Physiological activity of *E. coli* engineered to produce butyric acid

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Summary

*Faecalibacterium prausnitzii* (*F. prausnitzii*) is one of the most abundant bacteria in the human intestine, with its anti-inflammatory effects establishing it as a major effector in human intestinal health. However, its extreme sensitivity to oxygen makes its cultivation and physiological study difficult. *F. prausnitzii* produces butyric acid, which is beneficial to human gut health. Butyric acid is a short-chain fatty acid (SCFA) produced by the fermentation of carbohydrates, such as dietary fibre in the large bowel. The genes encoding butyryl-CoA dehydrogenase (BCD) and butyryl-CoA:acetate CoA transferase (BUT) in *F. prausnitzii* were cloned and expressed in *E. coli* to determine the effect of butyric acid production on intestinal health using DSS-induced colitis model mice. The results from the *E. coli* Nissle 1917 strain, expressing BCD, BUT, or both, showed that BCD was essential, while BUT was dispensable for producing butyric acid. The effects of different carbon sources, such as glucose, N-acetylglucosamine (NAG), N-acetylgalactosamine (NAGA), and inulin, were compared with results showing that the optimal carbon sources for butyric acid production were NAG, a major component of mucin in the human intestine, and glucose. Furthermore, the anti-inflammatory effects of butyric acid production were tested by administering these strains to DSS-induced colitis model mice. The oral administration of the *E. coli* Nissle 1917 strain, carrying the expression vector for BCD and BUT (*EcN-BCD-BUT*), was found to prevent DSS-induced damage. Introduction of the BCD expression vector into *E. coli* Nissle 1917 led to increased butyric acid production, which improved the strain’s health-beneficial effects.

Introduction

Human intestinal microbial ecosystems are diverse and directly impact human health. Microbial species are predominantly present in the distal gastrointestinal tract, and imbalance in their composition and function is often associated with various diseases, ranging from localized gastrointestinal disorders to the neurological, respiratory, metabolic, hepatic, and cardiovascular (Lynch and Pedersen, 2016). The homeostasis between a host and colonizing bacteria has several essential functional roles, including protection against pathogens, maturation and regulation of the immune system, maturation of the intestines, production of short-chain fatty acids (SCFAs), maintenance of mucosal physiology, and production of vitamin K and biotin (Tomás et al., 2013). Butyric acid is an SCFA commonly derived from bacterial fermentation of carbohydrates such as dietary fibres in the intestine (Offermanns, 2017). It can be utilized by intestinal epithelial cells as a primary energy source to stimulate their proliferation and differentiation, as well as to improve intestinal barrier function (Hamer et al., 2008). Moreover, as a signalling molecule in the immune system, it can be used for the medication of colonic inflammation and colorectal cancer by increasing the expression of tight junctions: Host defence proteins used to reduce infections through regulation of epithelial barrier permeability (Tralong et al., 2014). Butyric acid is produced by various anaerobes in the human gut, such as *Clostridium butyricum*, *Eubacterium limosum*, *Faecalibacterium prausnitzii*, and *Fusobacterium nucleatum*. Among these, *Faecalibacterium prausnitzii* is one of the main butyrate-producing bacteria, shown to produce high concentrations of butyrate from oligofructose and inulin when provided with acetate from bifidobacteria (Moens...
et al., 2016). Thus, a decrease in the intrinsic level of F. prausnitzii may cause ATP deficiency in the epithelial cells, which, in turn, may weaken the host’s ability to defend itself against the inflammatory response (Zhang et al., 2014).

*Escherichia coli* Nissle 1917 (EcN) is a gram-negative bacterium with lipopolysaccharide (LPS) as a structural component of its outer cell membrane and used therapeutically for over a century in Germany and other European countries (Scaldaferri et al., 2016). Certain characteristics of the LPS on EcN appear to explain the phenomenon of how strains do not exhibit immunotoxin effects, while still exhibiting characteristics of immune modulation (Behnsen et al., 2013). In addition, the antagonistic actions of EcN are, at least in part, due to the formation of microcins: Low molecular-weight antimicrobial peptides secreted by intestinal bacteria which are involved in microbial competition within the intestinal tract. Microcins utilize the iron uptake receptors of other Enterobacteriaceae to enter their cytoplasm and kill the cells (Vassiliadis et al., 2010). Through these roles, EcN may positively affect gastrointestinal and microbial balance. Indeed, their therapeutic benefits in inducing and maintaining the mitigation of intestinal diseases have been previously reported in various clinical trials (Souza et al., 2016; Abraham and Quigley, 2016; Losurdo et al., 2015).

Inflammatory bowel disease (IBD) refers to various chronic inflammatory conditions of the colon and small intestine, such as Crohn’s disease and ulcerative colitis, caused by an uncontrolled immune response (Duerr et al., 2006). IBD can be debilitating and is often characterized by severe diarrhoea, pain, fatigue, and weight loss, which may cause life-threatening complications (Perler et al., 2019). The aetiology and complexity of human IBD are generally studied through the development of various animal models. These models have become an indispensable tool for explaining histopathological, immunological, and morphological changes in the intestine, and for identifying potential therapeutic targets (Kiesler et al., 2015). The most widely used colitis mouse model involves the implementation of dextran sodium sulphate (DSS), a chemical colitogen with anticoagulant properties, to cause epithelial damage. This model is suitable for IBD research due to its quickness, simplicity, reproducibility, and controllability (Eichele and Kharbanda, 2017).

Oral administration of butyric acid can affect IBD since it acid does not reach the large intestine due to rapid gastrointestinal and duodenal absorption (Vieira et al., 2012). In this study, we engineered a butyric acid-producing EcN strain to determine whether increased butyric acid production exerts an anti-inflammatory effect in addition to the natural probiotic activity of the EcN strain using a DSS-induced colitis mouse model.

### Results

**Recombinant EcN for butyric acid production**

For butyric acid production, genes encoding butyryl-CoA dehydrogenase (BCD) and butyryl-CoA:acetate CoA transferase (BUT) from *F. prausnitzii* A2-165 were cloned under cat and crp promoters in pACYC184c and pBR322 vectors to make pACYC184-BCD and pBR322-BUT, respectively, and expressed in *E. coli* MG1655 and EcN. In pACYC184-BCD-BUT, the gene encoding BUT was added downstream of the gene encoding BCD to express both genes simultaneously from the cat promoter. BCD converts crotonyl-CoA to butyryl-CoA (Bennett and Rudolph, 1995), while BUT converts butyryl-CoA to butyric acid (Diez-Gonzalez et al., 1999) (Fig. S1). To confirm BCD and BUT expression, a real-time (RT)-PCR was conducted, while qPCR was used to determine the relative level of expression after induction (Fig. S2). BCD was expressed 2401.9–3590.6 folds, while BUT was induced 245.3–612.4 folds, compared to *E. coli* MG1655 wild-type strains. These gene expression levels indicated that the butyric acid pathway genes were successfully transcribed in *E. coli*.

The effect of BCD and BUT induction on butyric acid accumulation in the medium was then observed (Fig. 1), which showed that butyric acid production by *E. coli* strains significantly increased when transformed with pACYC184-BCD compared to the same strain transformed with the control vector pACYC184. However, the butyric acid production of *E. coli* strains carrying pBR322-BUT was similar to that of *E. coli* strains with pBR322. EcN strains were found to produce slightly more butyric acid compared to MG1655 strains, as EcN is known to produce SCFAs as the end products of carbohydrate metabolism (Singh et al., 2015). The EcN strain carrying pACYC184-BCD-BUT (EcN-BCD-BUT) produced a slightly higher amount of butyric acid compared to the same strain carrying pACYC184-BCD (EcN-BCD). Overall, EcN-BCD-BUT showed the highest butyric acid-producing ability (295.16 mg l⁻¹) among all *E. coli* strains engineered to produce butyric acid in this study. Notably, butyric acid was accumulated up to 355.12 mg l⁻¹ when *F. prausnitzii* was grown to the stationary phase in the RCM medium.

**Activity assay of BCD and BUT**

To investigate the relative contribution of BCD and BUT to butyric acid production, we measured their activities by coupling to that of *E. coli* FadB, which catalyses the formation of acetocetyl-CoA from crotonyl-CoA via 3-hydroxybutyryl-CoA (Fig. 2A). When the reverse pathway of butyric acid production proceeds, BCD and BUT will convert butyric acid into crotonyl-CoA via butyryl-CoA,
after which FadB will catalyse the conversion of crotonyl-CoA to acetoacetyl-CoA, with concomitant reduction of $\text{NAD}^+$ to NADH, which can be monitored at a wavelength of 340 nm. First, we confirmed that cell lysates of both wild-type EcN and EcN-BUT did not catalyse the formation of NADH from $\text{NAD}^+$, while the EcN-BCD lysate produced trace amounts of NADH (Fig. 2B). Notably, a detectable amount of NADH was formed from $\text{NAD}^+$ when both BCD and BUT were present in addition to acetyl-CoA and FAD. Furthermore, the FadB reaction

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did not proceed in the absence of butyrate or acetyl-CoA, indicating that the FadB-coupled assay used to investigate the BCD and BUT activities was reliable (Fig. 2C).

**Butyric acid production by engineered E. coli in various sugar conditions**

To evaluate the effects of BCD and BUT on butyric acid production, EcN, EcN-BCD, EcN-BUT, and EcN-BCD-BUT were cultured in an M9 medium containing 0.2% glucose (Fig. 3). The timelines of the EcN growth, depending on the presence of BCD and BUT, are shown in Fig. 3A. EcN can grow with glucose as the principal energy source, and all EcN strains reached the stationary phase after 5 h of incubation, even though glucose was depleted at 10 h (Fig. 3B). The butyric acid concentration reached 34.3 ± 5.98 mg l⁻¹ in the wild-type EcN culture medium, while EcN-BCD-BUT produced 297.3 ± 34.25 mg l⁻¹ after 10 h, which was approximately 8.6 times higher than the wild-type EcN (Fig. 3C). Although butyric acid production by EcN-BCD was slightly lower than that by EcN-BCD-BUT, overall butyric acid production was significantly improved compared to the wild-type strain. The concentration of acetic acid increased to 846.24 ± 51.32 mg l⁻¹ in the wild-type EcN culture medium after 11 h, while the acetic acid levels of EcN-BCD (698.36 ± 38.25), EcN-BUT (611.24 ± 27.36), and EcN-BCD-BUT (619.24 ± 16.36 mg l⁻¹) were lower than that of the wild-type EcN, suggesting that acetic acid could have been used for the production of butyric acid. (Fig. 4D). Next, we determined the effect of the glucose concentration on the growth and butyric acid production of EcN-BCD-BUT (Fig. S3). The EcN-BCD-BUT growth and production of butyric acid (and acetic acid) were not significantly different at various glucose concentrations (0.2%–2.0%), implying that 0.2% glucose is sufficient. To evaluate the effect of sugars in the mucosa of the large intestine on growth and butyric acid production, EcN-BCD-BUT was cultured in an M9 medium containing N-acetylglucosamine (NAG), N-acetylgalactosamine (NAGA), or inulin (Fig. 4). Growth analysis showed no difference between glucose and NAG, while the growth rate in NAGA was slower than that in glucose. Furthermore, only slight growth was obtained with inulin as the sole source of carbon, which is similar to the growth observed in the M9 medium with 0.1% casamino acid. The butyric acid yield from NAG was similar to that from glucose, with this result correlating with growth. The production rate and final concentration of butyric acid in NAGA was lower than those in glucose, with the rate of acetic acid production from NAGA also measuring slower than from glucose. Inulin produced higher yields.
of both butyric and acetic acid than casamino acid, despite similar growth. From these results, EcN-BCD-BUT may produce higher amounts of butyric acid, using sugars in the animal intestine, rather than EcN.

Measuring anti-inflammatory activity using a DSS-induced colitis model

Based on the butyric acid production results, the anti-inflammatory activities of MG1655-BCD-BUT and EcN-BCD-BUT were further assessed, with MG1655 and EcN used as respective controls. The effect of oral administration of these strains was evaluated in DSS-induced colitis model mice. DSS can destroy epithelial barriers, causing acute clinical symptoms when administered orally. As shown in Fig. 5A, the low level of butyric acid in the ileum is likely related to a low number of resident *E. coli*, as opposed to in this bacteria’s main habitat, the cecum. The concentration of butyric acid in the cecum content significantly increased in treatment groups with MG1655-BCD-BUT (9.73 mg g⁻¹) and EcN-BCD-BUT (10.92 mg g⁻¹), compared to that in wild-type groups (MG1655: 3.31 mg g⁻¹; EcN: 5.24 mg g⁻¹). The accumulation of butyric acid in the cecum of the mice was related with *E. coli* strains colonization and it was confirmed that the numbers of the administered strains in faeces increased with time (Fig. S4A). In Fig. S4B, it was confirmed that the amount of butyric acid increased as the colonization progressed for the recombinant strains, but the amount decreased for the wild-type strain.

After 7 days of DSS treatment, the bodyweight of the mice significantly decreased compared to the control mice (Fig. 5B). Although treatment with *E. coli* MG1655 was not effective in the DSS-induced weight loss, treatment with MG1655-BCD-BUT or EcN could alleviate this weight loss. In addition, the DSS-induced weight loss was more effectively reduced in the mice group treated with EcN-BCD-BUT than in the EcN-treated group, suggesting that butyric acid may be effective in weight gain. The Disease Activity Index (DAI) result also conformed with the result from the weight measurement (Fig. S5). In the DSS-treated group, a reduction in colon length at day 10 was also observed (Fig. 5C). However, oral treatment with EcN-BCD-BUT partially suppressed DSS-induced colon length shortening. The cytokine profile of colonic tissue was assessed to determine whether the
engineered *E. coli* strains reduced DSS-induced weight loss and colon's length shortening via butyric acid-mediated regulation of cytokine levels (Fig. 5D). Oral administration of MG1655-BCD-BUT, EcN, and EcN-BCD-BUT decreased IL-6 levels, compared to the DSS group (Fig. 5D). Although butyric acid helped to lower the IL-6 levels in the MG1655 strains, the IL-6 levels in EcN were reduced by other metabolites, such as microcin M and TepC protein (Guo *et al.*, 2019; Jo *et al.*, 2019). This may explain why the EcN or EcN-BCD-BUT
administered groups did not show a difference in IL-6 levels. In addition, EcN-BCD-BUT increased the level of TNF-α compared to EcN (Fig. 5E). Furthermore, treatment with MG1655-BCD-BUT also decreased levels of the inflammation marker, myeloperoxidase (MPO), indicating that butyric acid was effective against MPO (Fig. 5F).

The ability of engineered *E. coli* to prevent DSS-induced colonic damage was also evaluated at the histological level. Colon sections of the control mice showed an intact epithelium, a well-defined crypt length, and no neutrophil infiltration in the mucosal and submucosal layers (Fig. 6A). In contrast, colon tissues from DSS-treated mice showed severe inflammatory lesions throughout the mucosa and submucosa (Fig. 6B). Oral administration of MG1655 did not affect the DSS-induced damage, while treatment with MG1655-BCD-BUT significantly prevented the damage (Fig. 6C, and D). After 7 days, the EcN strains, especially EcN-BCD-BUT, ameliorated histological damage (Fig. 6E, and F). Histological scores showed that EcN had a greater anti-inflammatory effect, compared to the MG1655 strain, and that butyric acid further increased this effect (Fig. 6G).

**Discussion**

Herein, we investigated the anti-inflammatory effects of *E. coli* engineered to produce butyric acid to mitigate DSS-induced intestinal damage in mice when administered orally. A previous study has reported that EcN protects against trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats and LPS-induced organ damage in mice (Arribas *et al.*, 2009). Therefore, in this study, we sought to determine whether the anti-inflammatory effect of the EcN can be augmented by engineering to produce a higher level of butyric acid. To increase the butyric acid production, genes encoding BCD and BUT from *F. prausnitzii* were cloned and introduced into *E. coli*. While the transformation of *E. coli* strains with a BCD expression vector significantly increased the butyric acid production, transformation with a BUT expression vector showed little effect. These data suggest that while BCD activity is required to increase the butyric acid production in *E. coli*, the interconversion between butyryl-CoA and butyric acid can be catalysed by other enzymes in *E. coli*. We hypothesized that some *E. coli* enzymes such as ScpC and TesB might fulfil the thioesterase function in the conversion of butyryl CoA to butyric acid since ScpC transfers CoA between succinate and propionate and TesB is a thioesterase having a relatively broad substrate specificity, cleaving medium- and long-chain acyl-CoA substrates (Fig. S6A) (Nie *et al.*, 2008; Seto *et al.*, 2010; Mcmahon and Prather, 2014). As expected, butyric acid production was significantly decreased in ΔscpC and ΔtesB strains compared to wild-type strains (Fig. S6B), indicating that these enzymes contribute to the conversion of butyryl-CoA to butyric acid.

*E. coli* ΔscpC and ΔtesB strains carrying both pACYC184-BCD and pBR322-BUT produced a similar level of butyric acid to the same strains carrying pACYC184-BCD only. Whether *F. prausnitzii* BCD and BUT were expressed as active forms in *E. coli* remained to be determined, which prompted to develop the FadB-coupled activity assay for the two enzymes. In previous studies, BCD activity was measured via 2,6-dichlorophenolindophenol (DCPIP) reduction, and BUT activity was measured by determining the concentration of acetyl-CoA with citrate synthase assay (Lucas *et al.*, 2011; Sato *et al.*, 2016). However, since DCPIP is a redox dye reacting with a variety of chemicals and citrate synthase assay uses a quite complex procedure, including the detection of free thiol groups with dithiobis-2-nitrobenzoic acid, these approaches showed poor reproducibility. However, the FadB-coupled activity assay clearly showed that both BCD and BUT were expressed in active forms.

Mucins in the animal gastrointestinal tract consist of a peptide core and oligosaccharides with one or more of the four main sugars (NAG, NAGA, galactose, and fucose) and they can be used as nutrients by the microbiota (Derrien *et al.*, 2004; Bansil and Turner, 2018; Ouwerkerk *et al.*, 2016; Lievin-Le and Servin, 2006). Inulin is resistant to digestion in the human small intestine, yet is fermented in the large intestine (Niness, 1999; Shoaib *et al.*, 2016). Therefore, EcN-BCD-BUT was cultured with NAG, NAGA, or inulin to determine its growth and butyric acid production levels in the large intestine and the EcN-BCD-BUT strain may grow and produce butyric acid using the sugars in the animal intestinal mucin. The probiotic strain of EcN showed anti-inflammatory activity in DSS-induced colitis model, which was established to mimic ulcerative colitis (Garrido-Mesa *et al.*, 2011). EcN suppresses the attachment and invasion of other bacteria to the intestinal mucosa, a process that is vital to triggering the exacerbated immune response in IBD (Arribas *et al.*, 2009). In our experiments, the level of butyric acid in the cecum content was significantly increased in the engineered *E. coli* strain treatment groups compared to wild-type strains, although similar levels were observed in the ileum.

EcN treatment increased the butyric acid levels, which indicated a suppression in inflammatory reactions, such as mucosal infiltration. The adverse effects on the body weight, MPO level, and histological damage in the colitis model were alleviated by the increased butyric acid production from the BCD-BUT overexpressing strains, while the colon's length, IL-6 level, and TNF-α level showed decreased in ΔscpC and ΔtesB strains compared to wild-type strains (Fig. S6B), indicating that these enzymes contribute to the conversion of butyryl-CoA to butyric acid.
minor differences between the respective wild-type and overexpressing strains. These anti-inflammatory effectors were recovered from the DSS 2%-induced effects regardless of the strains, suggesting that they could be induced by other metabolites of MG1655 and EcN, as well as butyric acid. In this experiment, the mouse models were orally administered the E. coli strains for 9 days during which DSS was given from day 3 to the last day, and then sacrificed on day 10 for examination. Hence, the time window might not have been long enough to fully assess the recovery in these effectors. To better illustrate the effects on the colon’s length, IL-6 level, and TNF-α level, the strains should have been administered for 5 or more days following DSS feeding discontinuation (Breyner et al., 2017).

In conclusion, we have determined the potential benefit of the butyric acid production in the DSS colitis model and suggested that enhancing the butyric acid production capability could be effective in increasing the anti-inflammatory efficacy of the probiotic EcN strain.

Experimental procedures

E. coli strains, plasmid construction, and culture conditions

The strains and plasmids used in this study are listed in Table S1. EcN is a typical example of a non-pathogenic, commensal E. coli isolate. It forms the foundation of probiotics used in the treatment of various intestinal disorders and is a known colonizer of the human gut. E. coli MG1655, which originates from the K-12 wild-type strain, was used as the control for comparison against EcN. F. prausnitzii A2-165 was used as the source of the template DNA for cloning. Vector plasmids pACYC184, with chloramphenicol and tetracycline markers, and pBR322, with ampicillin and tetracycline markers, were used to construct protein overexpression vectors. For constitutive expression, pACYC184 and pBR322 were equipped with cat and crp promoters, respectively. Ribosome binding sites (RBS) were inserted directly after the constitutive promoters in both vectors.

Strains were cultured in LB medium, containing 10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract, and 10 g l⁻¹ NaCl, for seed culture with antibiotics, including 20 µg l⁻¹ chloramphenicol for pACYC184, and 100 µg l⁻¹ ampicillin for pBR322. The butyric acid production test for strains of E. coli and F. prausnitzii were performed in RCM containing 10.0 g l⁻¹ peptone, 10.0 g l⁻¹ beef extract, 3.0 g l⁻¹ yeast extract, 5.0 g l⁻¹ dextrose, 5.0 g l⁻¹ NaCl, 1.0 g l⁻¹ soluble starch, 0.5 g l⁻¹ cysteine HCl, 3.0 g l⁻¹ sodium acetate, and 0.5 g l⁻¹ agar. The dependence of the butyric acid production on sugar sources was determined by adding respective sugars to an M9 medium, containing 12.8 g l⁻¹ Na₂HPO₄·7H₂O, 3.0 g l⁻¹ KH₂PO₄, 0.5 g l⁻¹ NaCl, and 1.0 g l⁻¹ NH₄Cl, supplemented with 0.1% casamino acid, 0.1mM CaCl₂, and 1mM MgSO₄. Each strain was grown at 37 °C.

Gene amplification through PCR and gene cloning

To construct the butyric acid pathway in plasmids, BCD and BUT were amplified from the genomic DNA of F. prausnitzii A2-165, using PCR. To construct the BCD expression vector, primers possessing the synthetic restriction enzyme sites BamHI (restriction sites
underlined), located 4 bp upstream from the ATG start codon (in boldface type) (5’-GAC GTT GGA TCC AGA TAT GGA TTT TAC TCT GTC CAA G-3’), and SalI, located next to the TGA stop codon (in boldface type) (5’-GCA GGA GCA ACA AGC GCT GAG TCG AC-3’), were used to amplify the BCD gene from bacterial genomic DNA. To construct the BUT expression vector, primers possessing the synthetic restriction enzyme sites Ndel, which contain an ATG start codon (in boldface type) (5’-CAT ATG GAT TTT ACG GAA TTG TAT G-3’), and SalI, located next to the TGA stop codon (in boldface type) (5’-GCA GGA GCA ACA AGC GCT GAG TCG AC-3’), were used to amplify the BCD gene from the genomic DNA of F. prausnitzii. BCD was cloned into pACYC184 under a cat promoter, while, BUT was cloned into pBR322 under a crp promoter (Choe et al., 2017). In addition, to construct the BCD-BUT expression vector, BUT was cloned into the BCD-pACYC184 using Gibson assembly as an operon fusion. Expression of genes was performed in MG1655 and EcN E. coli.

Confirmation of BCD and BUT expression

Overexpression of BCD and BUT was confirmed using RT-PCR. Total RNA was purified from bacteria using the TaKaRa MiniBEST universal RNA extraction kit (Takara Bio, Shiga, Japan). Reverse transcription and PCR were subsequently performed using RNA to cDNA EcoDry™ premix (TaKaRa Bio, Mountain View, CA, USA), with 1 μg of RNA as the template. The primers utilized were 5’-TTA CTT CCC CAC CTC TGT CG-3’ and 3’-CGT CCA TGA AAC GGA AGA CG-5’, for BCD, and 5’-AGA ACT GGG TGC ACA TCT CCC A-3’ and 3’-GCT GTA TGT AGA GCC ACA CG-5’, for BUT. BCD and BUT expression levels were calculated by the cycle threshold numbers as 1.

Assays of BCD and BUT activity

For cell lysate preparation, frozen EcN, EcN-BCD, EcN-BUT, and EcN-BCD-BUT cells (3 mg wet weight) were suspended in 4 ml of 50 mM Tris-HCl (pH 7.5) containing 2 mM 1,4-Dithiothreitol (DTT) and were lysed using sonication for 10 min. Cell debris was removed using centrifugation at 100 000 rpm for 30 min at 4°C. The protein content was determined using the Bradford protein assay, with bovine serum albumin as the standard. Wild-type EcN, EcN-BCD, EcN-BUT, and EcN-BCD-BUT cell lysates were tested for BCD and BUT activities using assay mixtures containing butyric acid, acetyl-CoA, FAD, and NAD+ at 30°C. A UV-VIS spectrophotometer (Shimazu UV-1800 m) was used at a wavelength of 320 nm to measure the conversion of NAD to NADH by FadB.

Analytical methods

Cell growth during cultivation was measured at a wavelength of 600 nm using a UV-VIS spectrophotometer (Shimazu UV-1800 m) and microplate readers (TECAN Spark®, Tecan Trading AG, Zurich, Switzerland). Sugar and SCFA concentrations were measured using high-performance liquid chromatography (HPLC, Agilent 1100 series, USA), with a UV detector (210 nm) for both butyric and acetic acid and a refractive index detector for sugars. The analytical column used was the H+ cation-exchange column (Rezex™ ROA-Organic Acid, 300 x 7.8 mm; Phenomenex, Torrance, CA, USA), at a set temperature of 60°C. Elution of sugars and SCFAs was performed in 0.5 mM and 5 mM H2SO4, respectively, at a flow rate of 0.7 ml min⁻¹. The injected sample volume for HPLC analysis was 20 μl and the run time for each analysis was 30 min. All experiments were performed in triplicate and results were expressed as the mean value.

DSS-induced colitis model

Eight weeks old, male C57Bl/6J mice (Orient Bio, Seongnam, Korea) were housed in a cage maintained at 23°C with an alternating 12-hour light/dark cycle under specific pathogen-free conditions and were given 2% DSS in drinking water for 7 days. The concentrations of the E. coli strains MG1655, MG1655-BCD-BUT, EcN, and EcN-BCD-BUT, were 10⁸ CFU ml⁻¹ each in phosphate-buffered saline (PBS), containing 8.0 g l⁻¹ NaCl, 0.2 g l⁻¹ KCl, 1.44 g l⁻¹ Na₂HPO₄, and 0.24 g l⁻¹ KH₂PO₄. Next, 200 μl of the samples were administered to the mice via oral gavage, with starving 2 days before colitis induction and 7 days after. Mice were sacrificed after 10 days, and the clinical parameters and pathology were evaluated (Fig. S7). This study was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of South Korea. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University Bundang Hospital (Permission No. BA1506-178/027-01).

Measurement of SCFA concentration and CFU counts in caecal contents

Caecal contents were removed immediately after performing a laparotomy, and then butyric acid was measured using HPLC. Briefly, after the addition of an internal control, distilled water was added to produce
200 mg of each sample. Following incubation at 80°C for 15 min, mechanical shaking with beads and centrifugation at 13 000 rpm were performed for 10 min, and then the supernatant was filtered through a membrane filter (pore size 0.45 mm) (Mishiro et al., 2013).

The faecal samples were collected on days 5 and 10 and from each 1 g of sample homogenate, 10-fold serial dilutions were performed. The 200 μl of samples were cultured on macconkey agar containing 0.05 mg ml⁻¹ chloramphenicol or 0.1 mg ml⁻¹ ampicillin and incubated for 24 h at 37°C for colony counting. The lower limit of quantification is 10 CFU. To measure butyric acid in faecal samples, the same method as for the caecal sample was used.

**Measurement of inflammatory cytokines**

Levels of MPO and IL-6 in colonic tissue were measured using a mouse MPO ELISA kit (HK210; Hycult Biotechnology, Uden, The Netherlands), and a mouse IL-6 Quantikine ELISA kit (R&D Systems Inc., Minneapolis, MN, USA), respectively, according to manufacturer’s instructions. To analyse TNFα, total RNA was extracted from colonic tissues using TRizol™ Reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer’s instructions, and quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) and was analysed using quantitative real-time (qRT) PCR, with TNFα specific primers (Table S2) and Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific), in a ViA 7 instrument (Thermo Fisher Scientific). Every assay was performed in triplicates.

**Tissue processing and histopathology**

The colon was extracted from the peritoneum and divided into proximal and distal portions. The proximal colon portion, up to 1.5 cm from the ileocaecal valve, the rectum portion, up to 1.5 cm from the anal verge, and the colonic segments, containing any gross polyps, were all fixed with phosphate-buffered formalin and embedded in paraffin. The 5 mm sections were stained using haematoxylin and eosin (H&E) (Song et al., 2019). Histological severity was assessed using the microscopic damage score, which reflects colonic epithelial damage and depth of inflammatory cell infiltration, as previously described (Katakura et al., 2005). These parameters were evaluated by two researchers in a blind manner.

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