IECs, particularly within the stem cell compartment, and thus drives epithelial barrier development and maintenance during neonatal life stages. However, further clinical studies would be required to determine if our findings extrapolate to the human setting.

B. breve is known to confer beneficial effect on gut health, however our knowledge related to the mechanisms underlying these responses are limited. Most studies have focused on targeted immune cells or pathways (during disease and/or inflammation), and to our knowledge no studies have probed global transcriptomic changes within IECs - the frontline physical barrier between bacteria and host (Turroni et al., 2014, Gann, 2010). Our presented findings in a pre-clinical model: ~4,000 up-regulated DEGs and ~450 down-regulated DEGs within the *B. breve* group indicate that this *Bifidobacterium* strain modulates whole-scale changes within this critical single cell layer. Notably, we also examined how *B. breve* modulates adult IEC responses, however, we did not observe any significantly differentially regulated genes when compared to control animals. The striking differences in DEGs between these two life points indicates that *B. breve*-modulation of IECs is limited to the

neonatal window. Dominance of *Bifidobacterium* in early life (including strains of *B. breve*) overlaps with the development and maturation of many host responses, including epithelial barrier integrity. Therefore, presence of these strains would be expected to play an over-sized role in this initial homeostatic priming, that may afford protection against inflammatory insults in later-life, as has been shown previously in a mouse model of pathological epithelial cell shedding (Hughes et al., 2017). Further clinical studies would be required to probe these findings in detail to determine their importance during healthy infant development.

Exploring the murine transcriptional responses in more detail revealed that expressions of key genes associated with formation of epithelial barrier components were up-regulated, including major cell junction protein encoding genes (75%; 42/56 genes). More specifically, several integrin-associated genes were up-regulated in the presence of UCC2003. Integrins facilitates cell-cell and cell-extracellular matrix ECM adhesion and binding, and assembly of the fibronectin matrix that is pivotal for cell migration and cell differentiation (Harburger and Calderwood, 2009, Qin et al., 2004, Mosher et al., 1991). Integrins also play an important role in downstream intracellular signalling that controls cell differentiation, proliferation and cell survival, including the Raf-MEK-ERK signalling pathway (we also observed enrichment of genes involved in this pathway) (Chernyavsky et al., 2005, Li et al., 2016). Another key intestinal barrier component is represented by tight junctions; linking complexes between intercellular spaces, and comprise transmembrane proteins including occludins, claudins, zona occludens and junctional adhesion molecules (Edelblum and Turner, 2009, Groschwitz and Hogan, 2009). Dysfunctional tight junctions may lead to a ‘leaky’ gut, which is characteristic of numerous intestinal disorders including inflammatory bowel diseases (Krug et al., 2014). Notably, previous work has suggested early life microbiota disruptions (via antibiotic usage) and reductions in *Bifidobacterium* are correlated with increased risk and/or symptoms of ulcerative colitis and Crohn’s disease (Kronman et al., 2012, Hildebrand et al., 2008, Favier et al., 1997, Shaw et al., 2010, Ng et al., 2011). Several clinical studies have indicated that supplementation with certain *Bifidobacterium* strains positively modulate gastrointestinal symptoms of patients, which is corrected with reductions of inflammatory markers in colonic IEC-containing biopsies, however *B. breve* UCC2003 has not been used clinically in this patient setting (Furrie et al., 2005, Steed et al., 2010). Similar findings have also been reported in different animal models of intestinal inflammation (Philippe et al., 2011, Grimm et al., 2015, Zuo et al., 2014). A wide range of TJ-related genes were up-regulated after UCC2003 supplementation, particularly *Tjp1* (that encodes ZO-1), *Jam2* and

Claudin34c1, with a previous study indicating other *Bifidobacterium* species (i.e. *B. bifidum*) also modulate TJ expression via ZO-1 (Din et al., 2020). These data indicated that specific strains of *Bifidobacterium* may modulate key barrier integrity systems during the neonatal period, and therefore absence of this key initial bacterial-host crosstalk may correlate with increased risk of chronic intestinal disorders in later-life (Shaw et al., 2010). Intestinal mucus, encoded by *Muc* genes (up-regulated due to *B. breve* UCC2003 in this study), plays a crucial role in colonic protection via formation of a physical barrier between the gut lumen and IECs, and deficiencies in MUC-2 has been linked with experimental colitis and increased inflammation in IBD patients (Shirazi et al., 2000, Van der Sluis et al., 2006). We have also observed that *B. breve* UCC2003 significantly increases goblet cell numbers and mucus production (in gnotobiotic and SPF mice; data not shown). Although the mucus layer may impact direct *Bifidobacterium*-IEC interactions, previous studies have indicated that *B. breve* UCC2003 surface molecules, such as EPS and the Tad pilus may modulate IEC function via signaling through TLRs (O'Connell Motherway et al., 2019, Hughes et al., 2017). Moreover, bifidobacterial metabolites, such as short-chain fatty acids may also act to modulate the IEC transcriptome, with previous studies indicating enhanced expression of TJs and cadherins via acetate (Hsieh et al., 2015, Ling et al., 2016, Ewaschuk et al., 2008, Lewis et al., 2017).

Further network and functional analysis indicated clusters of up-regulated DEGs were associated with cell maturation and cell differentiation (as confirmed by cell type specific analysis), suggesting neonatal *B. breve* exposure positively modulates IEC cell differentiation, growth and maturation. Somewhat surprisingly, we did not observe the same type of striking responses in immune pathways, which may be masked by the sheer number of DEGs involved in cellular differentiation and processes. However, pathways such as Toll-like Receptor TLR1 or TLR2 pathways do appear to be enriched (cluster 2 of signalling network analysis). This may link to previous work indicating that the UCC2003 EPS directly signals via TLR2 to induce MyD88 signalling cascades to protect IECs during intestinal inflammation (Hughes et al., 2017). *B. breve* M-16V was also shown to interact with TLR2 to up-regulate ubiquitin-editing enzyme A20 expression that correlated with increased tolerance to a TLR4 cascade in porcine IECs, further supporting the involvement of *B. breve* in programming key host immunoregulation receptors (Tomosada et al., 2013).

Cell type specific analysis of DEGs revealed stem cells as the IEC type most affected by *B. breve,* with absorptive enterocytes least affected despite being most accessible to bacteria in the gut. It could be hypothesised that *B. breve* or their secreted metabolites may reach the

crypts of the small intestinal epithelium. This has been previously suggested by *in situ* hybridisation histology *in vivo* and by *Bifidobacterium*-conditioned media altering the expression of hundreds of host epithelial genes linked to immune response, cell adhesion, cell cycle and development in IECs *in vitro* (Hughes et al., 2017, Guo et al., 2015). However, the direct impact of bifidobacterial-associated metabolites on these responses would require further studies to confirm metabolic activity of *B. breve* within the small intestine (via transcriptomics and metabolomics), although daily supplementation with live bacteria may also provide a source of these metabolites in our model. Interestingly, certain *Bifidobacterium* and *Lactobacillus* strains that have been heat-killed have also been shown to induce host responses, indicating that surface structures alone may play a role in downstream effects (Pique et al., 2019). All but two of the 37 differentially expressed stem cell marker genes were up-regulated in the presence of *B. breve* UCC2003, indicating an activating effect resulting in increased pluripotency of stem cells, increased quantity of stem cells and/or an increased quantity of semi-differentiated cells. Single cell sequencing of IECs could be used to further investigate this finding. Thirty-two TFs were predicted to regulate these stem cell signature genes, providing possible targets for future investigation of the mechanisms underlying these responses. Functional analysis of the stem cell signature genes and their regulators suggests *B. breve* increases pluripotency of stem cells and/or semi-differentiated epithelial cells through WNT signalling and nuclear hormone signalling (Jeong and Mangelsdorf, 2009). Furthermore, the overrepresentation of the process “RUNX1 regulates transcription of genes involved in differentiation of HSCs” indicates a possible role for histone methylation in response to *B. breve* UCC2003 (Imperato et al., 2015). Further determination of host and bacterial metabolome and proteome after *B. breve* exposure, may allow identification of the specific underlying molecular mechanisms (Guo et al., 2015).

In conclusion, *B. breve* UCC2003 plays a central role in orchestrating global neonatal IEC gene responses in a distinct manner as shown in our murine model; modulating genes involved in epithelial barrier development, and driving universal transcriptomic alteration that facilitates cell replication, differentiation and growth, particularly within the stem cell compartment. This study enhances our overall understanding of the benefits of specific early life microbiota members in intestinal epithelium development, with prospective avenues to probe further health-promoting mechanisms of *Bifidobacterium* in humans. Further work exploring time-dependent transcriptional responses, impact of other *Bifidobacterium* species and strains (and use of mutants and transcriptionally active strains as positive controls), in

tandem with metabolomic and proteomic approaches are required to advance our understanding on the key host pathways and bifidobacterial molecules governing development and maturation of the intestinal barrier during the early life window. Nevertheless, further clinical studies would be essential to explore if these responses and findings are similar to those observed in humans.

Limitations of the Study

As we only observed low relative abundance of *Bifidobacterium* in our control neonatal animals this may suggest induction of responses may be linked to 'introduction' of a new microbiota member (i.e. *B. breve* UCC2003), therefore results should be carefully interpreted. However, we did not observe associated global transcriptional inflammatory immune changes that would be expected if this was the case, but rather global changes in barrier function transcripts and pathways. Furthermore, *Bifidobacterium* has previously been isolated from C57BL/6 mice (including from our mouse colony), and therefore appears to be a resident rodent gut microbiota member, although it is found at varying abundances in different animal units and suppliers (Grimm et al., 2015, Hughes et al., 2020). Indeed, one particular study has shown that high levels of resident *Bifidobacterium* in mice directly correlated with improved immune responses to cancer immunotherapies (Sivan et al., 2015). In addition, we did not explore if *B. breve* UCC2003 is potentially driving more nuanced microbe-microbe interactions, and that, in-directly, these may also be stimulating IEC responses. Therefore, further studies probing these aspects in more detail, and comparing other *Bifidobacterium* strains, to compare and contract responses, would be of interest.

B. breve UCC2003 is a model strain that was previously isolated from the stool of a breast-fed infant (National Collection of Industrial Food and Marine Bacteria (NCIMB), 2020, Sheehan et al., 2007). Although a human-associated strain, it has not been used in clinical studies, so directly extrapolating to human-specific settings should be cautiously considered. Further large-scale clinical studies would be required to confirm any positive strain-level impacts, however in-depth analysis of e.g. small IECs would be unethical in a healthy infant cohort, which emphasises the importance of preclinical models.

Previous studies have shown this strain can efficiently colonise (long-term) the mouse gastrointestinal tract, however, we could not confirm this in our short-term, daily supplementation study (Cronin et al., 2008, O'Connell Motherway et al., 2011). Therefore, the IEC responses observed may occur as a result of transient interactions with *B. breve*

UCC2003 as it passes through the small intestine. Nevertheless, although at lower levels (~10^5 CFU/g), we did observe viable *B. breve* UCC2003 in the small intestine, linking to our subsequent observations of significant impacts on the IEC transcriptome from this intestinal region.

Very low abundance microbiota members (<2% relative abundance) including *Streptococcus*, *Ruminococcus*, *Prevotella*, and *Coprococcus* were significantly reduced in relative abundance compared to controls, raising the question whether supplementation of *Bifidobacterium* could have reduced these taxa. Regrettably, we could not determine if this is a bifidobacterial effect due to the lack of longitudinal samples, and we did not quantify bacterial titres, which is an important consideration for future work. We also did not profile microbial community composition within the small intestines which is known to differ from fecal samples.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Lindsay J. Hall (Lindsay.Hall@quadram.ac.uk).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The code generated for RNA-Seq analysis during this study are available at GitHub https://github.com/raymondkiu/Bifidobacterium-IEC-transcriptomics. The raw sequencing reads (both RNA-Seq and 16S rRNA amplicon sequencing) are available at European Nucleotide Archive (ENA) accession number PRJEB36661.

Methods

All methods can be found in the accompanying Transparent Methods supplemental file.

Ethics Approval

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.

Supplemental Information

Supplemental Information can be found online at

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Author Contributions

Conceptualisation, R.K., L.C.H. and L.J.H.; Methodology, R.K., A.T., L.C.H. and L.J.H.; Software, R.K., A.T. and S.C.; Validation, R.K., A.T., T.K. and L.J.H.; Formal analysis, R.K. and A.T.; Investigation, R.K., A.T., L.C.H. and C.L.; Resources, S.C.; Data curation, R.K.; Writing - original draft preparation, R.K., A.T. and L.J.H.; Writing - review and editing, R.K., A.T., D.vS, T.K. and L.J.H.; Visualisation, R.K. and A.T.; Supervision, T.K. and L.J.H.; Project administration, R.K.; Funding acquisition, T.K., D.vS. and L.J.H.

Declaration of Interests

The authors declare no competing interests.

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Figure and Scheme Legends

Figure 1. Schematic representation of the study design and *in silico* analysis workflow

Figure 2. 16S rRNA amplicon sequencing analysis of murine intestinal microbiota
(A) Genus-level 16S rRNA gene profiling of mice gut microbiota on Day 4 (control vs UCC2003).
(B) Dynamics of *B. breve* UCC2003 load (CFU/g) from Day 1 (prior to *B. breve* administration) through Day 4. *B. breve* was present in intestines throughout (small intestines and colon; on Day 4). ND: Non-detectable. Data are represented as mean ± SD.
(C) Relative abundance of genus *Bifidobacterium* in UCC2003 group is significantly increased.
(D) Principal Component Analysis on mice gut microbiota (control vs UCC2003 based on genus-level metataxonomics).
(E) Shannon diversity index on mice gut microbiota (control vs UCC2003). Data are represented as mean ± SD. Significance test: *t*-test (* $p<0.05$; two-sided).
(F) Linear Discriminant Analysis (LDA) showing enriched taxa in each group (control vs UCC2003).
(G) Relative abundance comparison of all genera. * $p<0.05$ (LDA).

Figure 3. RNA-Seq analysis and statistics

(A) Principal component analysis showing distinct overall gene expression profiles across all individual samples based on 12,965 highly-expressed genes. See also Table S1.
(B) Clustering of individual RNA-Seq samples based on Jensen-Shannon distance. Distinct gene expression profiles were demonstrated between these two groups of samples (control vs UCC2003).
(C) Total number of differentially expressed genes (DEGs) in UCC2003 group.
(D) Volcano plot on global gene expression. Up-regulated DEGs are labelled as red dots whilst down-regulated DEGs in blue.
(E) MA plot on global gene expression.

Figure 4. Gene expression analysis
(A) Heatmap comparison of gene expression profiles of 4,461 DEGs (control vs UCC2003). See also Table S2.
(B) Top 20 DEGs ranked by FDR-adjusted p values (q values).
(C) Top 20 up-regulated DEGs ranked by log_2FC values.
(D) Top 20 down-regulated DEGs ranked by log_2FC values.
(E) Expression of epithelial integrity associated genes in UCC2003 group ($q<0.05$).
(F) Expression of integrin-associated genes in UCC2003 group. Grey dotted lines in the bar charts indicate the threshold of absolute $Log_2FC>1.0$. Data are represented as Mean ± SE.

Figure 5. Signalling network analysis, IEC subtyping and key regulator analysis
(A) Cluster analysis of signaling network for significantly up-regulated genes (*n*=3,996). Representative enriched pathways (Reactome) and GO terms (Biological Process) identified in each individual cluster were listed alongside. See also Table S10.
(B) Heat plot showing percentage of cell type signature genes in DEG and expressed genes (both control and UCC2003 groups). All expressed genes are well represented in IEC cell type signature genes.
(C) Cell type analysis on IEC DEGs using known cell-specific signature genes. Stem cells were statistically over-represented in DEGs. * $p<0.05$. See also Table S11.
(D) Key regulators of stem cell DEGs.

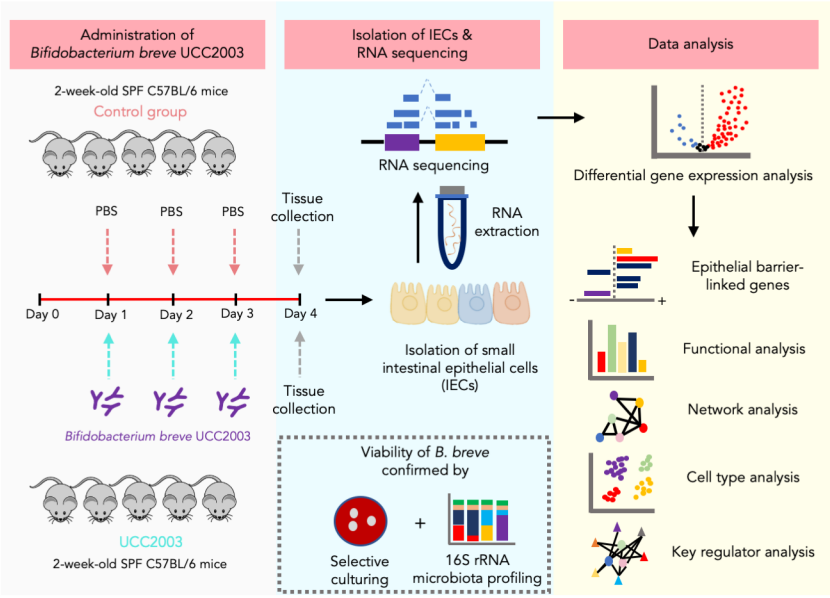
Supplemental table titles

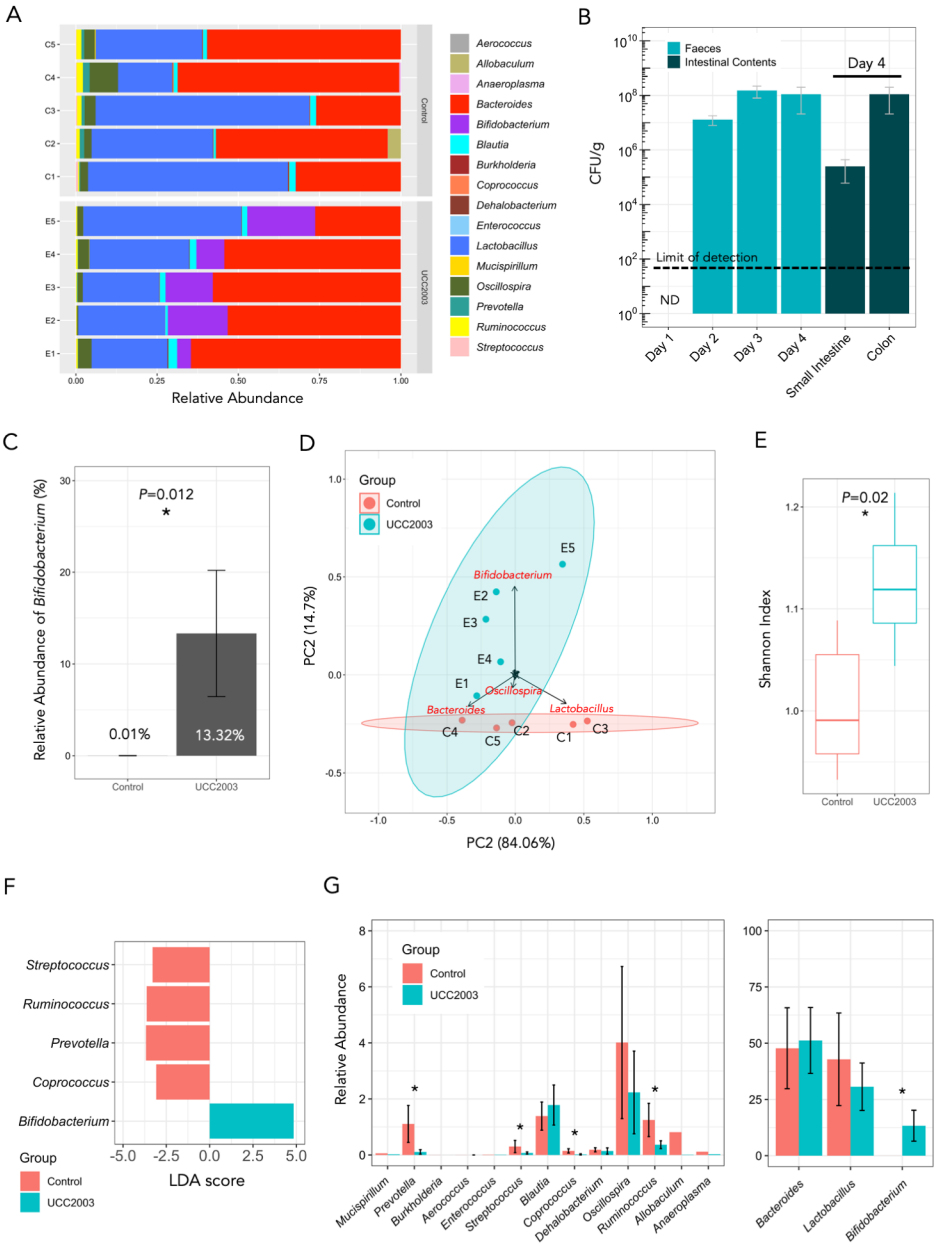
Table S1. Highly expressed genes (n=12,965 genes). Related to Figure 3.

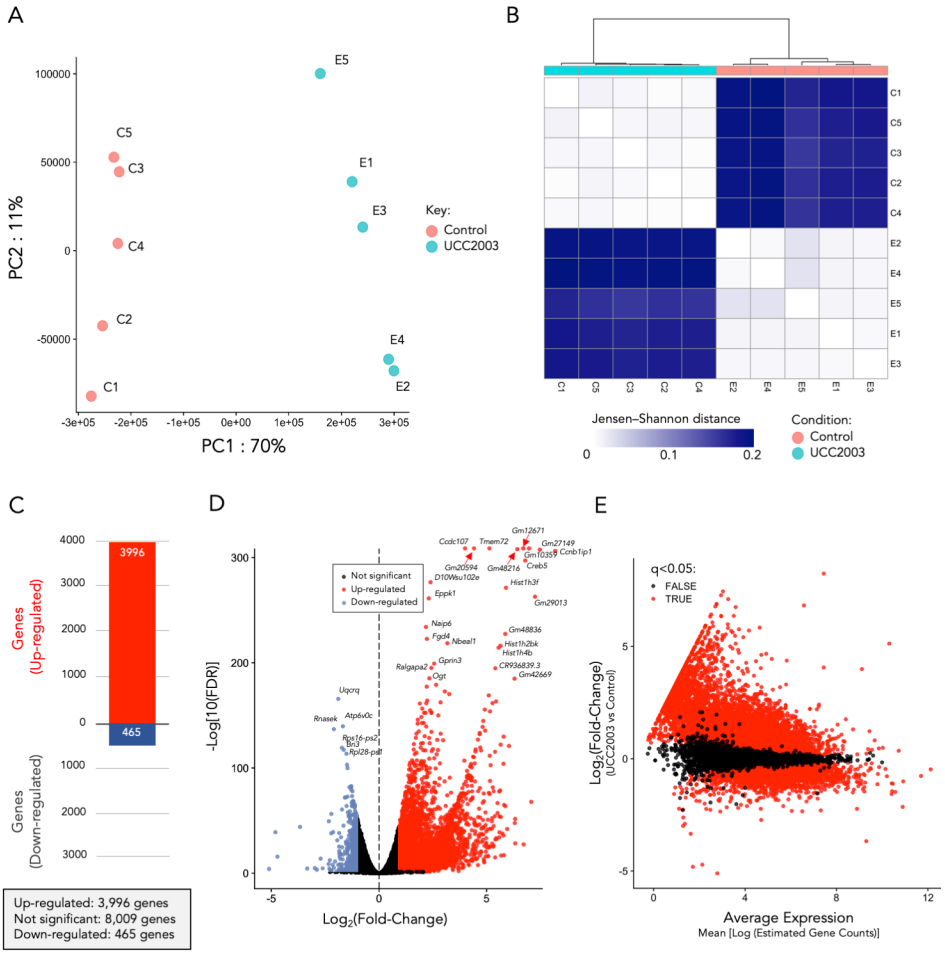
Table S2. Significantly regulated genes (n=4,461 genes). Related to Figure 4.

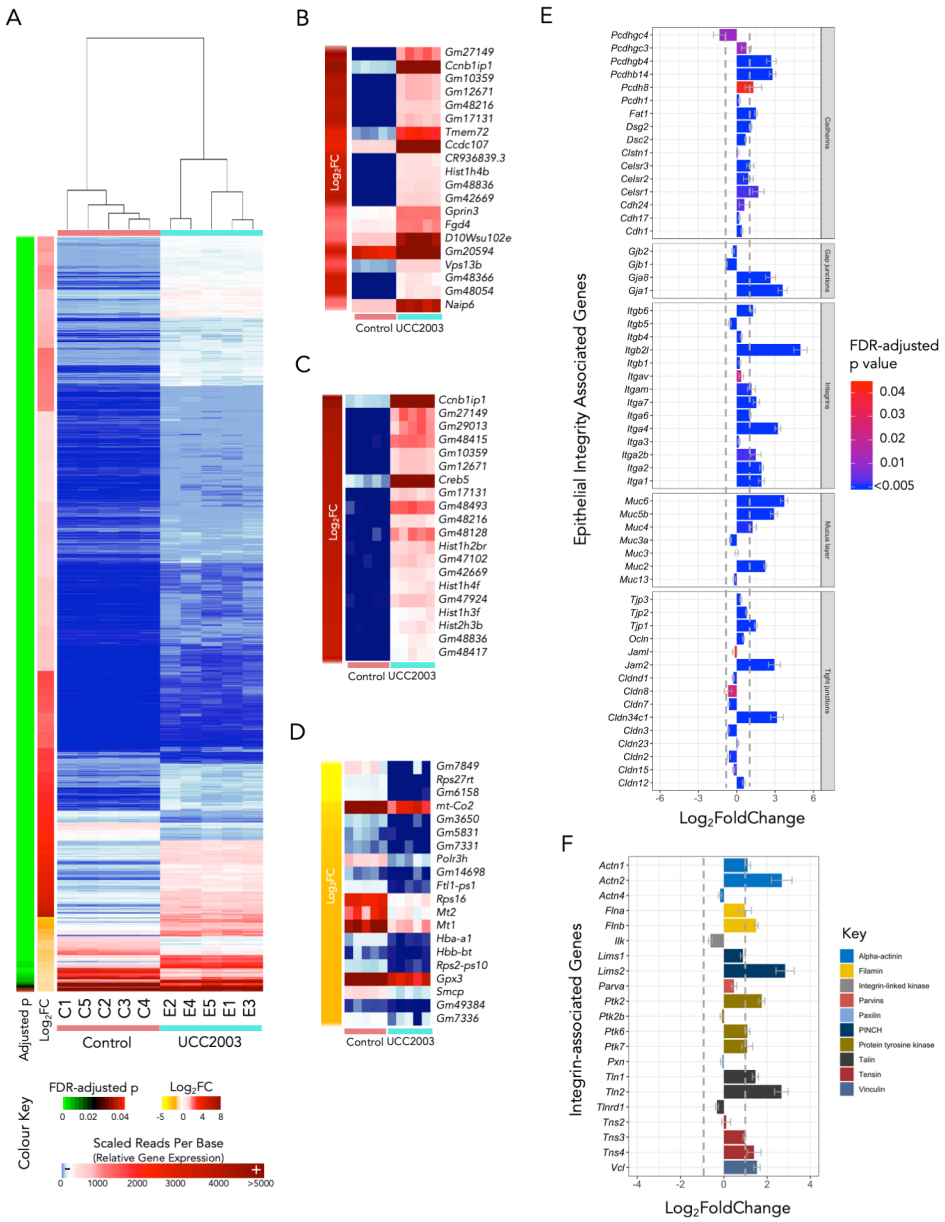
Table S10. Cluster analysis on DEGs and related genes. Related to Figure 5.

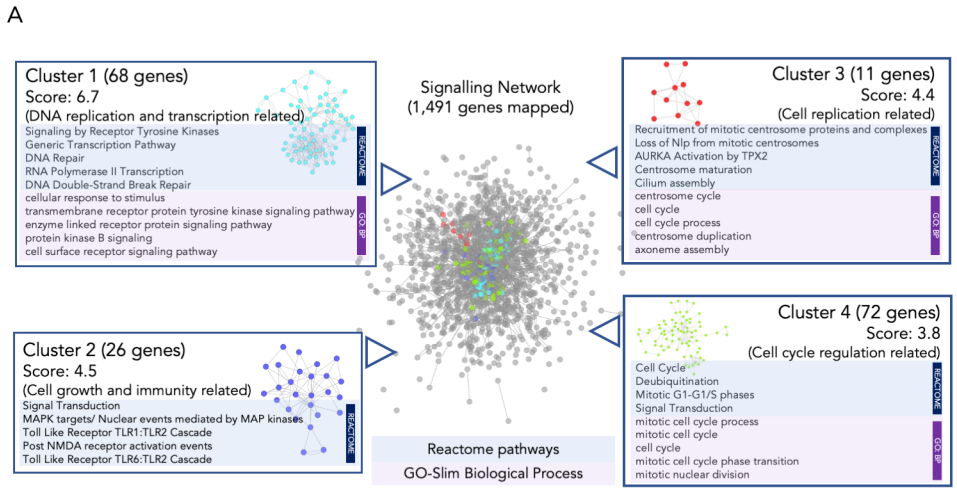
Table S11. Overlap between cell type signature genes and differentially expressed genes. Related to Figure 5.

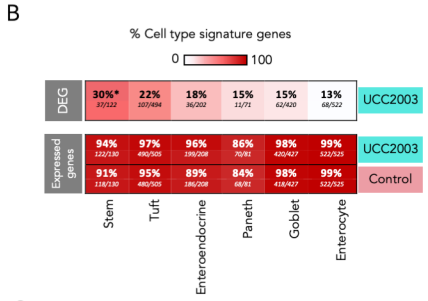

Administration of
Bifidobacterium breve UCC2003
2-week-old SPF C57BL/6 mice
Control group
PBS
PBS
PBS
Day 0
Day 1
Day 2
Day 3
Day 4
Bifidobacterium breve UCC2003
UCC2003
2-week-old SPF C57BL/6 mice
Isolation of IECs &
RNA sequencing
RNA sequencing
Tissue collection
RNA extraction
Isolation of small intestinal epithelial cells (IECs)
Tissue collection
Viability of B. breve confirmed by
Selective culturing
+
16S rRNA microbiota profiling
Data analysis
Differential gene expression analysis
Epithelial barrier-linked genes
Functional analysis
Network analysis
Cell type analysis
Key regulator analysis

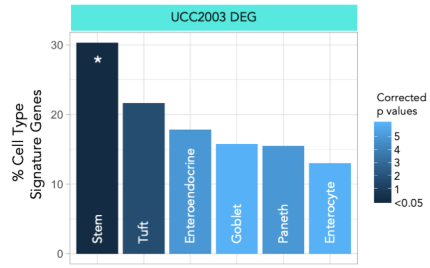

A
C5
C4
C3
C2
C1
E5
E4
E3
E2
E1
Control
UCC2003
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Aerococcus
Allobaculum
Anaeroplasma
Bacteroides
Bifidobacterium
Blautia
Burkholderia
Coprococcus
Dehalobacterium
Enterococcus
Lactobacillus
Mucispirillum
Oscillospira
Prevotella
Ruminococcus
Streptococcus
B
Faeces
Intestinal Contents
Day 4
CFU/g
Limit of detection
ND
Day 1
Day 2
Day 3
Day 4
Small Intestine
Colon
C
Relative Abundance of Bifidobacterium (%)
P=0.012
*
0.01%
13.32%
Control
UCC2003
D
Group
Control
UCC2003
E5
Bifidobacterium
E2
E3
E4
E1
Oscillospira
Bacteroides
Lactobacillus
C4
C5
C2
C1
C3
PC2 (14.7%)
PC2 (84.06%)
E
P=0.02
*
Shannon Index
Control
UCC2003
F
Streptococcus
Ruminococcus
Prevotella
Coprococcus
Bifidobacterium
Group
Control
UCC2003
LDA score
G
Group
Control
UCC2003
Relative Abundance
Mucispirillum
Prevotella
Burkholderia
Aerococcus
Enterococcus
Streptococcus
Blautia
Coprococcus
Dehalobacterium
Oscillospira
Ruminococcus
Allobaculum
Anaeroplasma
Bacteroides
Lactobacillus
Bifidobacterium

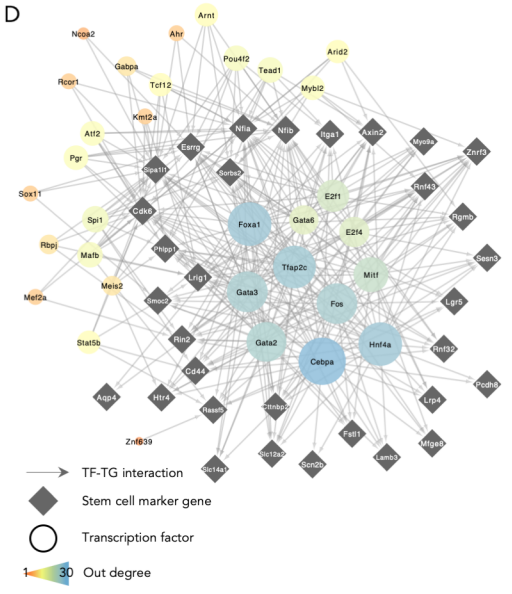

A
PC2 : 11%
PC1 : 70%
E5
C5
C3
E1
E3
C4
C2
E4
E2
C1
Key:
Control
UCC2003
B
Jensen–Shannon distance
Condition:
Control
UCC2003
C
Genes (Up-regulated)
Genes (Down-regulated)
3996
465
Up-regulated: 3,996 genes
Not significant: 8,009 genes
Down-regulated: 465 genes
D
Not significant
Up-regulated
Down-regulated
-Log[10(FDR)]
Log2(Fold-Change)
E
q<0.05:
FALSE
TRUE
Log2(Fold-Change)
(UCC2003 vs Control)
Average Expression
Mean [Log (Estimated Gene Counts)]


A
Adjusted p
Log2FC
C1 C5 C2 C3 C4 E2 E4 E5 E1 E3
Control
UCC2003
Colour Key
FDR-adjusted p
0 0.02 0.04
Log2FC
-5 0 4 8
Scaled Reads Per Base
(Relative Gene Expression)
0 1000 2000 3000 4000 >5000
B
Log2FC
Gm27149
Ccnb1ip1
Gm10359
Gm12671
Gm48216
Gm17131
Tmem72
Ccdc107
CR936839.3
Hist1h4b
Gm48836
Gm42669
Gprin3
Fgd4
D10Wsu102e
Gm20594
Vps13b
Gm48366
Gm48054
Naip6
Control UCC2003
C
Log2FC
Ccnb1ip1
Gm27149
Gm29013
Gm48415
Gm10359
Gm12671
Creb5
Gm17131
Gm48493
Gm48216
Gm48128
Hist1h2br
Gm47102
Gm42669
Hist1h4f
Gm47924
Hist1h3f
Hist2h3b
Gm48836
Gm48417
Control UCC2003
D
Log2FC
Gm7849
Rps27rt
Gm6158
mt-Co2
Gm3650
Gm5831
Gm7331
Polr3h
Gm14698
Ftl1-ps1
Rps16
Mt2
Mt1
Hba-a1
Hbb-bt
Rps2-ps10
Gpx3
Smcp
Gm49384
Gm7336
Control UCC2003
E
Epithelial Integrity Associated Genes
Pcdhgc4
Pcdhgc3
Pcdhgb4
Pcdhb14
Pcdh8
Pcdh1
Fat1
Dsg2
Dsc2
Clstn1
Celsr3
Celsr2
Celsr1
Cdh24
Cdh17
Cdh1
Cadherins
Gjb2
Gjb1
Gja8
Gja1
Gap junctions
Itgb6
Itgb5
Itgb4
Itgb2l
Itgb1
Itgav
Itgam
Itga7
Itga6
Itga4
Itga3
Itga2b
Itga2
Itga1
Integrins
Muc6
Muc5b
Muc4
Muc3a
Muc3
Muc2
Muc13
Mucus layer
Tjp3
Tjp2
Tjp1
Ocln
Jaml
Jam2
Cldnd1
Cldn8
Cldn7
Cldn34c1
Cldn3
Cldn23
Cldn2
Cldn15
Cldn12
Tight junctions
-6 -3 0 3 6
Log2FoldChange
FDR-adjusted p value
0.04
0.03
0.02
0.01
<0.005
F
Integrin-associated Genes
Actn1
Actn2
Actn4
Flna
Flnb
Ilk
Lims1
Lims2
Parva
Ptk2
Ptk2b
Ptk6
Ptk7
Pxn
Tln1
Tln2
Tlnrd1
Tns2
Tns3
Tns4
Vcl
-4 -2 0 2 4
Log2FoldChange
Key
Alpha-actinin
Filamin
Integrin-linked kinase
Parvins
Paxillin
PINCH
Protein tyrosine kinase
Talin
Tensin
Vinculin


A
Cluster 1 (68 genes)
Score: 6.7
(DNA replication and transcription related)
Signaling by Receptor Tyrosine Kinases
Generic Transcription Pathway
DNA Repair
RNA Polymerase II Transcription
DNA Double-Strand Break Repair
cellular response to stimulus
transmembrane receptor protein tyrosine kinase signaling pathway
enzyme linked receptor protein signaling pathway
protein kinase B signaling
cell surface receptor signaling pathway
Signalling Network
(1,491 genes mapped)
Cluster 3 (11 genes)
Score: 4.4
(Cell replication related)
Recruitment of mitotic centrosome proteins and complexes
Loss of Nlp from mitotic centrosomes
AURKA Activation by TPX2
Centrosome maturation
Cilium assembly
centrosome cycle
cell cycle
cell cycle process
centrosome duplication
axoneme assembly
Cluster 2 (26 genes)
Score: 4.5
(Cell growth and immunity related)
Signal Transduction
MAPK targets/ Nuclear events mediated by MAP kinases
Toll Like Receptor TLR1:TLR2 Cascade
Post NMDA receptor activation events
Toll Like Receptor TLR6:TLR2 Cascade
Cluster 4 (72 genes)
Score: 3.8
(Cell cycle regulation related)
Cell Cycle
Deubiquitination
Mitotic G1-G1/S phases
Signal Transduction
mitotic cell cycle process
mitotic cell cycle
cell cycle
mitotic cell cycle phase transition
mitotic nuclear division
Reactome pathways
GO-Slim Biological Process


B
% Cell type signature genes
0
100
DEG
Expressed genes
UCC2003
UCC2003
Control
Stem
Tuft
Enteroendocrine
Paneth
Goblet
Enterocyte


C
UCC2003 DEG
% Cell Type Signature Genes
Stem
Tuft
Enteroendocrine
Goblet
Paneth
Enterocyte
Corrected p values
<0.05


D
TF-TG interaction
Stem cell marker gene
Transcription factor
Out degree

Highlights are 3–4 bullet points of no more than 85 characters in length, including spaces, and they summarize the core results of the paper in order to allow readers to quickly gain an understanding of the main take-home messages.

- *B. breve* administration significantly alters the murine neonatal IEC transcriptome
- Genes/pathways involved in epithelial barrier function are particularly impacted
- *Bifidobacterium* may target the IEC stem cell compartment to induce regeneration