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Physiological activity of butyrate-producing gut bacteria

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Physiological activity of butyrate-producing gut bacteria

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Seoul National University

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ABSTRACT

Physiological activity of butyrate-producing gut bacteria

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Faecalibacterium prausnitzii (*F. prausnitzii*) is one of the most abundant bacteria in the human intestinal microbiota and regarded as a major effector in human intestinal health because of its anti-inflammatory effects. It produces butyric acid, which has a beneficial effect on human gut health. However, the extreme oxygen sensitivity has been a significant obstacle to cultivate and study the physiological characteristics of this organism. To investigate physiological characteristics of *F. prausnitzii*, its growth on various types of sugars was tested and the results showed that glucose, fructose, N-acetylglucosamine (NAG), galactose and N-acetylgalactosamine (NAGA) were consumed as major carbon sources. Butyric acid was accumulated to 315.18 ± 14.2 ~ 355.66 ± 21.4 mg/L in the growth supernatant, when glucose, fructose, or N-acetylglucosamine was supplemented. 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. The two genes were cloned individually or together under the control of the

constitutive *cat* and *crp* promoter into the plasmid pACYC184 and pBR322. According to the results of butyric acid production in wild-type *E. coli* expressing BCD, BUT or both, BCD was shown to be essential, while BUT was dispensable, for the production of butyric acid. The effect of a carbon source, such as glucose, N-acetylglucosamine, N-acetylgalactosamine and inulin was compared and the results showed that optimal carbon sources for butyric acid production were glucose and NAG, which is one of major components of mucin in the human intestine. The anti-inflammatory effects of butyric acid production were tested by administrating these strains into DSS-induced colitis model mice. The oral administration of *E. coli* Nissle 1917 strain carrying the expression vector for BCD and BUT (EcN-BCD-BUT) prevented damage induced by DSS.

Keywords :

***Faecalibacterium prausnitzii*, Sugar, Butyric acid, *E.coli* Nissle1917, DSS- induced colitis model**

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ABBREVIATIONS

F. prausnitzii: *Faecalibacterium prausnitzii*

GIT: gastrointestinal tract

EOS: extremely oxygen sensitive

5-ASA: aminosalicylic acid

DNBS: dinitrobenzene sulfonic acid

RCM: Reinforced Clostridial Medium

PTS: Phosphoenolpyruvate: sugar phosphotransferase system

ABC: ATP-binding cassette

NAG: N-acetylglucosamine

NAGA: N-acetylgalactosamine

SCFAs: short-chain fatty acids

EcN: *Escherichia coli* Nissle 1917

LPS: Lipopolysaccharide

DSS: Dextran Sulfate Sodium

BCD: Butyryl-CoA dehydrogenase

BUT: Butyryl-CoA: acetate CoA-transferase

scpC: Propionyl-CoA:succinate CoA transferase

tesB: acyl-CoA thioesterase II (*tesB*)

Chapter I

Literature Review

1. Gut microbiome

In the human gut, approximately 10^{14} microbial cells are present. The number of gut microbiota is ten times the number of cells in our bodies and their collective genomes are quantitatively larger than our genome in orders of magnitude (1). The gut microbiota participates in a diverse range of biochemical and metabolic activities that are essential to the host. For example, these microbes break down indigestible dietary polysaccharides and produce micronutrients and vitamins; they regulate the function of the host's intestinal epithelium and immune system; they provide protection against invasion by potentially harmful pathogens (2). The human intestine is a stable, protected, and nutrient-rich environment for the bacterial growth, and thus the composition of the adult gut microbiota is relatively stable over time. However, this habitat can also be changed due to antibiotic exposure, enteric infection, or a long-term change in diet, all of which can lead to alterations in the gut microbial composition. For example, a study with healthy volunteers has shown that repeated antibiotic administration can result in the development of a new state of stability in the gut microbiota (3).

Recent studies have revealed that there are significant variations in the composition of the gut microbiota in healthy individuals (Human Microbiome Project Consortium, 2012), and several factors have been identified to affect the gut microbial composition. For instance, aging is closely associated with changes in the gut microbiota. The relative abundance of Bifidobacteria was shown to decrease with increasing age in three different populations significantly. Geography is another factor in shaping the gut microbiota, as significant inter-personal variations were observed in the gut microbial communities

among humans living in geographically and culturally distinct environments. This fact indicates that the differences in host ethnicity, geographic location of residence, lifestyle, and diet may lead to the differences in gut microbiota (4). More recently, it has been reported that host genetics also influence the composition of the microbiota (5). Taken together, the intro- and inter-personal variations driven by those various factors may be related to the development of the gut microbiome-related diseases such as obesity, diabetes, and inflammatory bowel diseases. Therefore, identifying specific disease-associated taxa and the factors that affect the abundances of such taxa will help optimize strategies to prevent or treat the related diseases.

1.1 The interaction of host and microbiota

The beneficial relationship between the host and the microbiota produces a microbial metabolite that contributes to the evolutionary fitness of the host (6). The diversity and composition of gut microbiota in host species are influenced by the topographic and temporal variations of the microbial population linked to specific niches in the body habitat as well as growth or maturation stages of the host (7). Additionally, the formation of microbiota is driven by a complex and dynamic series of interactions among lifestyle, such as diet, obtainment of diseases and usage of antibiotics.

Interactions between the microbiota and the host immune system begin at birth. Microorganisms form the development of the immune system, and the immune system in turn forms the microbial system. This interaction between the microorganism and the host immune system is transmitted through a vast signal pathway that encompasses a

wide variety of molecules and extends beyond the immune system. These immune-mediated signaling processes act directly on the microbes and the host, as well as on the internal organs such as liver, muscle, and brain (8). The profound effects of gut microbiota on the host immune system are strongly associated with long-term health. The core gut microbiota composition is considered inherently stable throughout adulthood (9). There are also dynamic, biologically and metabolically flexible components corresponding to perturbations such as environmental stress or alteration in dietary habits, causing changes in species composition that can affect the host health or the risk of developing a disease (10).

1.2 The interaction of microbiota and pathogen

Health and disease development reflects the overall balance among host reactions, indigenous microbiota and potential pathogens. This balance is maintained by a colonial resistance mechanism that can be directly or indirectly mediated by microbiota. Microorganisms can indirectly mediate colonial resistance by stimulating host mucous membrane for immune defenses, preventing intrusions and infections of non-native microorganisms (11). The mucous membrane immune system and its healthy development and function are affected by the presence of native microorganisms (12). Alternatively, aboriginal microbes can directly inhibit non-native microbes through various mechanisms. A confusion in established community structures and follow-up functions changes the overall balance between microorganisms and hosts, thereby altering the sensitivity of the host against infection.

2. Short chain fatty acids (SCFAs)

SCFA is an organic fatty acid with 2 to 6 carbon atoms and is a significant anion caused by the bacterial fermentation of polysaccharides, oligosaccharides, proteins, peptides and protein precursors in the colon (13). Fermentation involves a variety of reactions and metabolic processes in the anaerobic microbial decomposition of organic matter to generate metabolic energy for microbial growth and maintenance of the host and other metabolic end products. The major SCFA found in colonial lumens are acetic acid, propionic acid, and butyric acid. The dominant luminal anion of the colonic liquid has a typical concentration range of 70-100 mM and a relative charge of 60 acetic acid: 25 propionic acid: 15 butyric acid (14). Although there are differences in the role of nutrients and biological modifiers, SCFA has a very similar effect on bicarbonate transportation, and SCFA production can also be regarded as the reflection of diet. It is clear that the low level of SCFA with normal carbohydrates consumption rises with diets high in fiber, soy, resistant green tea, and complex carbohydrates as it leads to higher rates of SCFA formation. (15). Recognizing the beneficial effects of dietary fiber, many investigators have hypothesized that this effect is associated with SCFA.

In recent years, colon health has become increasingly associated with maintaining overall health and reducing the risk of various diseases by changes in diet and lifestyle. At the forefront are other dietary ingredients such as "prebiotics" and "probiotics" that target functional foods, diets and fiber to affect the colonic environment, improving SCFA production, and promoting or delaying the absorption of the same biologically active substances (16).

The role of SCFA has been expanded to include its role as a colonic epithelial nutrient, the regulator of the colon and intracellular pH, regulators of cell function and other functions associated with ion transport, as well as its role as a regulator of spread, differentiation and gene expression (16). The decrease in pH indirectly affects the composition of colon microflora, reducing the solubility of bile acids, increasing mineral uptake, and reducing ammonia uptake by definite separation of ammonia. (17)

2.1 Acetic acid

Acetic acid, the major SCFA of the colon, is easily absorbed and transported to the liver to reduce colon metabolism (18). In human studies, acetic acid is often used to monitor colonial events because it is the major SCFA in the blood. Acetic acid is the primary substrate of cholesterol synthesis. The direct injection of acetic acid and propionic acid resulted in a dose-dependent increase in serum total cholesterol and triglyceride levels, providing indirect evidence that SCFA is used for lipid synthesis (19). However, the methodology used in this study was a rapid intake of acetic acid which may have resulted in a non-physiological level of acetyl-CoA. This may have led to the conversion of SCFA to lipid composition rather than oxidation (20). Substrate-dependent SCFA produced by fermentation may inhibit cholesterol synthesis (21). Besides, acetic acid has been shown to play a key role in the ability of bifidobacteria to control intestinal pathogens (22).

2.2 Propionic acid

Colonic bacteria produced propionic acid using three different pathway; succinate pathway, acrylate pathway and propanediol pathway (23). Propionic acid is a substrate for the glycolysis in liver and has been reported to inhibit cholesterol synthesis in liver tissue (24). The generation of SCFAs especially propionic acid which is the main precursor for gluconeogenesis constitutes the primary source of ruminant animal energy. And propionic acid is reported to reduce food intake by inducing the production of built-in hormones (25). Decreased levels of propionic acid producers were detected in children at risk of asthma (26).

2.3 Butyric acid

Butyric acid is the preferred source of the colonic epithelial cells and it also plays an essential role in cell proliferation and differentiation control (27). It is the most critical SCFA in colonocyte metabolism and 70 to 90 percent of butyric acid is metabolized by colon cells (18). Butyric acid is used in a ratio of 90 : 30 : 50 over propionic acid and acetic acid, and is preferable to glucose or glutamine supplied by blood (27). Butyric acid oxidation has been shown to make up more than 70% of the oxygen consumed by human colonic tissue. Acetic and propionic acids are also shown to induce angiostasis in the tumor cell of colorectal cancer but are much less than butyric acid (28). Butyric acid also stimulates the immunogenicity of cancer cells.

3. Proteins related to butyric acid production pathway

3.1. Butyryl CoA dehydrogenase (BCD)

Butyryl-CoA dehydrogenase catalyzes the reduction of carbon-carbon double bond in crotonyl-CoA to yield butyryl-CoA, a key branch point in acid and solvent formation. Butyryl-CoA can direct acid formation and ATP production mediated by phosphotransbutyrylase and butyrate kinase or can also be converted to butanol catalyzed by butyrylaldehyde dehydrogenase and butanol dehydrogenase with consumption of NADH. In mammalian systems, similar enzymes involved in step-wise fatty acid oxidation are found in mitochondria (29). BCD can also participate in the reverse direction, from the decomposition of fatty acids to the reduction of butyryl CoA (30).

3.2. Butyryl CoA: acetate CoA transferase (BUT)

Butyryl CoA: acetate CoA-transferase presents two different mechanisms. It reclaims CoA from butyryl-CoA at the end of butyrate biosynthesis (an important step performed by some *E. coli*) and transfers CoA to acetate. Alternatively, BUT delivers butyrate to inorganic phosphate, allowing butyrate kinase to transfer the phosphate to ADP to produce ATP (31)

Chapter II

Characterization of the growth of *Faecalibacterium prausnitzii* and its butyric acid production

1. Introduction

Faecalibacterium prausnitzii (F. prausnitzii) was initially classified as *Fusobacterium prausnitzii*. However, the complete sequences of 16s rRNA genes identified from the two strains of *F. prausnitzii* in 1966 showed that the bacterium was closer to *Clostridium cluster IV* (the *Clostridium leptum* group) than *Fusobacterium* (32). The new nomenclature was definitively adopted in 2002 when a new genus *Faecalibacterium* was created to include the non-spore-forming and non-motile gram-positive bacterium named *Faecalibacterium prausnitzii* (33). Nowadays, *F. prausnitzii* species are the major representatives of *Firmicutes* phylum, *Clostridium* class, and *Ruminococcaceae* family. *F. prausnitzii* strains appear to lack plasmids, have circular chromosomes of 2.93 to 3.32 Mb with an average GC content between 47 and 57%, and are predicted to encode around 3,000 proteins (34). In humans, the *Faecalibacterium* genus exists in two different phylogroups, yet the difference between their physiological functions has not been identified. *F. prausnitzii* is localized in the gastrointestinal tract (GIT) of pigs (35), mice (36), calves (37), poultry (38) and cockroaches (39). The localization of *F. prausnitzii* along the GIT may result from a combination of environmental factors such as other commensal species, redox mediators, oxygen concentration, mucus layer, bile salt concentrations and pH (34).

F. prausnitzii is one of the beneficial bacteria in the human gut and it accounts for more than 5% of the total human gut microbiota in healthy adults (32). Although *F. prausnitzii* is predominant in healthy adults, its intestinal population is regulated by a

variety of factors. Recent studies suggest that the amount of *F. prausnitzii* in the intestinal flora depends on the gender of the host, as researchers found out that females carry less than males (female-to-male ratio: 0.41, intestinal flora) (40). In addition, the population of this bacterium changes with age (41). At first, the amount of *F. prausnitzii*-specific RNAs in stools collected from infants up to 6 months of age is below the detection threshold. Its value increases between 6 to 24 months, yet it still remains low until early childhood (2 ~ 3 years) (42). Also in seniors, the amount of *F. prausnitzii* decreases significantly to 0.3% (43). The low population of *F. prausnitzii* in the early infancy suggests that the arrival of early colonizers facilitates the subsequent implantation of the bacteria. Presumably, consumption of available oxygen by facultative anaerobic bacteria may have been required to create an anaerobic environment favorable for the growth of *F. prausnitzii*. Hence the transplantation of extremely oxygen sensitive (EOS) bacteria, in particular *F.prausnitzii*, may rely on physicochemical conditions formed beforehand by other commensal bacteria (44).

F. prausnitzii plays critical roles in supplying energy to colon cells and maintaining intestinal health (45) and further influences human health primarily through inhibiting gut inflammation (46). For example, *F. prausnitzii* has been linked to dysbiosis in several human disorders such as inflammatory bowel disease (IBD), in which a low abundance was found in patients exhibiting endoscopic recurrence six months after surgery (47). Both in vitro and in vivo experimental evidences on strong anti-inflammatory effect of *F. prausnitzii* are emerging, and hence the lack of *F. prausnitzii* may induce and strengthen inflammation. In particular, a significant inverse correlation was found between the

activity of the disease and the number of *F. prausnitzii* in patients with ulcerative colitis (UC) or even latent diseases (48). In addition, the depletion of *F. prausnitzii* was observed in Crohn's disease (CD) patients without treatment, yet not in patients with chronic diarrhea, suggesting the involvement of the bacteria in the pathogenesis of CD (49). Finally, by analyzing stool samples collected from IBD patients, decreasing morbidity of IBD in contrast to the increasing abundance of *F. prausnitzii* was identified (50). In addition, a negative correlation was observed between the abundance of *F. prausnitzii* and various diseases such as atopic dermatitis, asthma, obesity and depression (51-53).

F. prausnitzii achieves its anti-inflammatory effect through producing and supplying butyric acid in the colon mucosa. Butyric acid is the final product of intestinal bacterial carbohydrate metabolism and affects the main function of colon epithelium. In the colon, butyric acid is present at the concentration of millimolar (54). In addition to the established role in regulating viability, differentiation and diffusion, butyric acid was reported to be effective in cancer treatment (55). Furthermore, butyric acid can help prevent inflammation in the mucous membrane, since the degradation of the available butyric acid is associated with the distinct form of colitis (56). Moreover, butyric acid enemas or a high-fiber diet that increases colonic butyric acid concentration was shown to be an effective treatment for mucosal inflammation in both humans and animal models with colitis (57). The other metabolites of *F. prausnitzii* included salicylic acid, shikimic acid and raffinose (46). Salicylic acid is used in the pharmaceutical industry to produce the amine derivative 5-aminosalicylic acid (5-ASA or mesalamine) for the treatment of

patients suffering from IBD. Shikimic acid is a precursor molecule of several aromatic compounds including salicylic acid, produced through chorismate synthase pathway (58). Hence, the anti-inflammatory effects observed in mice treated with *F. prausnitzii* may be associated with the role of *F. prausnitzii* in producing salicylic acid. In contrast, raffinose, an oligosaccharide, is not associated with antimicrobial activity but is fermented by mucosal permeable microorganisms, improving the gut permeability (59). In this sense, *F. prausnitzii* was able to balance the enhancement of intestinal barrier permeability in a murine model of gut dysfunction induced by dinitrobenzene sulfonic acid (DNBS) (60). However, studies need to be performed to clarify these metabolites involved in barrier permeability produced either by the host or bacteria and their specific functions.

F. prausnitzii is an EOS bacterium making it difficult to cultivate even in anaerobic conditions. Thus most of the data on its physiology is based on its metagenomics (32). In this study, we investigated whether *F. prausnitzii* uses carbon sources present in the intestine to maintain a high ratio in the intestinal microbial community and under what conditions it produces butyric acid, a metabolite that promotes intestinal health.

2. Materials and Methods

2.1. Bacterial strains

F. prausnitzii A2-165 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Göttingen, Germany) and the DSM number is 17677.

2.2. Medium and culture conditions

For seed culture, *F. prausnitzii* was grown in Reinforced Clostridial Medium (RCM) (Difco, USA) for 48 h at 37 °C in the anaerobic chamber. The composition of the RCM is described in Table 1. The components of YCFA medium used for growth comparison study is shown in Table.2 (DSMZ_medium 1611). The YCFA medium was manufactured by first dissolving ingredients in water except for $NaHCO_3$, hemin, and cysteine. After boiling for 10 min it was cooled down to room temperature. The $NaHCO_3$, hemin, and cysteine were then added and the pH was adjusted to 6.7 - 6.8. Fatty acid and vitamin solution were sterilized by syringe filters. The vitamin solution was added to YCFA medium 100 μl per liter. Finally, the manufactured RCM and YCFA medium were stored in an anaerobic chamber for a day to remove oxygen.

The sugar preference of *F. prausnitzii* was determined by adding a final concentration of 1.0% (wt/vol) sugar to RCM without carbohydrate (10 g/L peptone, 10 g/L Beef extract, 3 g/L Yeast Extract, 5 g/L Sodium Chloride, 3 g/L Cysteine HCl,).

The sugar preference of *F. prausnitzii* was alternatively determined by using anaerobe identification test panel (AN Microplate^{tm}, Biolog catalog #1007, Biolog, USA). A pure culture of *F. prausnitzii* was grown on RCM agar plate at 37 ℃ for 2 days. The colonies on RCM agar plate were swabbed from the surface of the agar plate and suspended in AN Inoculating Fluid (Biolog Catalog #72007, Biolog, USA) and RCM base medium. The optical density (600nm) of *F. prausnitzii* was 0.01 and 100 µl of suspension was pipetted into each well of the AN Microplate. The microplate was incubated in Anaerobic Pouch (GasPak^{Tm} EZ Pouch System, BD catalog# 260683, BD, USA) without hydrogen at 37 ℃ for 24 h. Every step was performed in the anaerobic chamber. After incubation, the An plate was read with a microplate reader (Powerwave X, BioTek, USA) at the wavelength of 490 nm for AN inoculating fluid and 700 nm for RCM base medium.

Cell growth during the cultivation was measured at 600 nm wavelength using UV-VIS spectrophotometer (Shimazu UV-1800m, Japan) and microplate readers (TECAN Spark®, Switzerland). The sample measured in microplate reader was added to 96well plate and the plate was sealed with a film (Microseal® 'B' PCR Plate Sealing, Bio-RAD, United Kingdom) for the maintenance of the anaerobic condition.

2.3. Identification of microorganism

For liquid cultured cells, samples of 50 μl were centrifuged in 6,000 rpm for 5 min. After the removal of the supernatant, the pellet was dissolved in 50 μl of sterilized distilled water. For a colony obtained from an agar plate, the colony was dissolved in 50 μl of sterilized distilled water. After boiling the cells for 8 minutes, 5 μl of the boiled samples were used as a template. Each primer (Table. 3) was added to 0.1 μl and 5 μl of 2X mix dye (2X TOP simple^{TM} dye mix-tenuto. Enzynomics, Republic of Korea) was added. The PCR was run under conditions of annealing temperature at 50 ° C and elongation time of 1 minute.

2.4. Analytical methods

Sugar and SCFA concentrations were measured by high-performance liquid chromatography (HPLC, Agilent 1100 series, USA) with a UV detector (210nm) and refractive index detector. For analysis, the H^+ cation-exchange column (Rezex^{TM} ROA-Organic Acid, 300 × 7.8mm, USA) was used and 0.5 mM H_2SO_4 for sugar and 5 mM H_2SO_4 for SCFA were used as an eluent.

Table 1. Composition of RCM.

Components	Concentration (g/L)
Peptone	10.0
Beef extract	10.0
Yeast extract	3.0
Dextrose	5.0
Sodium Chloride	5.0
Soluble Starch	1.0
Cysteine HCl	0.5
Sodium Acetate	3.0
Agar	0.5

Table 2. Composition of YCFA.

Components	Concentration (g/L)
Casitone	10.0
Yeast extract	2.5
Glucose	5.0
$MgSO_4$ x 7 H_2O	0.045
$CaCl_2$ x 2 H_2O	0.09
K_2HPO_4	0.45
KH_2PO_4	0.45
NaCl	0.9
Resazurin	0.001
$NaHCO_3$	4.0
L-Cysteine-HCl	1.0
Hemin	0.01

Volatile fatty acid	Concentration (ml/L)
Acetic acid	10.0
Propionic acid	2.5
iso-Butyric acid	5.0
n-Valeric acid	0.045
iso-Valeric acid	0.09

Vitamin solution	Concentration (mg)
Biotin	2.0
Folic acid	2.0
Pyridoxine-HCl	10.0
Thiamine-HCl x 2 H_2O	5.0
Riboflavin	5.0
Nicotinic acid	5.0
D-Ca-pantothenate	5.0

Vitamin B_{12}	0.1
p-Aminobenzoic acid	5.0
Lipoic acid	5.0
Distilled water	1,000 ml

Table 3. Primer sequences for the identification of *Enterococcus a*nd *F. prausnitzii.*

	Forward primer	Reverse primer
Universal Primer	GTGCCAGGCAGCCGCGG	CCGTCAATTCCTTTGAGTTT
F. prausnitzii-specific primer	TTCTTTCCTCCCGAGTGC	TCTATCTCTAGAGTGGTC
Enterococcus-specific primer	AGTTTATGCCGCATGGCAT	GAAACCCTCCAACACTTAGCAC

3. Results and Discussion

3.1. Identification of *F. prausnitzii*

For this study, *F. prausnitzii A2-165* was purchased from DSMZ and cultured in YCFA medium. However, the images obtained from the microscope and Scanning Electron Microscope (SEM) indicated that the culture was contaminated with *Enterococcus faecalis* (Figure 1 and 2). The presence of *Enterococcus faecalis* was confirmed by sequencing its 16S rRNA using the universal primer (Table 3.) *Enterococcus* is a large genus of lactic acid bacteria related to retinopathy and it is gram-positive bacteria that often occur in pairs or short chains. Its physical characteristics alone make it difficult to distinguish from streptococci (61). There are two species of common symbiotic in human intestines; *E. faecalis* (90–95%) and *E. faecium* (5–10%). Notable clinical infections by Enterococcus include urinary tract infection, bacterium endometritis, gecilitis, meningitis, spontaneous bacterial peritonitis, and etc. (62, 63). The antibiotic-sensitive strains can be treated with ampicillin, penicillin, and anti-comycin. Urinary tract infection can be treated specifically with nitropurantoin even with anti-comycin resistance.

To separate these two strains, colonies were streaked on YCFA agar plate, and a single colony was picked. The colony selected was observed under a microscope and specific 16S rRNA sequence was confirmed through PCR using *F. prausnitzii*-specific primer (Table 3). As the anaerobic chamber is easily exposed to contamination, we examined the contamination status of our samples by microscopic observation and PCR before every experiment to obtain pure F. *prausnitzii* (Figure 3).

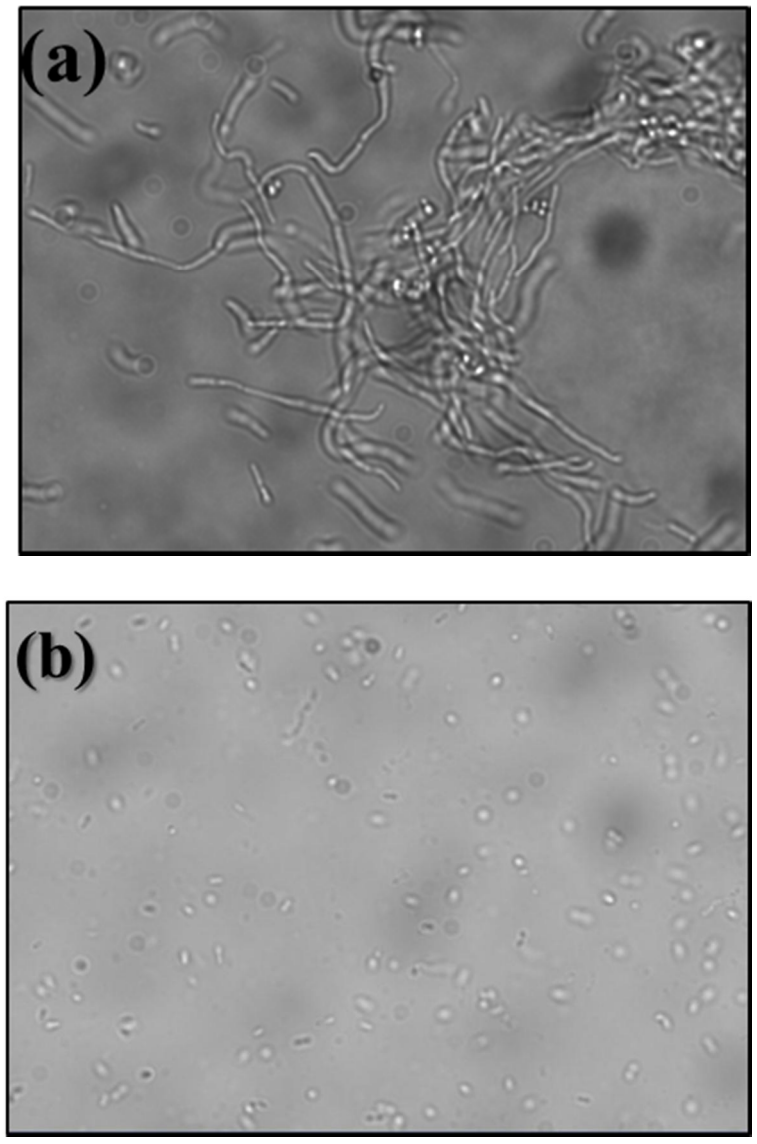


Figure 1. Microscopic images (x 400) on (a) *F. prausnitzii* and (b) *Enterococcus faecalis*.

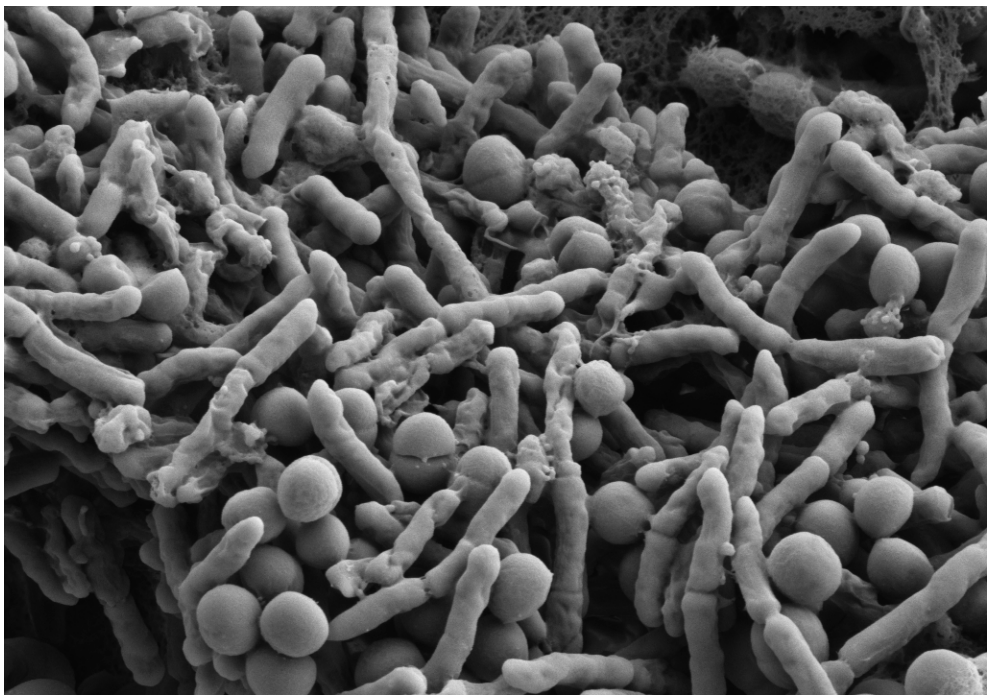


Figure 2. Scanning electron microscopy (SEM) image of *F. prausnitzii (rod-shaped)* and *Enterococcus faecalis* (globular shape).

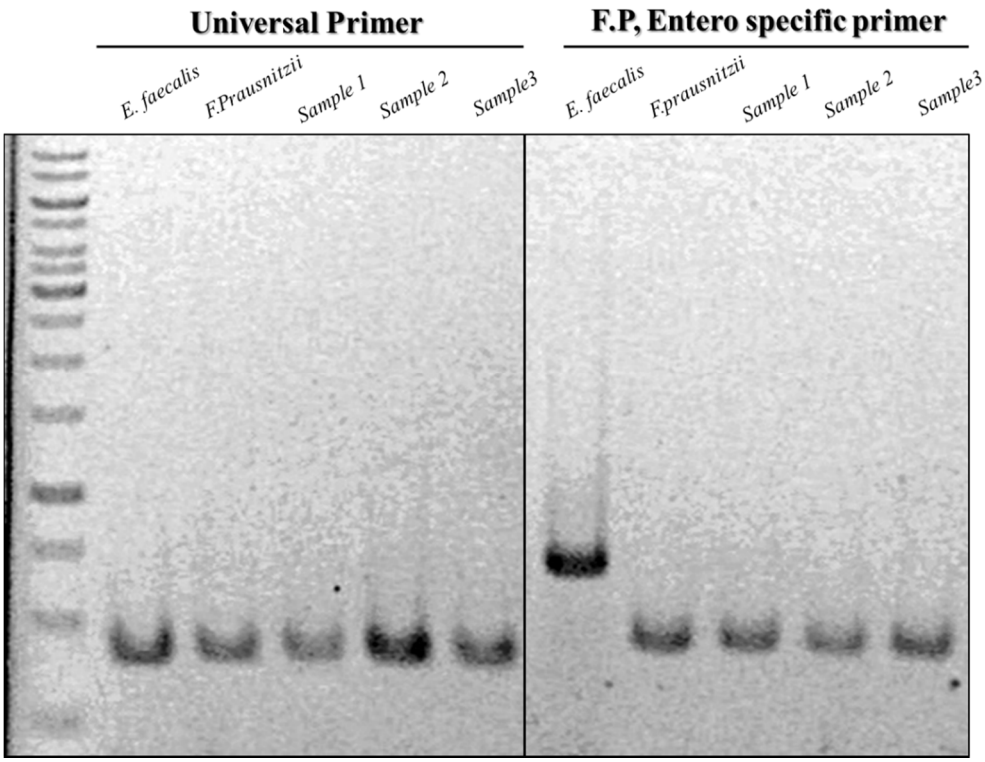


Figure 3. Identification of *F. prausnitzii* and *Enterococcus faecalis* by 16S rRNA sequencing (universal primer) and *Enterococcus* and *F. prausnitzii*-specific primers (F.p, Entero specific primer).

3.2. Selection for culture medium of *F. prausnitzii*

In order to select an optimized culture medium for *F. prausnitzii,* YCFA and RCM were tested for 48 h at 37°C. Several studies have used YCFA medium(64, 65) while others have used RCM (66, 67) to culture *F. prausnitzii*. Figure 4 shows that the growth of *F. prausnitzii* in RCM was higher than in YCFA. The most significant difference between RCM and YCFA was the presence of beef extract. To investigate the effect of beef extract, *F. prausnitzii* was cultured in M9, YCFA, YCFA with beef extract and RCM (Figure 5). Since the growth of *F. prausnitzii* in M9 displays minimal growth, it was used as the negative control. Even though the growth varied depending on the types of sugars, the results showed that the growth of *F. prausnitzii* in RCM was better than YCFA under all conditions. However, YCFA medium supplemented with beef extract could support the growth of *F. prausnitzii* as well as the RCM. The addition of N-acetylglucosamine in YCFA also increased the growth of the bacterium yet not as much as the RCM. These results indicated that *F. prausnitzii* prefers beef extract, which is made by extracting the water-soluble fraction from the muscle part of beef. It provides salt, nucleic acid, sugar, amino acid, and non-immune protein. Overall, we chose RCM to culture *F. prausnitzii*

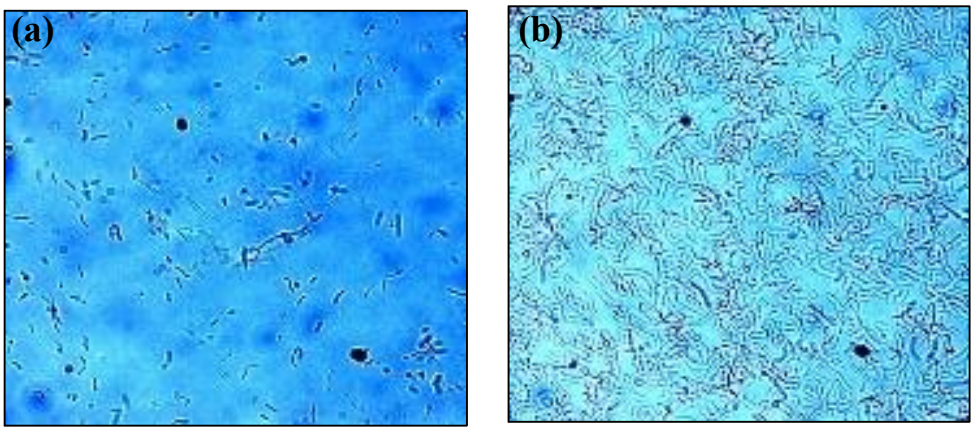
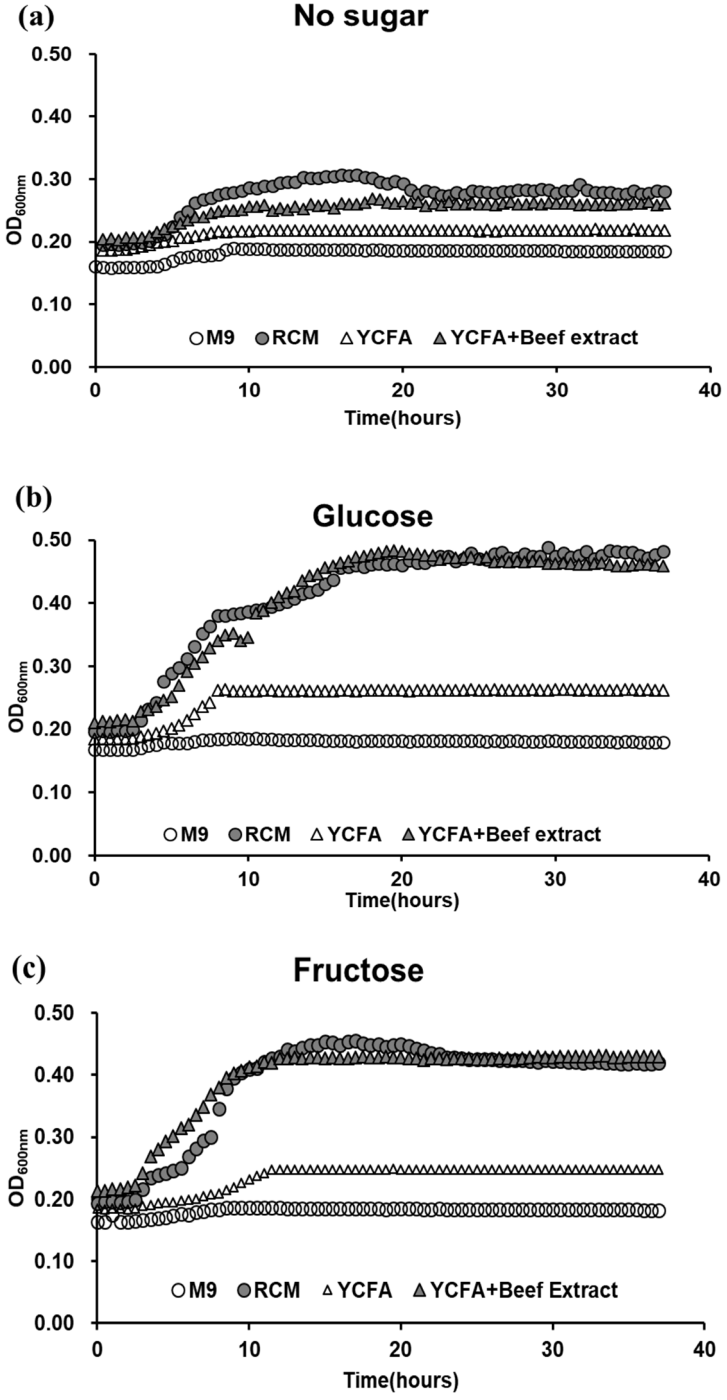


Figure 4. The micrograph of *F. prausnitzii* grown in YCFA medium (a) and RCM (b)


(a)
No sugar
0.50
0.40
0.30
0.20
0.10
0.00
OD600nm
M9 RCM YCFA YCFA+Beef extract
0
10
20
30
40
Time(hours)
(b)
Glucose
0.50
0.40
0.30
0.20
0.10
0.00
OD600nm
M9 RCM YCFA YCFA+Beef extract
0
10
20
30
40
Time(hours)
(c)
Fructose
0.50
0.40
0.30
0.20
0.10
0.00
OD600nm
M9 RCM YCFA YCFA+Beef Extract
0
10
20
30
40
Time(hours)

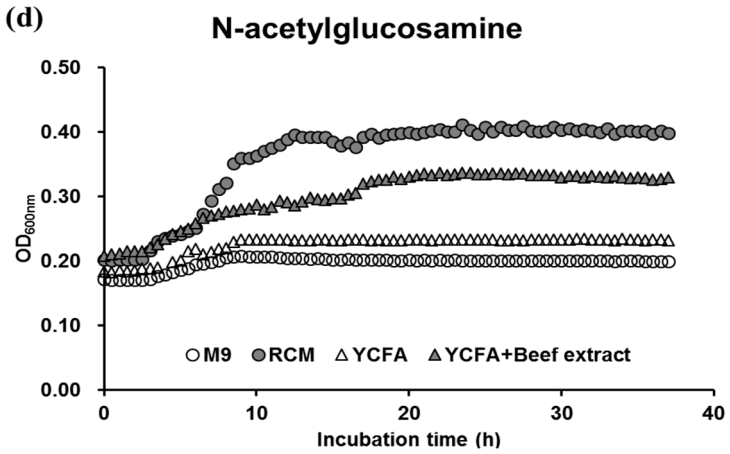


Figure 5. The time course growth of *F. prausnitzii* in M9, RCM, YCFA and YCFA with beef extract under the anaerobic condition for 36 h at 37°C. (a) No sugar, (b) 0.2% Glucose, (c) 0.2% Fructose, (d) 0.2% N-acetylglucosamine.

3.3. Effect of sugar on *F. prausnitzii* growth

Phosphoenolpyruvate: sugar phosphotransferase system (PTS) is one of the sugar transport systems and PTS sugars are preferred over non-PTS carbon sources (68). ATP-binding cassette (ABC) transport mediates the uptake of sugars by coupling ATP hydrolysis to a physical movement. *F. prausnitzii* A2-165 has PTS connected to the uptake of glucose, fructose and N-acetylglucosamine while ABC transport is associated with galactose, inulin and maltose, based on genome annotation (65).

In addition, we assumed that *F. prausnitzii* has a mannose PTS based on the data obtained from whole genome sequencing. N-acetylgalactosamine is one of the sugars constituting mucin that affects intestinal microbiota (69). Based on these facts, 8 kinds of sugars were selected for the measurement of the relative growths of the bacteria over the time-course as shown in Figure 6. The exponential growth phase of *F. prausnitzii* started after 10 to 12 h and *F. prausnitzii* could grow even without sugar as RCM contained beef extract, helping the bacteria to grow (Figure 5a). *F. prausnitzii* exhibited optimal growth and obtained higher cell density in media with carbon sources of glucose (OD_{600nm} 0.562), fructose (OD_{600nm} 0.479), N-acetylglucosamine (NAG) (OD_{600nm} 0.486) and galactose (OD_{600nm} 0.421). On the other hand, *F. prausnitzii* did not grow in N-acetylgalactosamine (NAGA) and inulin which are also present in the human intestine. However *F. prausnitzii* in microbial communities with bifidobacterial species grew in inulin because bifidobacteria utilized inulin and the metabolite of bifidobacteria lead to microbial communities. (70). In addition, we observed that mannose and maltose were not utilized by *F. prausnitzii*, despite being a PTS sugar and being related to ABC

transport in the bacteria, respectively. In addition, the growths of *F. prausnitzii* in 49 carbon sources including the 8 sugars were examined (Figure 7). The result showed that under glucose, fructose, NAG and galactose conditions, *F. prausnitzii* kept growing throughout the experiment indicating that these sugars can be utilized as carbon sources and sufficient to support its growth. Other sugars except glucose, fructose, NAG and galactose was not sufficient to support the growth of *F. prausnitzii* after a certain point (OD 0.42) which is 2-times higher than growth without sugar.

The effect of sugar concentrations on the growth of *F. prausnitzii* is shown in Figure 8. Varying concentrations of glucose, fructose or NAG showed minimal effects on the growth of *F. prausnitzii*. Yet the optimal condition for the growth of the bacterium was identified as 1.0% of glucose concentration. In the culture medium with glucose or NAG, the growth of F. *prausnitzii* was increased when the concentration ranged between 0.2% to 1.0 %. At 2.0 % glucose concentration the growth decreased (Figure 8a). However, in the fructose culture medium, the growth of *F. prausnitzii* was not significantly different. (Figure 8b).

The AN microplate was used to identify the preferred carbon source of *F. prausnitzii* and the results were shown in Figure 9. Total 95 types of carbon sources were applied on AN microplate and water was used as a control. The principle of AN microplate is based on the reduction of tetrazolium shown by the purple color gradient in accordance with the number of microorganisms. The AN inoculating fluid was used for colony suspension and RCM also used to make optimal conditions of *F. prausnitzii* growth. In the inoculating fluid, the OD value of control (water) was 0.217. Samples that displayed values more

than twice higher than control were samples with N-acetyl-D-galactosamine, N-acetylglucosamine, D-fructose, D-galactose, α-D-glucose, Palatinose, L-rhamnose, α-hydroxybutyric acid, D,L-lactic acid, L-lactic acid, D-lactic acid methyl ester, D-malic acid and m-tartaric acid. In RCM base medium, the OD value of the control was 0.235. The samples that were more than twice as the OD value of the control were samples with N-acetylglucosamine, D-fructose, D-galactose, D-gluconic acid and α-D-glucose. From these results, it was proved that *F. prausnitzii* prefers glucose, fructose, N-acetylglucosamine and galactose over other carbon sources.

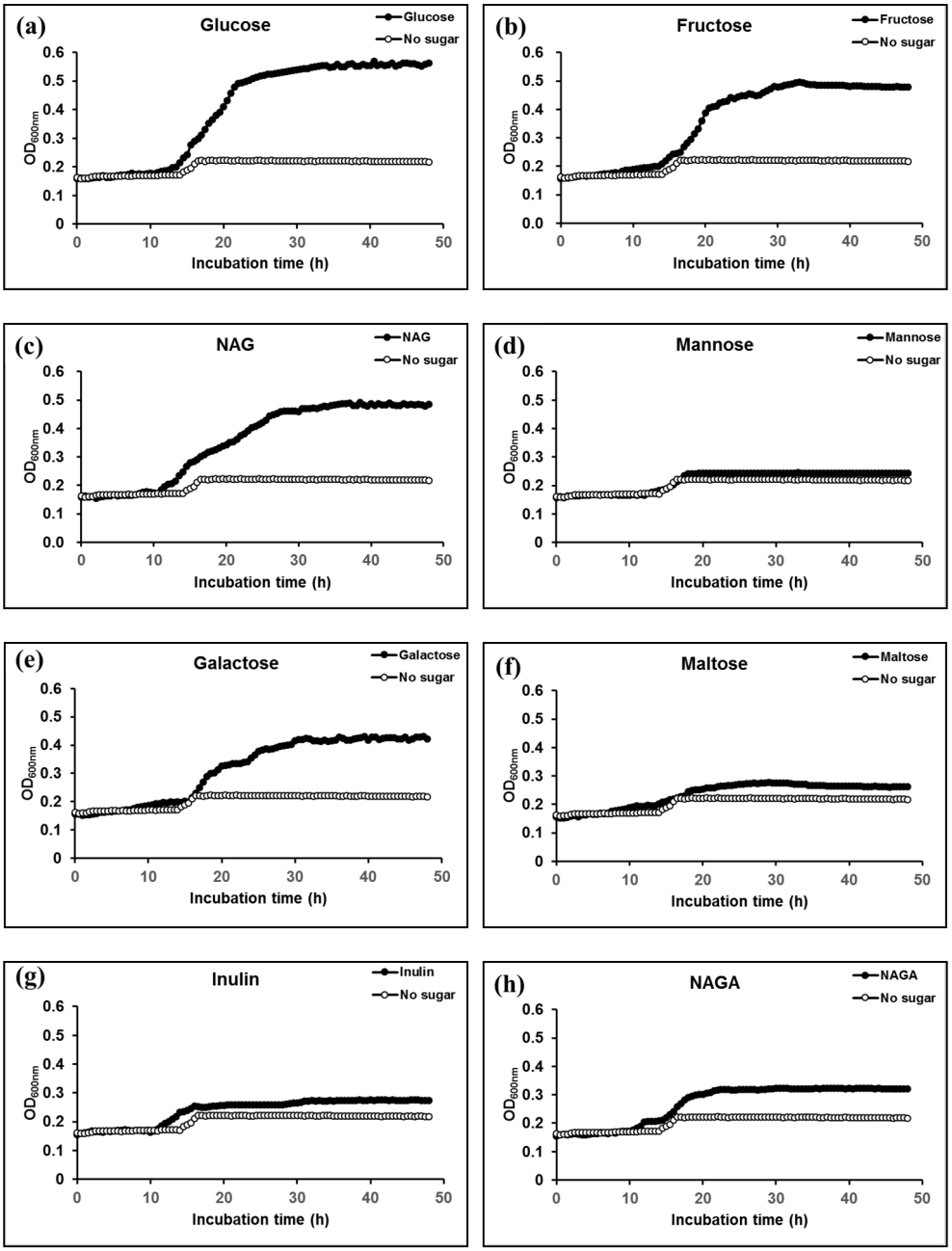

(a)
Glucose
Glucose
No sugar
(b)
Fructose
Fructose
No sugar
(c)
NAG
NAG
No sugar
(d)
Mannose
Mannose
No sugar
(e)
Galactose
Galactose
No sugar
(f)
Maltose
Maltose
No sugar
(g)
Inulin
Inulin
No sugar
(h)
NAGA
NAGA
No sugar
OD600nm
Incubation time (h)

Figure 6. The time course growth of *F. prausnitzii* in RCM supplied with various sugars measured under the anaerobic condition for 48 h at 37°C. PTS sugar; (a) 1.0% Glucose, (b) 1.0% Fructose, (c) 1.0% N-acetylglucosamine (NAG), (d) 1.0% Mannose, ABC transport sugar; (e) 1.0% Galactose, (f) 1.0% Maltose, (g) 1.0% Inulin, Component of mucin; (h) 1.0% N-acetylgalactosamine (NAGA)

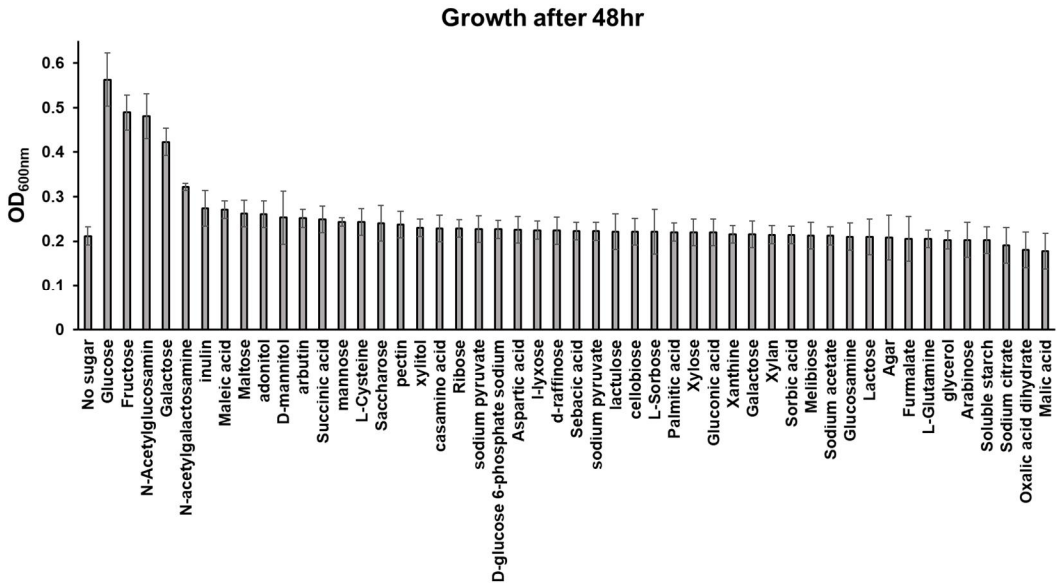


Figure 7. The growths of *F. prausnitzii* in RCM supplemented with 49 types of carbon sources measured under the anaerobic condition for 48 h at 37°C.

The concentration of all carbon sources were 0.2%

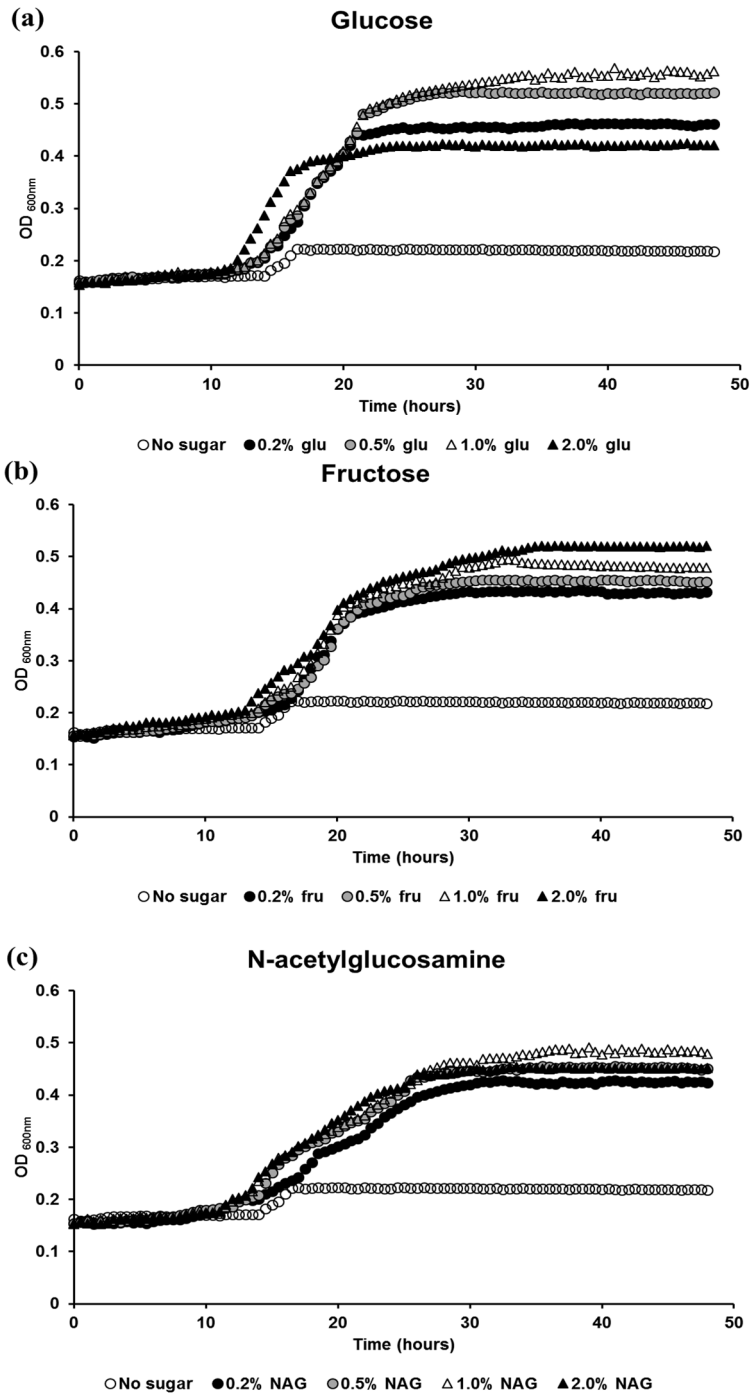
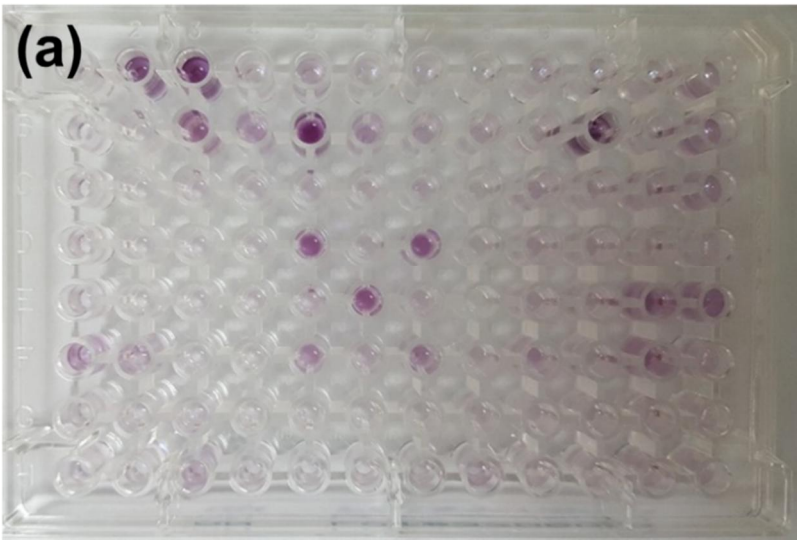
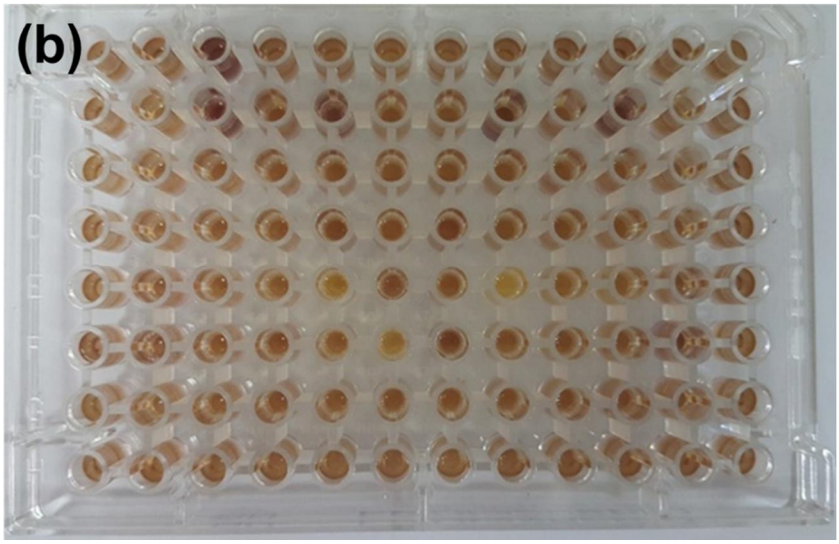


Figure 8. The time course growth of *F. prausnitzii* in RCM supplied with various

concentrations of sugars observed under the anaerobic condition for 48 h at 37°C. (a) Glucose, (b) Fructose, (c) N-acetylglucosamine



Water	N-Acetyl-D-Galactosamine	N-Acetyl-D-Glucosamine	N-Acetyl-β-D-Mannosamine	Adonitol	Amygdalin	D-Arabitol	Arbutin	D-Cellobiose	α-Cyclodextrin	β-Cyclodextrin	Dextrin
0.217	0.941	1.11	0.202	0.277	0.256	0.21	0.185	0.216	0.285	0.255	0.435
Dulcitol	i-Erythritol	D-Fructose	L-Fucose	D-Galactose	D-Galacturonic Acid	Gentiobiose	D-Gluconic Acid	D-Glucosaminic Acid	α-D-Glucose	α-D-Glucose-1-Phosphate	D-Glucose-6-Phosphate
0.244	0.22	0.79	0.351	1.173	0.344	0.305	0.259	0.23	1.472	0.22	0.34
Glycerol	D,L-α-Glycerol Phosphate	m-Inositol	α-D-Lactose	Lactulose	Maltose	Maltotriose	D-Mannitol	D-Mannose	D-Melezitose	D-Melibiose	3-Methyl-D-Glucose
0.208	0.19	0.201	0.218	0.244	0.218	0.324	0.206	0.249	0.295	0.278	0.307
α-Methyl-D-Galactoside	β-Methyl-D-Galactoside	α-Methyl-D-Glucoside	β-Methyl-D-Glucoside	Palatinose	D-Raffinose	L-Rhamnose	Salicin	D-Sorbitol	Stachyose	Sucrose	D-Trehalose
0.297	0.203	0.193	0.211	0.606	0.223	0.577	0.235	0.244	0.218	0.265	0.207
Turanose	Acetic Acid	Formic Acid	Fumaric Acid	Glyoxylic Acid	α-Hydroxybutyric Acid	β-Hydroxybutyric Acid	Itaconic Acid	α-Ketobutyric Acid	α-Ketovaleric Acid	D,L-Lactic Acid	L-Lactic Acid
0.211	0.173	0.224	0.265	0.194	0.743	0.289	0.166	0.38	0.279	0.436	0.414
D-Lactic Acid Methyl Ester	D-Malic Acid	L-Malic Acid	Propionic Acid	Pyruvic Acid	Pyruvic Acid Methyl Ester	D-Saccharic Acid	Succinamic Acid	Succinic Acid	Succinic Acid Mono-Methyl Ester	m-Tartaric Acid	Urocanic Acid
0.476	0.436	0.181	0.2	0.382	0.3	0.374	0.235	0.337	0.321	0.469	0.378
Alaninamide	L-Alanine	L-Alanyl-L-Glutamine	L-Alanyl-L-Histidine	L-Alanyl-L-Threonine	L-Asparagine	L-Glutamic Acid	L-Glutamine	Glycyl-L-Aspartic Acid	Glycyl-L-Glutamine	Glycyl-L-Methionine	Glycyl-L- Proline
0.197	0.358	0.267	0.212	0.331	0.191	0.202	0.248	0.2	0.2	0.258	0.203
L-Methionine	L-Phenylalanine	L-Serine	L-Threonine	L-Valine	L-Valine plus L-Aspartic Acid	2'-Deoxy Adenosine	Inosine	Thymidine	Uridine	Thymidine-5'-Mono-phosphate	Uridine-5'-Mono-phosphate
0.241	0.233	0.294	0.221	0.235	0.233	0.216	0.243	0.201	0.198	0.263	0.241



Water	N-Acetyl-D-Galactosamine	N-Acetyl-D-Glucosamine	N-Acetyl-β-D-Mannosamine	Adonitol	Amygdalin	D-Arabitol	Arbutin	D-Cellobiose	α-Cyclodextrin	β-Cyclodextrin	Dextrin
0.235	0.241	0.797	0.24	0.238	0.203	0.234	0.257	0.26	0.206	0.218	0.246
Dulcitol	i-Erythritol	D-Fructose	L-Fucose	D-Galactose	D-Galacturonic Acid	Gentiobiose	D-Gluconic Acid	D-Glucosaminic Acid	α-D-Glucose	α-D-Glucose-1-Phosphate	D-Glucose-6-Phosphate
0.236	0.213	0.808	0.306	0.719	0.269	0.267	0.53	0.268	0.830	0.262	0.252
Glycerol	D,L-α-Glycerol Phosphate	m-Inositol	α-D-Lactose	Lactulose	Maltose	Maltotriose	D-Mannitol	D-Mannose	D-Melezitose	D-Melibiose	3-Methyl-D-Glucose
0.192	0.236	0.243	0.242	0.252	0.237	0.247	0.54	0.25	0.248	0.253	0.239
α-Methyl-D-Galactoside	β-Methyl-D-Galactoside	α-Methyl-D-Glucoside	β-Methyl-D-Glucoside	Palatinose	D-Raffinose	L-Rhamnose	Salicin	D-Sorbitol	Stachyose	Sucrose	D-Trehalose
0.226	0.228	0.241	0.238	0.323	0.255	0.338	0.242	0.248	0.242	0.255	0.234
Turanose	Acetic Acid	Formic Acid	Fumaric Acid	Glyoxylic Acid	α-Hydroxybutyric Acid	β-Hydroxybutyric Acid	Itaconic Acid	α-Ketobutyric Acid	α-Ketovaleric Acid	D,L-Lactic Acid	L-Lactic Acid
0.223	0.247	0.229	0.202	0.152	0.362	0.228	0.315	0.165	0.182	0.257	0.252
D-Lactic Acid Methyl Ester	D-Malic Acid	L-Malic Acid	Propionic Acid	Pyruvic Acid	Pyruvic Acid Methyl Ester	D-Saccharic Acid	Succinamic Acid	Succinic Acid	Succinic Acid Mono-Methyl Ester	m-Tartaric Acid	Urocanic Acid
0.276	0.251	0.237	0.226	0.184	0.14	0.345	0.22	0.238	0.218	0.423	0.205
Alaninamide	L-Alanine	L-Alanyl-L-Glutamine	L-Alanyl-L-Histidine	L-Alanyl-L-Threonine	L-Asparagine	L-Glutamic Acid	L-Glutamine	Glycyl-L-Aspartic Acid	Glycyl-L-Glutamine	Glycyl-L-Methionine	Glycyl-L- Proline
0.211	0.217	0.227	0.244	0.22	0.239	0.232	0.223	0.242	0.247	0.218	0.216
L-Methionine	L-Phenylalanine	L-Serine	L-Threonine	L-Valine	L-Valine plus L-Aspartic Acid	2'-Deoxy Adenosine	Inosine	Thymidine	Uridine	Thymidine-5'-Mono-phosphate	Uridine-5'-Mono-phosphate
0.227	0.198	0.228	0.222	0.23	0.238	0.248	0.25	0.235	0.224	0.223	0.232

Figure 9. The sugar preference test on *F. prausnitzii* using AN microplate under the anaerobic condition for 24 h at 37°C. (a) Microplate and measured OD values in inoculating fluid, (b) Microplate and OD values in RCM base medium

3.4. Effect of sugars on butyric acid production by *F. prausnitzii*

When *F. prausnitzii* was grown on different carbohydrate sources for 48 hours, it produced butyric acid as a metabolite as shown in Figure 10. The optimal type of sugar for butyric acid production of *F. prausnitzii* was 1% glucose, resulting in 355.66 ± 21.4 mg/L butyric acid in supernatant of culture medium, while the lowest concentration of the butyric acid production was the supernatant of mannose-grown culture medium and the concentration of butyric acid was 112.64 ± 16.65 mg/L. It can be seen from the result that butyric acid production is closely associated with the growth of *F. Prausnitzii*. In addition, the efficiency of butyric acid production was investigated to identify whether PTS sugars (glucose, fructose, NAG) could generate butyric acid in higher quantity than non-PTS sugars (galactose, insulin and maltose)

As glucose, fructose, NAG and galactose engendered significant butyric acid production, medium with 1% of these 4 sugars were prepared to observe time course changes in growth rate, consumption of carbohydrates and metabolites production of the bacteria (Figure. 11) The provided sugars were consumed constantly from the initial time point of culture with notable increase in consumption after 12 h when *F. prausnitzii* started its growth (Figure.11 a and b). The addition of glucose ranked highest in growth followed by fructose, then N-acetylglucosamine and finally galactose. The growth rate appeared to be corresponding to the consumption of sugars. However, the bacteria consumed NAG faster than fructose in contrast to its growth rate in these carbohydrates. The amount of butyric acid and acetic acid production also corresponded to sugars

displaying higher growth rates. The quantity of metabolites increased after 12h of culture when *F. prausnitzii* started to grow and correlated to the growth rate in the exponential phase. Yet around the end point of the exponential phase, acetic acid was utilized for cell growth and hence decreased in quantity after 30 h, while butyric acid continuously increased until 36 h in all sugar conditions (Figure.11 c and d)

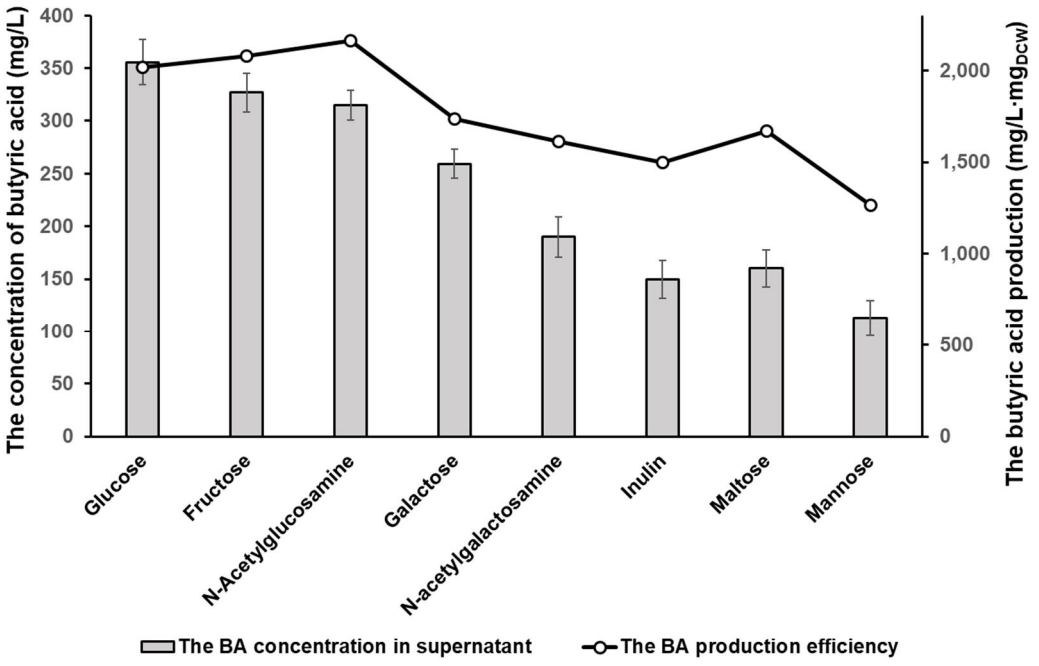
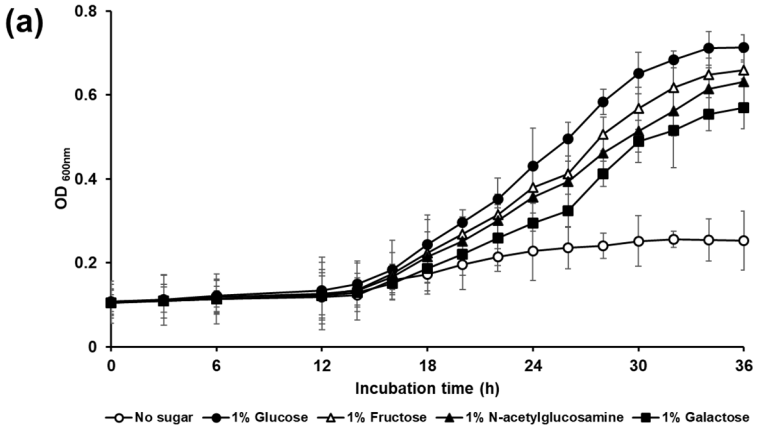
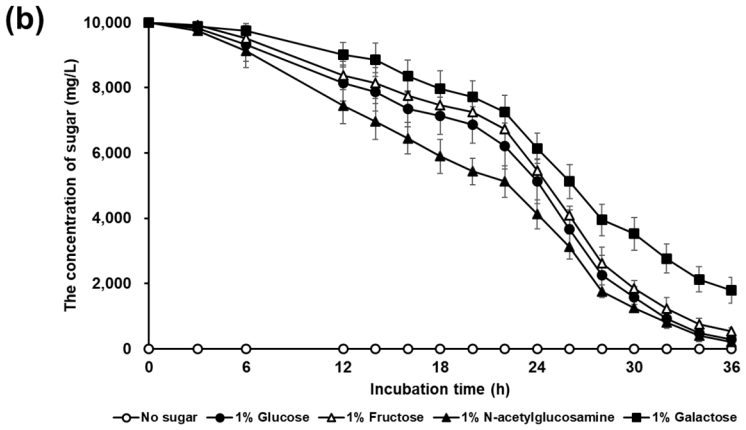
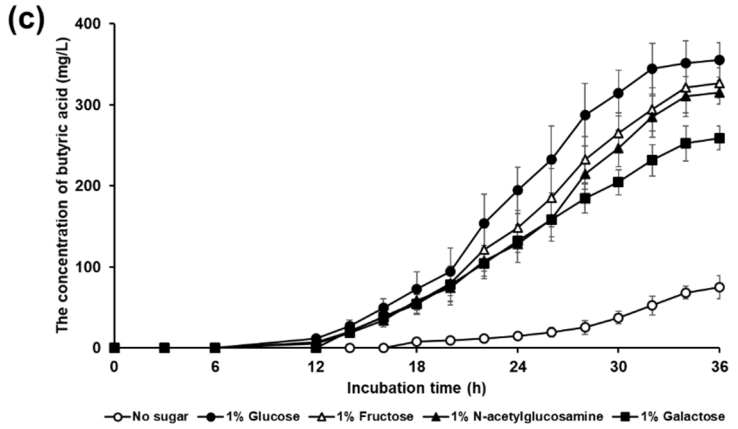


Figure 10. Effect of sugars on butyric acid production by *F. prausnitzii* and production efficiency.


(a)
0.8
0.6
0.4
0.2
0
OD 600nm
0
6
12
18
24
30
36
Incubation time (h)
No sugar
1% Glucose
1% Fructose
1% N-acetylglucosamine
1% Galactose


(b)
10,000
8,000
6,000
4,000
2,000
0
The concentration of sugar (mg/L)
0
6
12
18
24
30
36
Incubation time (h)
No sugar
1% Glucose
1% Fructose
1% N-acetylglucosamine
1% Galactose


(c)
400
300
200
100
0
The concentration of butyric acid (mg/L)
0
6
12
18
24
30
36
Incubation time (h)
No sugar
1% Glucose
1% Fructose
1% N-acetylglucosamine
1% Galactose

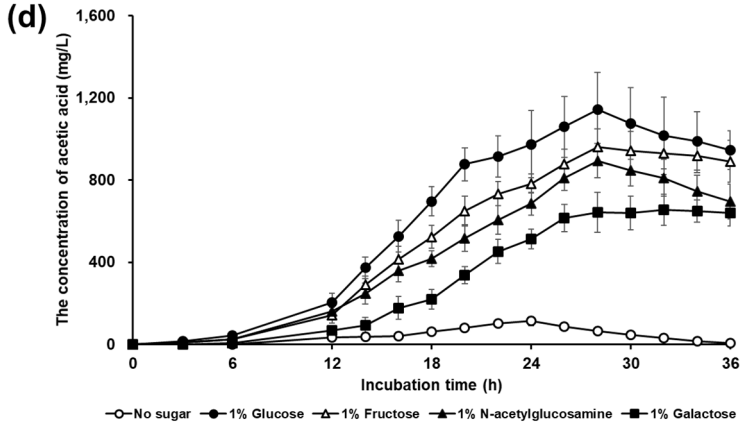


Figure 11. The effect of sugars on growth and metabolites production of *F.prausnitzii*; (a) Cell growth, (b) Sugar consumption, (c) Butyric acid production, (d) Acetic acid production

4. Conclusion

In this chapter, the effect of sugars on the growth of the *F. prausnitzii* and its butyric acid production were examined. *F. prausnitzii* is challenging to cultivate even in the anaerobic chamber and is contaminated easily. For this reason, a method for pure culture was needed to carry out this study. Therefore, microscopic observation and PCR using manufactured primers were used to confirm the successful incubation of *F. prausnitzii* for the proceeding experiments.

We confirmed that the beef extract was necessary for the successful growth of *F. prausnitzii*. Hence the RCM, a medium containing beef extract, was selected to culture *F. prausnitzii* in this study. *F. prausnitzii* requires a carbohydrate energy source like sugar for its growth and butyrate formation. The optimal condition of *F. prausnitzii* growth was identified as RCM containing 0.1% glucose. The bacterium could also use fructose, N-acetylglucosamine and galactose. The growth of *F. prausnitzii* appeared to be proportional to sugar concentration except for glucose.

The highest amount of total butyric acid in the culture supernatant was obtained from samples cultivated under the glucose and the amount of butyric acid in the culture supernatant increased along with the growth of the microorganism. In addition, butyric acid production by *F. prausnitzii* during its culture was dominant within PTS sugars (glucose, fructose, NAG) than with non-PTS sugars (galactose, inulin, maltose)

In conclusion, *F. prausnitzii* in human intestine grows by ingesting N-acetylglucosamine, N-acetylgalactosamine and galactose from mucin. The bacteria consumes glucose and fructose hydrolyzed from inulin upon digestion by another gut

microbiota, though it does not utilize pectin, xylose and inulin itself (Figure 12).

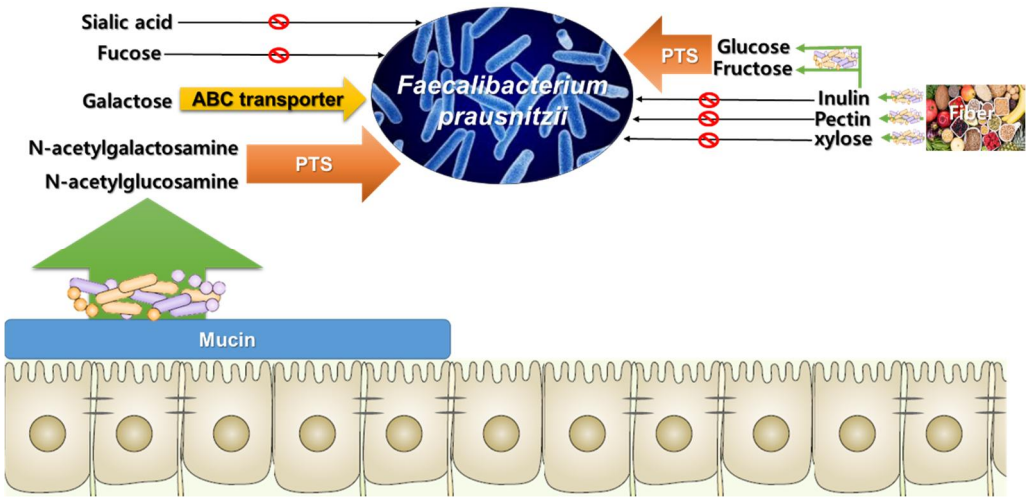


Figure 12. The model for carbon source utilization by *F. prausnitzii* in the intestine

Mucin in the human large intestine is degraded by gut microbiota, generating various metabolites such as sialic acid, fucose, galactose, N-acetylglucosamine (NAG), and N-acetylgalactosamine (NAGA). *F.prausnitzii* ingests NAG and NAGA through the PTS and galactose through an ABC transporter. Undigested polysaccharides in the host body are degraded into inulin, pectin and xylose by microorganisms in the large intestine. *F.prausnitzii* is unable to digest these products except for glucose and fructose which might be released from inulin by activities of other gut microorganisms.

Chapter III

Physiological activity of *E. coli* engineered for butyric acid production

1. Introduction

Human intestinal microbial ecosystems are extensive, diverse, and directly related to human health. Microbial species are present in the distal gastrointestinal tract and imbalances in the composition and function of microorganisms in the digestive tract are associated with diseases ranging from localized gastrointestinal disorders to the nervous system, respiratory, metabolic, hepatic and cardiovascular diseases. The homeostasis formed between a host and the colonizing bacteria plays essential functional roles such as protection against pathogens, maturation and regulation of the immune system, maturation of the intestine, production of SCFAs, mucosal physiology and production of vitamin K and biotin (44).

Butyric acid belongs to short-chain fatty acids (SCFAs) and is the most plentiful metabolite derived from bacterial fermentation of carbohydrates such as dietary fibers in the intestine. It can be utilized by intestinal epithelial cells as the main energy source to stimulate their proliferation and differentiation and to improve intestinal barrier function (71). Moreover, acting as a signal in the immune system, it can be used for the medication of colonic inflammation and colorectal cancer to increase expression of tight junctions, which are the host defense proteins used to reduce infections by regulating the permeability of epithelial barriers (72, 73). Butyric acid is produced by anaerobes in the human gut such as *Clostridium butyricum, Eubacterium limosum, Fusobacterium nucleatum* and etc. Among them, *F. prausnitzii* is one of the major butyric acid-producing bacteria by metabolizing the intestinal lactose, which is a significant source of ATP production in the intestinal epithelium (74) . Hence, a decrease in the intrinsic level

of *F. prausnitzii* can lead to ATP deficiency in the epithelial cells, which in turn can weaken the self-defense ability against the inflammatory response.

Escherichia coli Nissle 1917 (EcN) is an active ingredient in the pharmaceutical preparation Mutaflor®, a microbial agent approved for medical use in Germany and some European countries today. This drug has traditionally been used in the treatment of various diseases and disorders in the intestinal tract since 1917, when Alfred Nissle had screened the human intestinal *E. coli* strains to inhibit the growth of *Salmonella Shigella*, and other intestinal pathogens. Although the strain does not have factors similar to a virus, clusters of genes located on genetic islands (GEI) in the chromosomes are responsible for the synthesis of 'Pi-Tins factors' that contribute to the probiological properties of this strain. The strain ecologically belongs to the *E. coli* O6 group, with serotype O6:K5:H1 (75, 76). EcN is a typical gram-negative bacteria that exhibit lipopolysaccharide (LPS) as a structural component on the outer cell membrane. The surface antigen O6 represents the outer part of the LPS of the strain and shows several unusual features. For example, the complete molecular structure of LPS on EcN (76) differs from the LPS of all other *E. coli* family in a number of molecular aspects (77). For example, the O6 polysaccharide side chain is notably short as it consists only of a single 'repeat unit' of a typical oligosaccharide building block on the O6 antigen due to a point mutation that introduces a stop codon in the gene of an O6 antigenic enzyme. This molecular feature gives the strain a 'semi-transparent' phenotype when growing on a solid nutrition medium. Certain characteristics of the LPS on EcN appear to explain the phenomenon of how strains do not exhibit immunotoxin effects yet exhibit characteristics

of immune modulation. The antagonistic actions of EcN are at least in part due to the formation of microcins against the producer strain which itself is immune to. One of these microcins (microcin H47) had already been found from another *E. coli* strain whereas the second microcin was identified as microcin M. The microcin H47 and microcin M utilize iron uptake receptors of other enterobacteriaceae to enter the cytoplasm and kill these bacteria. Another strain-specific feature of EcN is a special lipopolysaccharide (LPS) on its outer cell membrane, being responsible for the immunomodulating properties of EcN (78) (Figure 13).

In this study, we have engineered EcN for butyric acid production to observe corresponding changes in the anti-inflammatory effect of EcN. For this purpose, two enzymes in the butyric acid biosynthesis pathway of *F. prausnitzii* were overexpressed in EcN. The engineered *E.coli* was then tested for its anti-inflammatory effect in Dextran Sulfate Sodium (DSS)-induced colitis model.

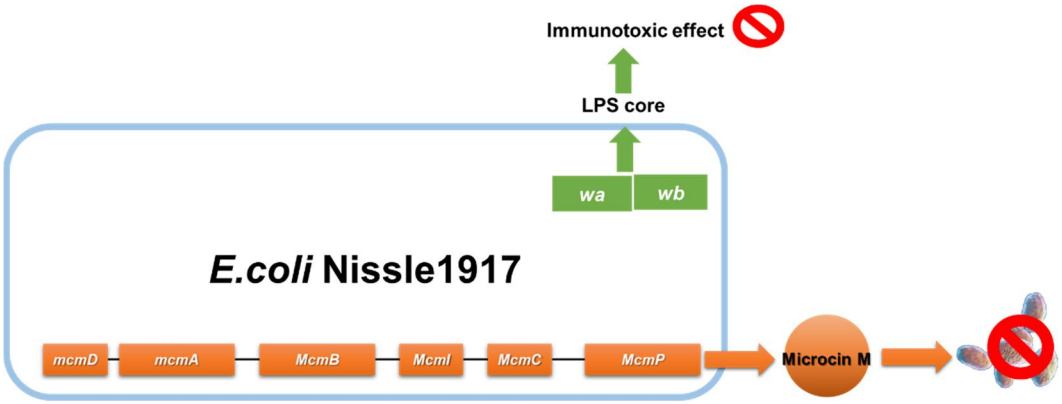


Figure 13. The genetic difference between EcN and other *E.coli* strains. Among the genes that are present in Nissle1917 while absent in other *E.coli* strains, the *mcm* gene that produces microcin M contributes to the strain's bacteriocidal activity. The *wa* and *wb* form the LPS core on the outer membrane, protecting the bacteria from the host's immunotoxic effect.

2. Materials and Methods

2.1. *E. coli* Strains, plasmids construction and culture condition

The strains and plasmids used in this study are listed in Table 4. EcN is a typical example of a nonpathogenic, commensal *E. coli* isolate. It forms the basis of probiotic preparation used for the treatment of various intestinal disorders and is known to be a successful colonizer of the human gut. *E. coli* MG1655, which originates from the wild-type K-12 strain, was used as the control for comparing with EcN. *F. prausnitzii* A2-165 was used as the source for template DNA for cloning. Vector plasmids pACYC184 with chloramphenicol and tetracycline marker and pBR 322 with ampicillin and tetracycline marker were used to construct the overexpression vector of proteins. pACYC184 was equipped with the *cat* promoter and pBR322 was equipped with the *crp* promoter for constitutive expression. The ribosome binding sites (RBS) were inserted after constitutive promoters in both vectors.

Strains were cultured in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) for seed culture with antibiotics including 20 μg/L chloramphenicol for pACYC184 and 100 μg/L ampicillin for pBR322. Butyric acid production test for *E.coli* strains and *F. prausnitzii* was performed in RCM. The dependence of butyric acid production on sugar sources was determined by adding respective sugars to M9 medium (12.8 g/L $Na_2HPO_4{\cdot}7H_2O$, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl and 1.0 g/L NH_4Cl) supplemented with 0.1% casamino acid, 0.1mM $Cacl_2$ and 1mM $MgSO_4$. Every strain

was grown at 37 ℃.

Table 4. Strains and plasmids used in this study

Strains	Genotype or phenotype	Source or Reference
E.coli MG1655	Wild-type *E. coli,* F^-, $lambda^-$, rph-1	(79)
E.coli Nissle1917	O6:K5:H1	(80)
F. prausnitzii A2-165	*Clostridium.* cluster IV	(81)

Plasmids	Genotype or phenotype	Source or Reference
pACYC184	Cmr Tcr; pl5A Ori; 4,245 bp	(82)
pBR322	Ap^r , Tc^r ColEl Ori; 4,361 bp	(83)

Ap^r: Resistance to ampicillin; Tc^r: Resistance to tetracycline; Cm^r: Resistance to chloramphenicol

2.2. Gene amplification through PCR and cloning of the genes

To construct the butyric acid pathway in plasmids, Butyryl-CoA dehydrogenase (BCD) and Butyryl-CoA: acetate CoA-transferase (BUT) were amplified through PCR from genomic DNA of *F. prausnitzii* A2-165. 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 bold) (5'-GAC GTT GGA TCC AGA T**AT G**GA TTT TAC TCT GTC CAA G-3'), and SalI, located next to the TGA stop codon (in bold) (5'-GCA GGA GCA ACA AGC GC**T GA**G TCG AC-3'), were used to amplify the BCD gene from the genomic DNA of the bacteria. To construct BUT expression vector, primers possessing the synthetic restriction enzyme sites NdeI, which contain 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 GC**T GA**G TCG AC-3') of the BCD gene, were used to amplify the BUT gene from *F. prausnitzii*'s genomic DNA. BCD was cloned into pACYC184 with a *cat* promoter and BUT was cloned into pBR322 with a *crp* promoter. In addition, to construct the BCD-BUT expression vector, BUT was cloned into BCD-pACYC184 by Gibson assembly. The expression of genes was performed in *E. coli* MG1655 and EcN.

2.3. Confirmation of BCD and BUT expression

The overexpression of BCD and BUT genes were confirmed with RT-PCR. Total RNA was purified from the bacteria with TaKaRa MiniBEST universal RNA extraction kit (Takara, Japan). The reverse transcription and PCR was subsequently performed using RNA to cDNA Eco DryTM premix (TaKaRa Bio, USA) with 1mg of RNAs as the templates. 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'-GGT GTA TGT AGA GCC ACA CG-5' for BUT. Expression levels of the BCD and BUT genes were calculated from the obtained threshold cycle numbers in RT-PCR (CFX96TM Real-Time System, Bio-Rad, USA)

2.4. Assays of BCD and BUT activities

For cell lysate preparation, frozen cells of *F. prausnitzii*, EcN, EcN-BCD, EcN-BUT and EcN-BCD-BUT (3 g 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 through sonication for 10 min. Cell debris was removed by centrifugation at 100,000 rpm for 30 min at 4°C. The protein content was determined by the Bradford assay with bovine serum albumin as a standard.

The cell lysates of EcN wild-type, EcN-BCD, EcN-BUT and EcN-BCD-BUT were tested with assay mixtures containing butyric acid, Acetyl-CoA, FAD and NAD^+.

2.5. *scpC* and *tesB* deletion

Propionyl-CoA:succinate CoA transferase (*scpC*) and acyl-CoA thioesterase II (*tesB*)

knockouts were introduced into *E. coli* MG1655 and EcN by tetracycline-inducible deletion (84). Linear DNAs including tetracycline resistant gene were produced by PCR amplification using primers in Table 5. The generated linear DNAs were transformed into *E. coli* MG1655 and EcN containing pKD46 after induction with 0.4% L-arabinose. The deletion mutants were selected on LB plates containing 20 μg/ml tetracycline. The gene deletions were confirmed with colony PCR using a confirmation primer. pKD46 was eliminated by heat treatment as descry previously (84). The resulting strains were transformed with BCD and BUT.

2.6. Analytical Methods

Cell growth during the cultivation was measured at 600 nm wavelength using UV-VIS spectrophotometer (Shimazu UV-1800m, Japan) and Microplate readers (TECAN Spark®, Switzerland). Sugar and SCFA concentrations were measured by high-performance liquid chromatography (HPLC, Agilent 1100 series, USA) with a UV detector (210 nm) and a refractive index detector. The analytical column used was the H^+ cation-exchange column (RezexTM ROA-Organic Acid, 300 × 7.8 mm, USA) with the temperature of the column set at 60℃. H_2SO_4 solution was used as eluent at 0.5 mM for sugars and 5 mM for SCFAs, and the flow rate was 0.7 ml/min.

2.7. DSS-induced colitis model

Male C57Bl/6J mice, 8 weeks of age, were given 2% dextran sulfate sodium salt (DSS) in drinking water for 8 days. The concentration of *E. coli* strains, MG1655, MG1655-

BCD-BUT, Nissle1917 and EcN-BCD-BUT was 10^9 CFU/ml in Phosphate buffered saline (PBS) (8.0 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4). Samples were administered 200 μL by oral gavage, starving 2 days prior to the induction of colitis and for 8 days thereafter. Mice were sacrificed after 10 day and the clinical parameters and pathology were evaluated.

2.8. Measurement of inflammatory cytokines

The levels of myeloperoxidase (MPO) and IL-6 in colonic tissues were measured using a mouse MPO ELISA kit (HK210, Hycult Biotechnology, The Netherlands) and a mouse IL-6 Quantikine ELISA kit (R&D Systems Inc., Minneapolis, MN), following the manufacturer's instructions. Every assay was performed in triplicates.

2.9. Tissue processing and histopathology

The colon was extracted from the peritoneum and was divided into proximal and distal portions. The proximal colon portion up to 1.5 cm from the ileocecal valve, the rectum portion up to 1.5 cm from the anal verge, and the colonic segments containing any gross polyps were fixed with phosphate buffered formalin and embedded in paraffin. The 5-mm sections were stained with hematoxylin and eosin (H&E).

The tumor incidence (%) was determined by the percentage of mice having more than one tumor. The classification of adenoma and adenocarcinoma was performed, and the depth of adenocarcinoma invasion in colonic tissues was specified as mucosa or submucosa invasion (85). Histological severity was assessed using the microscopic

damage score reflecting colonic epithelial damage and the depth of inflammatory cell infiltration as previously described (86). These parameters were evaluated by two researchers in a blinded manner.

Table 5. The primer for *scpC* and *tesB* deletion; tetracycline resistance sequence in boldface type

Deletion gene		**Primer sequence**
scpC	Forward primer	CGTAAACCTA ATTTCGTTGG TCATTAATCC CTGCGAACGA AGGAGTAAAA **TTAAGACCAC TTTCACATTT AAGTTG**
	Reverse primer	CCGCCACGAT GCTTCAGCAT ATTGCTGAAG ATCGTGACGG GACGAGTCA **CTAAGCATTGTCTCCTGTTAC**
tesB	Forward primer	TACTCAACTC ACTTTGGCTT GCTGCGGCAG CTTTGTTACT GGAGAGTTAT **TTAAGACCCAC TTTCACATTT AAGTTG**
	Reverse primer	AACAAGCACT GCAAAAAACA GCCGGACGGT TTTCACCTC C GGCTATTTTT **CTAAGCACTT GTCTCCTGTT TAC**

3. Results and discussion

3.1 Recombinant *E. coli* for butyric acid production

The butyryl-CoA dehydrogenase (BCD) and butyryl-CoA: acetate CoA transferase (BUT) genes from *F. prausnitzii* were cloned for butyric acid production under *cat* and *crp* promoter in pACYC184c and PBR322 vectors, respectively, and expressed in *E. coli* MG1655 and EcN. BCD converts crotonoyl CoA to butyryl CoA and BUT converts butyryl CoA to butyric acid (Figure 14). The constructed strains of BCD and BUT were shown in Figure 15. To confirm BCD and BUT gene expression, real-time RT-PCR was conducted, and relative expression levels after induction were determined by qPCR (Figure 16). The expression level of BCD was 2,401.9 ~ 3,590.6 fold and BUT was induced 245.3~612.4 fold compared to the wild-type cell. These gene expression levels proved that the butyric acid pathway genes were successfully transcribed.

The effect of *bcd* and *but* gene induction on the butyric acid accumulation in the medium was then observed (Figure 17). It shows that the butyric acid production by *E.coli* strains with the *bcd* gene was increased compared to the production by the wild-type strains. However butyric acid production by *E.coli* strains with the *but* gene was similar to that of the *E. coli* wild-type. In addition, butyric acid production by *E.coli*

strains with both *bcd* and *but* gene insertion increased slightly. Moreover, EcN strains were found to produce more butyric acid compared to MG1655 strains as it is already known that EcN produces SCFAs as end products of carbohydrate metabolism (87). Overall, EcN-BCD-BUT showed the highest butyric acid producing ability (295.16 mg/L) in this study. The accumulation of butyric acid from *F. prausnitzii* (355.12 mg/L) was higher than EcN-BCD-BUT, yet the incubation time of *F.prausnitzii*(42 h) was 4.2 times longer than EcN-BCD-BUT (10 h). Hence the efficiency of butyric acid production was higher in EcN-BCD-BUT (29.51 mg/L ·h**)** than in *F. prausnitzii* (8.45 mg/L ·h**)**.

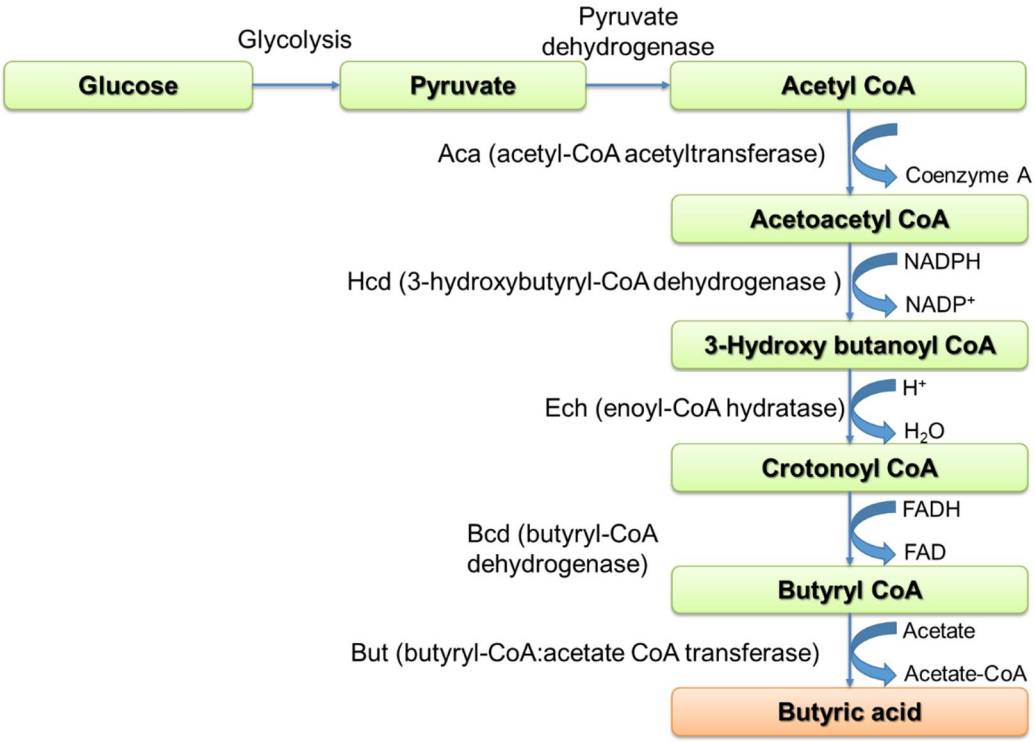

Glycolysis
Pyruvate dehydrogenase
Glucose
Pyruvate
Acetyl CoA
Aca (acetyl-CoA acetyltransferase)
Coenzyme A
Acetoacetyl CoA
Hcd (3-hydroxybutyryl-CoA dehydrogenase)
NADPH
NADP+
3-Hydroxy butanoyl CoA
Ech (enoyl-CoA hydratase)
H+
H2O
Crotonoyl CoA
Bcd (butyryl-CoA dehydrogenase)
FADH
FAD
Butyryl CoA
But (butyryl-CoA:acetate CoA transferase)
Acetate
Acetate-CoA
Butyric acid

Figure 14. Butyric acid production pathway of *F. prausnitzii*

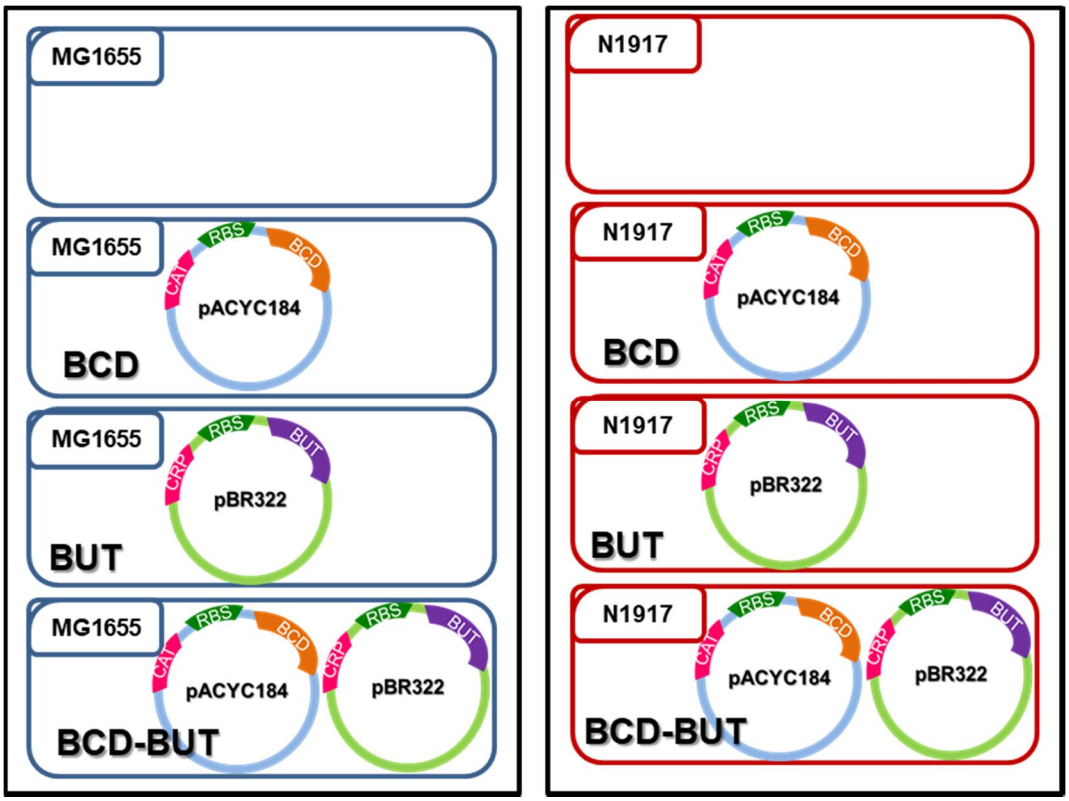


Figure 15. Engineered *E. coli* strains in this study

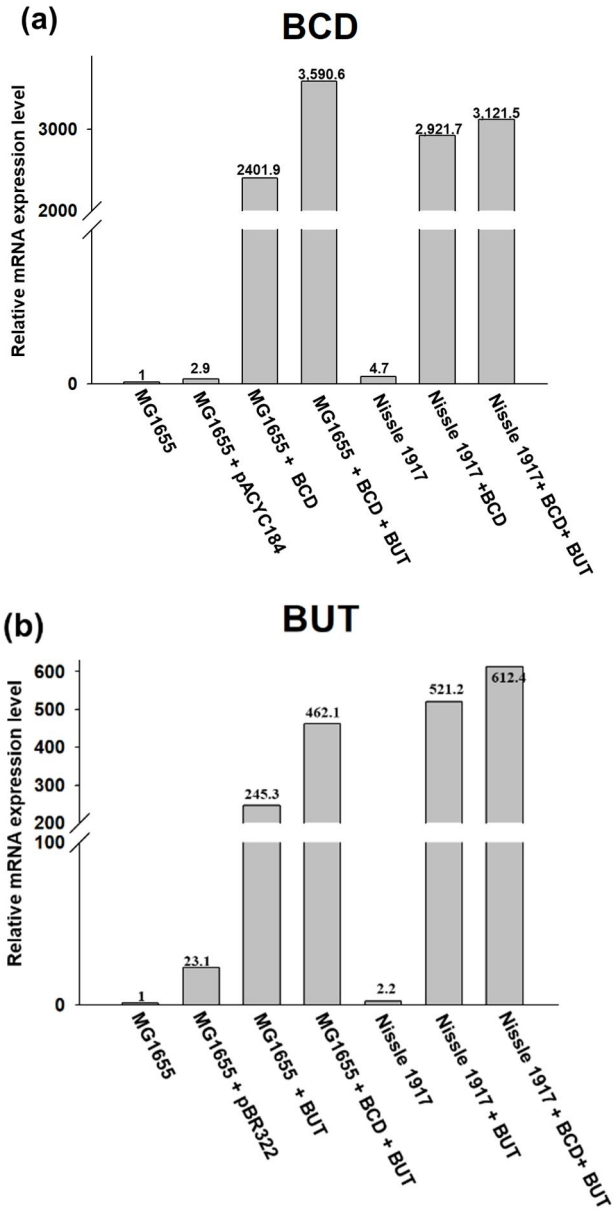


Figure 16. Expression levels of BCD and BUT genes in engineered *E. coli* strains (a) BCD and (b) BUT

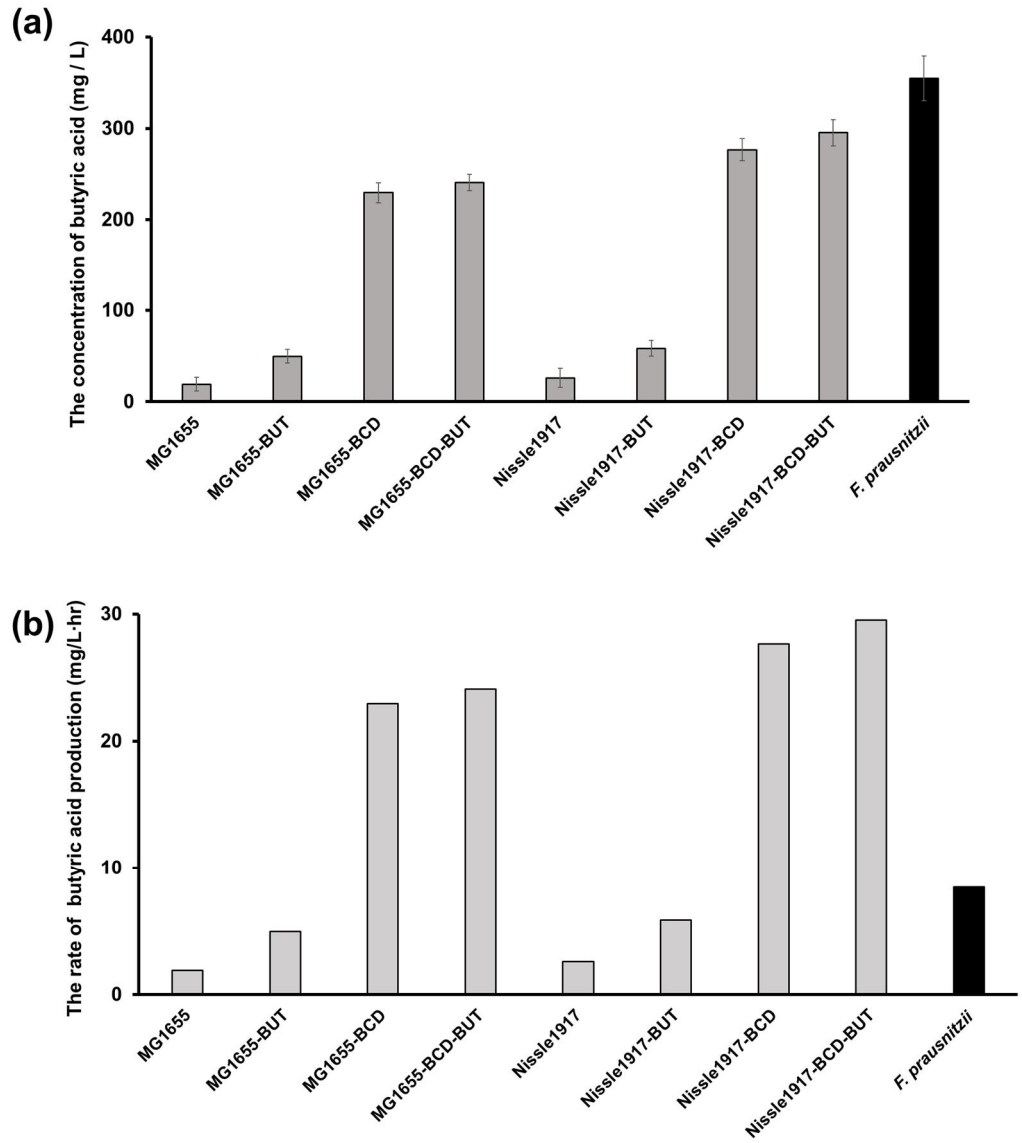


Figure 17. The butyric acid production by recombinant *E.coli* strains; (a) The butyric acid concentration in supernatants after culture (b) The butyric acid

production efficiency of engineered strains over time

3.2. Activity assay of BCD and BUT

To investigate the relative contribution of BCD and BUT to butyric acid production, we measured the NADH level through the reaction of FadB involved in the reverse pathway of butyric acid production in EcN-BCD-BUT (Figure 18). When the reverse pathway of butyric acid production proceeds, butyric acid is converted to crotonyl-CoA by BCD and BUT. After this reaction, NAD^+ is reduced to NADH while crotonyl-CoA is converted to acetoacetyl-CoA by FadB. It was confirmed that cell lysates of EcN wild-type, EcN-BCD and EcN-BUT did not catalyze the formation of NADH from NAD^+ (Figure 19(a)). However, NADH was formed from NAD^+ when BCD and BUT were present in addition to acetyl-CoA and FAD. To confirm that the reverse pathway of butyric acid production proceeded, an assay was carried out without acetyl-CoA or butyric acid and the results showed that reverse pathway of butyric acid had proceeded (Figure 19(b)).

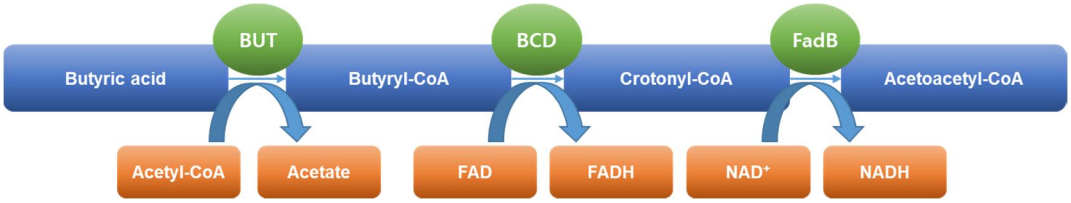


Figure 18. Scheme for the FadB-coupled assay of the BUT and BCD activities in the EcN strain carrying an expression vector for BCD and BUT.

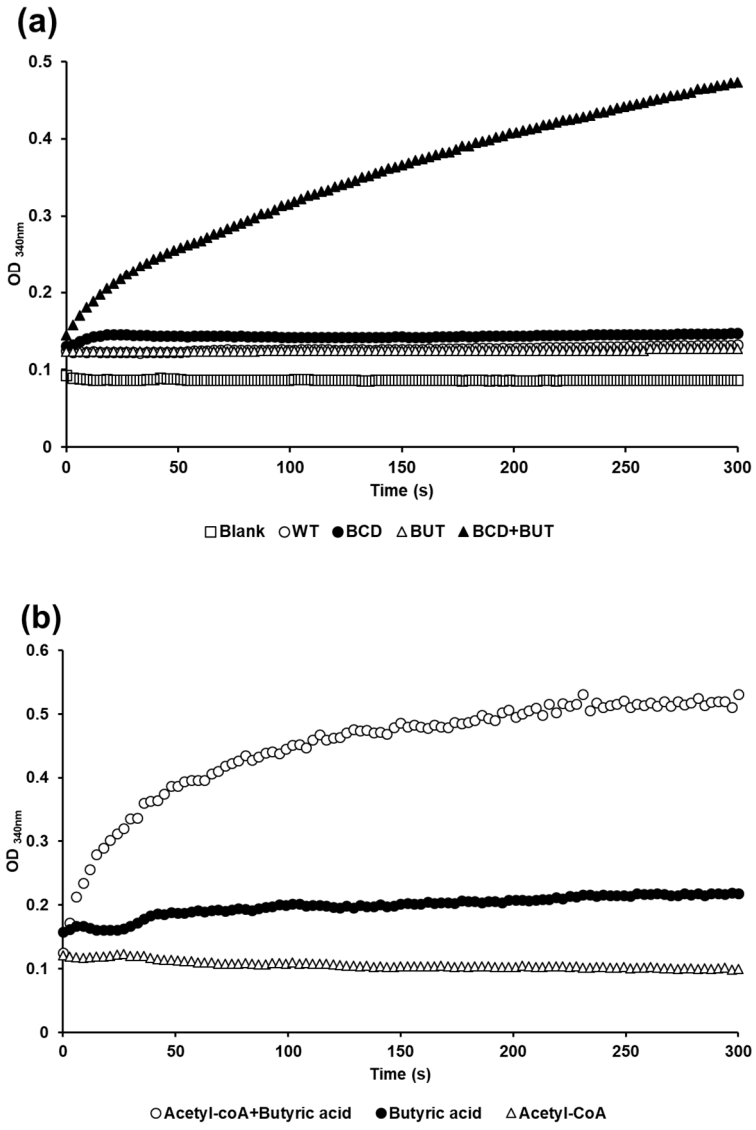


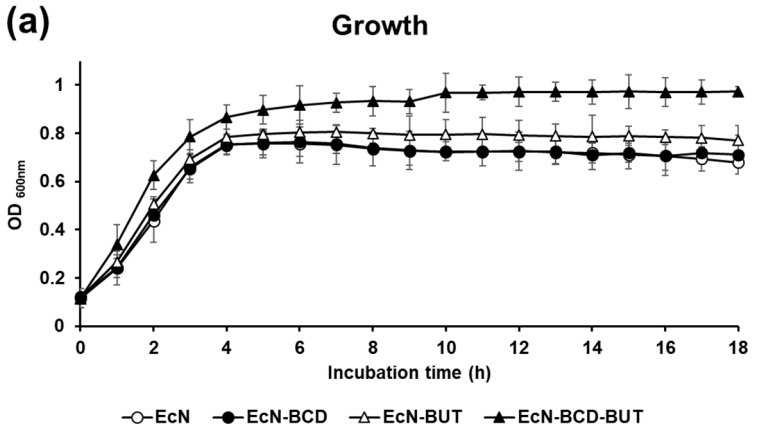
Figure 19. Protein activity test in engineered EcN with BCD and BUT; (a)depending on the protein, (b) depending on the substrate

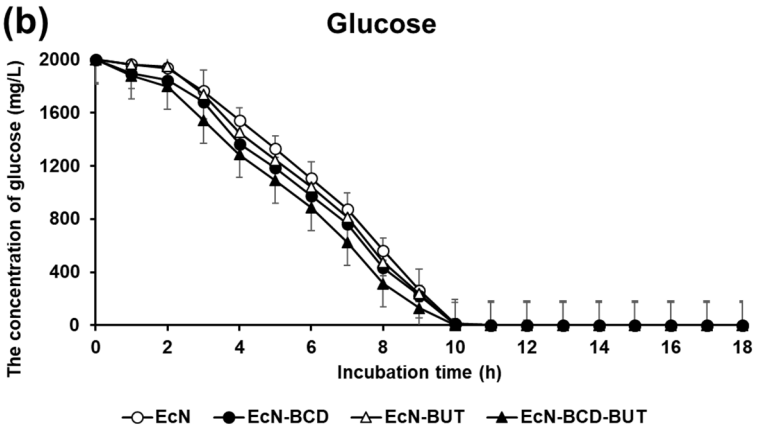
3.3. Butyric acid production by engineered *E. coli* in various sugar conditions

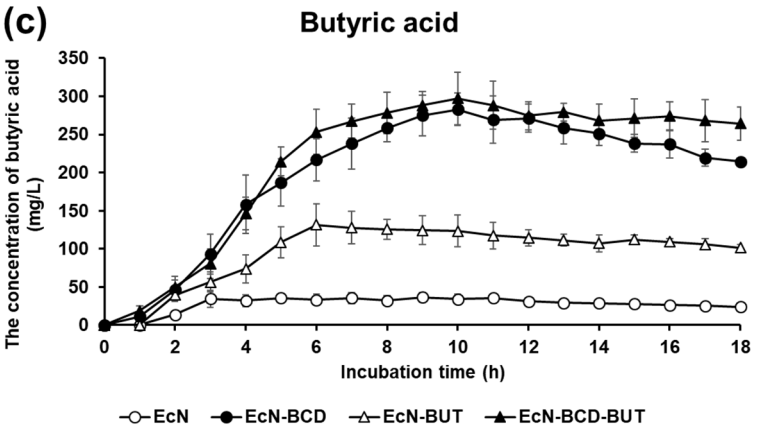
To evaluate the effects of BCD and BUT genes on EcN growth, glucose metabolism and SCFA fermentation, EcN, EcN-BCD, EcN-BUT and EcN-BCD-BUT were cultured in M9 medium containing 0.2% glucose (Figure 20). Time courses of EcN growth depending on the presence of BCD and BUT are shown in Figure 20 (a). EcN is capable of growing with glucose as the principal source of energy and all EcN strains reached an OD_{600nm} of 0.7 after 5 h of incubation. The growth did not increase after 6 h even though glucose was depleted after 10 h (Figure 20 b). Butyric acid production of EcN wild-type was 34.3 mg/L after 10 h while EcN-BCD-BUT produced 297.3 mg/L after 10 h, which is 8.6 times higher than EcN wild-type (Figure 20 c). It seemed that the butyric acid production of EcN-BCD and EcN-BCD-BUT correlated with cell counts. Although butyric acid production of EcN-BCD was slightly lower than that of EcN-BCD-BUT, butyric acid production was significantly improved compared to the wild-type. The acetic acid production also increased with growth. EcN wild-type produced 813.2 mg/L of acetic acid after 11 h and EcN –BCD-BUT produced 643.2 mg/L of the metabolite since acetic acid was used by BUT. In addition, after the depletion of glucose, acetate was consumed until 18 h.

The effects of glucose concentrations (0.2% ~ 2.0%) on growth and butyric acid production of EcN-BCD-BUT are shown in Fig. 21. The growth and acetic acid and butyric acid production of EcN-BCD-BUT were not significantly different in various glucose concentrations, implying that 0.2% glucose is enough for EcN-BCD-BUT growth and butyric acid production.

Mucins are present in human intestinal epithelial cells and consist of oligosaccharides containing N-Acetylglucosamine and N-acetylgalactosamine that act as nutrients for microbiota. Inulin is considered to be widely present in various plants because it exists in more than 3,000 vegetables and belongs to fructans which are a group of non-digestible carbohydrates (88). Inulin is resistant to digestion in the human small intestine due to the composition of anomeric C-2, yet is fermented in the large intestine. To evaluate the effect of sugar in the large intestine on EcN-BCD-BUT growth and acetic acid and butyric acid production, EcN-BCD-BUT was cultured in M9 containing N-acetylglucosamine (NAG), N-acetylgalactosamine (NAGA) and inulin (Figure 22). The growth analysis showed that there is no difference between glucose and NAG while the growth rate in NAGA was lower than in glucose. In addition, the growth result in inulin showed that EcN-BCD-BUT appeared to be unable to use. The butyric acid yield from NAG was similar to that from glucose and this result could be correlated with growth. The final concentration of butyric acid production in NAGA was lower than in glucose and butyric acid production rate was also lower than glucose as the rate of acetic acid production of EcN-BCD-BUT in NAGA was slower than in glucose. The acetic acid and butyric acid production from inulin were significantly lower than glucose because inulin was not utilized and glycolysis did not proceed. From these results, it was confirmed that EcN-BCD-BUT could produce sufficient butyric acid in the human intestine with NAG.


(a)
Growth
OD 600nm
Incubation time (h)
EcN
EcN-BCD
EcN-BUT
EcN-BCD-BUT


(b)
Glucose
The concentration of glucose (mg/L)
Incubation time (h)
EcN
EcN-BCD
EcN-BUT
EcN-BCD-BUT


(c)
Butyric acid
The concentration of butyric acid (mg/L)
Incubation time (h)
EcN
EcN-BCD
EcN-BUT
EcN-BCD-BUT

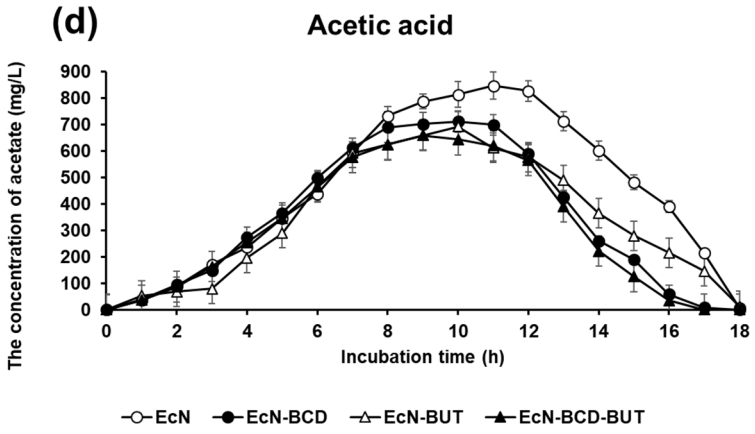
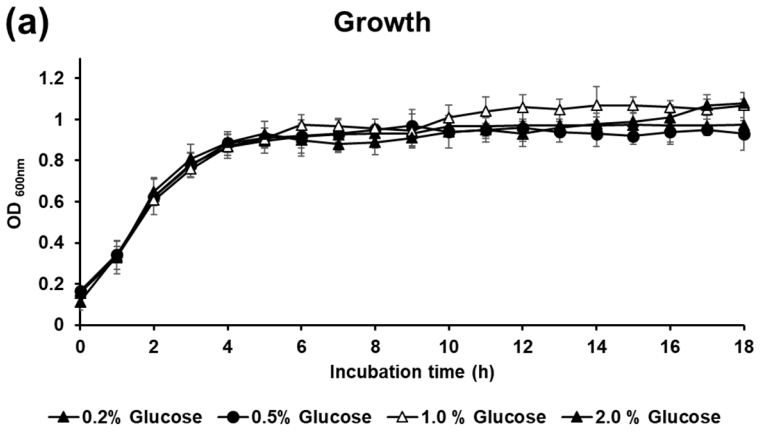
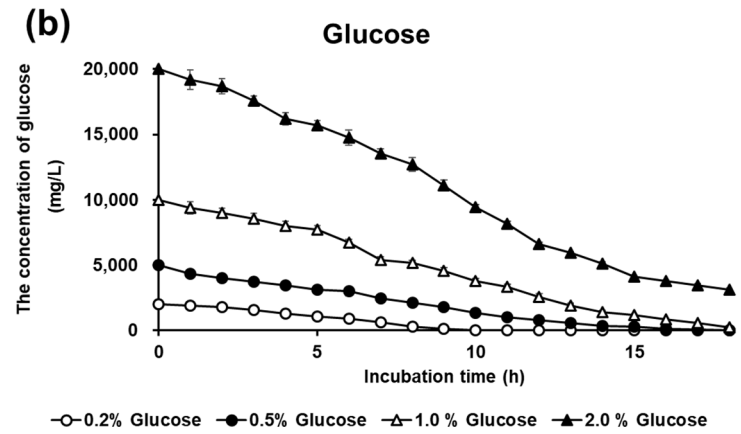
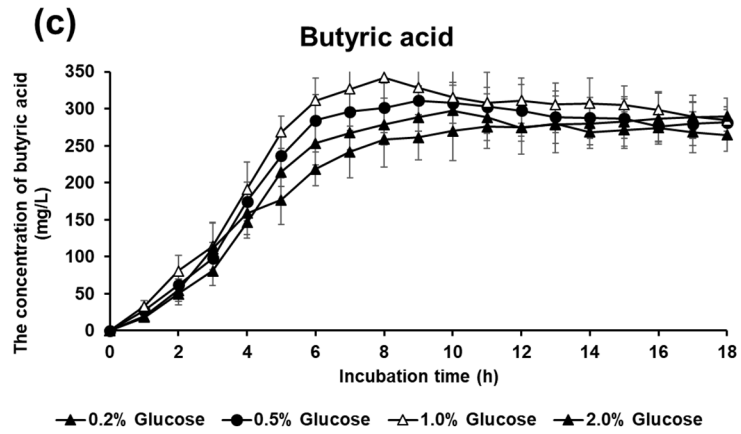


Figure 20. Butyric acid production by recombinant *E.coli* strains; EcN was transformed with BCD and BUT; (a) Cell growth, (b) Glucose consumption, (c) Butyric acid production, (d) Variation of acetic acid


(a)
Growth
OD 600nm
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
18
Incubation time (h)
0.2% Glucose
0.5% Glucose
1.0 % Glucose
2.0 % Glucose


(b)
Glucose
The concentration of glucose (mg/L)
20,000
15,000
10,000
5,000
0
0
5
10
15
Incubation time (h)
0.2% Glucose
0.5% Glucose
1.0 % Glucose
2.0 % Glucose


(c)
Butyric acid
The concentration of butyric acid (mg/L)
350
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
16
18
Incubation time (h)
0.2% Glucose
0.5% Glucose
1.0% Glucose
2.0% Glucose

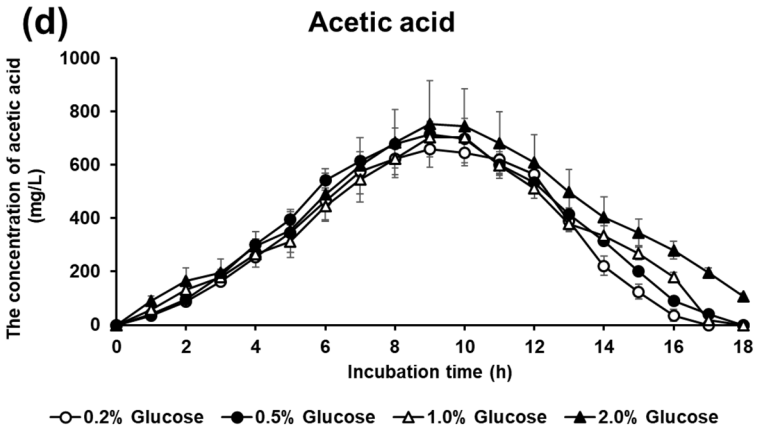


Figure 21. The effect of glucose concentration on growth and butyric acid production of EcN-BCD-BUT; (a) Cell growth, (b) Glucose consumption, (c) Butyric acid production, (d) Variation of acetic acid

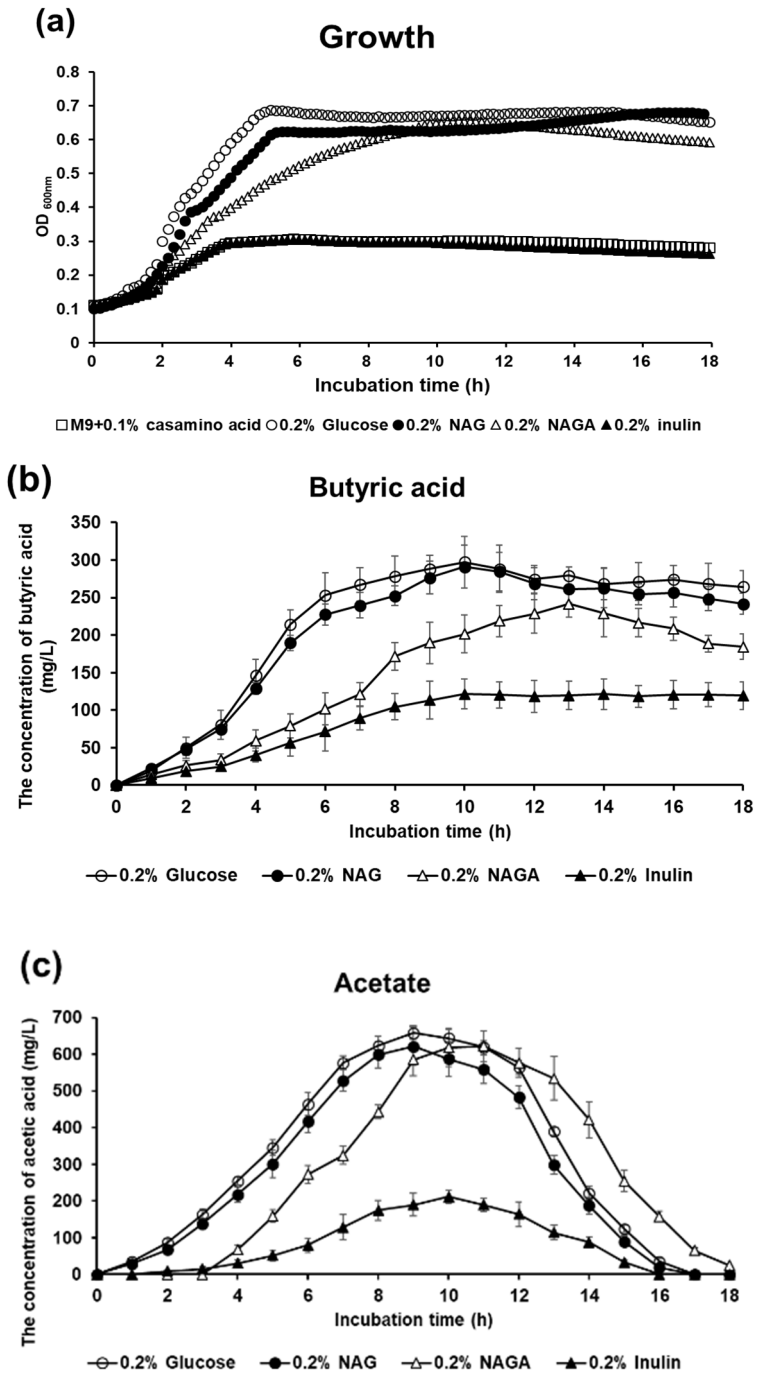


Figure 22 The effect of sugar on growth and butyric acid production of EcN-BCD—

BUT; (a) cell growth, (b) butyric acid production, (c) variation of acetic acid

3.4. Butyric acid production in *scpC* and *tesB* deletion strains

The butyric acid production level of EcN-BUT was not higher than EcN. We hypothesized that *scpC* and *tesB* could substitute BUT in converting butyryl CoA to butyric acid. ScpC works in the metabolic pathway to produce succinyl-CoA from propanoyl-CoA and this metabolic function is similar to BUT in that it transfers the CoA group to another molecule. TesB was known to effectively catalyze butyryl-CoA to butyric acid (Figure 23). As expected, butyric acid produced by Δ*scpC* and Δ*tesB* strains was reduced to 12 %(MG1655-ΔscpC) ~ 53 % (EcN-ΔtesB-BCD) compared to the non-deletion type of EcN (Figure.24). As a result, ScpC and TesB were able to partially replace the function of BUT and TesB was more efficiency that it could catalyze the production of butyric acid from butyryl CoA.

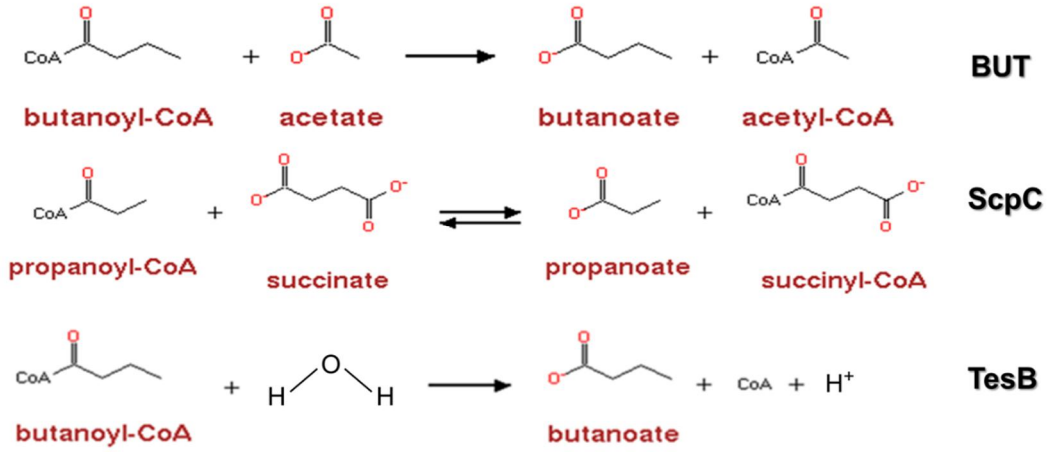


Figure 23. The chemical reactions of BUT, *scpC* and *tesB*;

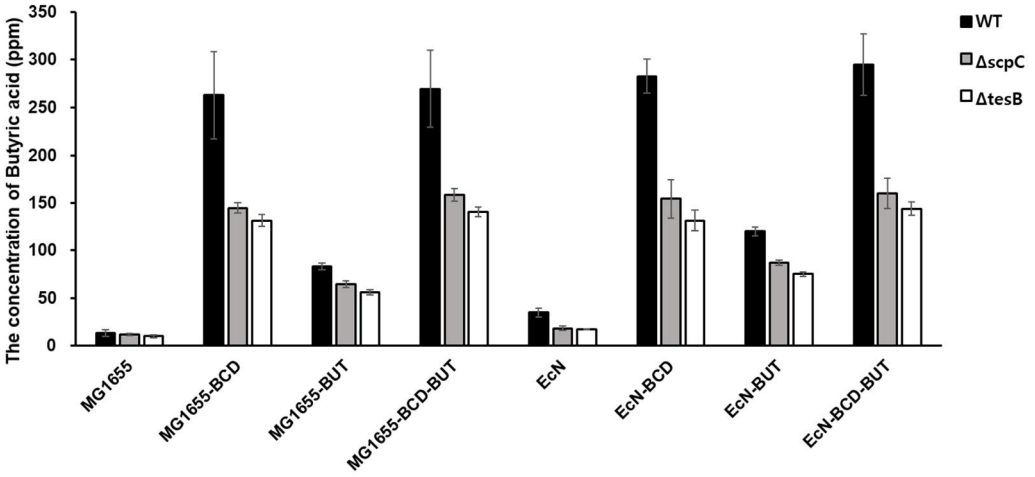


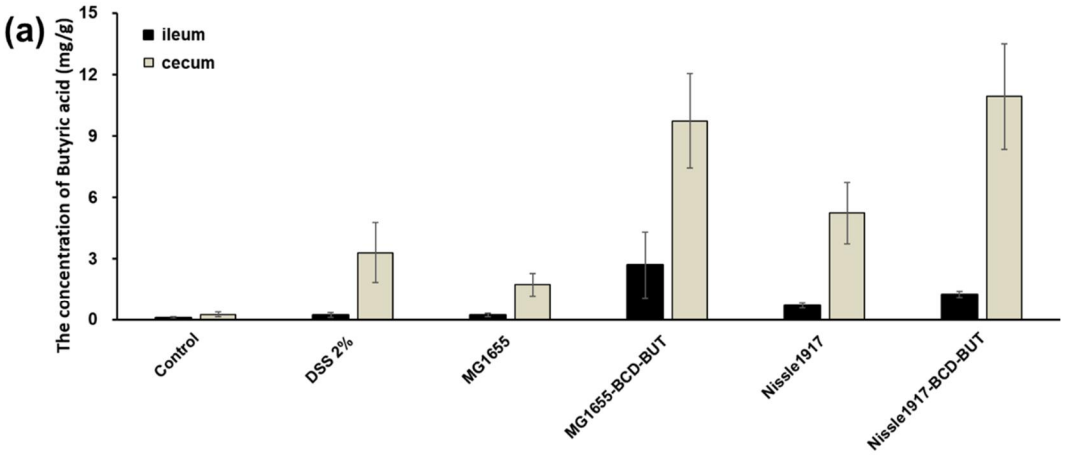
Figure 24. The butyric acid productions of deletion strains; Black bar (■) indicates the wild-type, grey bar shows the *scp* ( ) deletion mutant and white bar (□) shows the -*s* deletion mutant

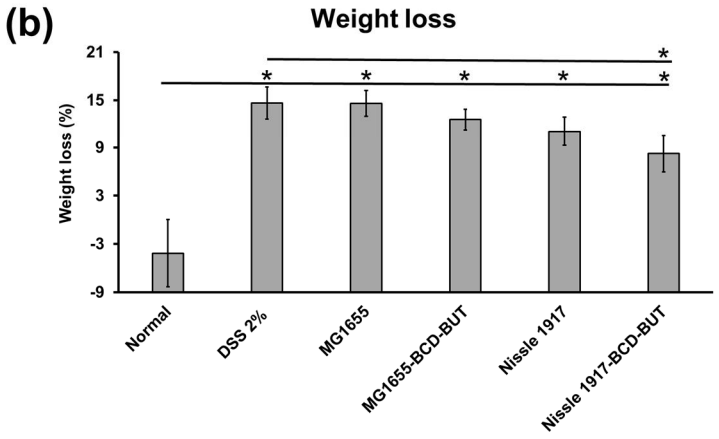
3.5. Anti-inflammatory activity by DSS-induced colitis model

Based on butyric acid production results, MG1655-BCD-BUT and EcN-BCD-BUT were tested for their anti-inflammatory activities with MG1655 and EcN as controls, respectively. The effect of oral administration of these strains was tested in DSS-induced colitis model mice. DSS (Dextran Sulfate Sodium Salt) is a physical agent that destroys epithelial barriers causing acute clinical symptoms when given orally. As shown in Figure 25 (a), low level of butyric acid in the ileum is related to the low number of *E. coli* residing in ileum opposed to its major habitat, cecum. The concentration of butyric acid in cecum content increased significantly in the treatment groups with MG1655-BCD-BUT (9.73 mg/g) and EcN-BCD-BUT (10.92 mg/g) compared to wild-type group (MG1655: 3.31 mg/g, EcN: 5.24 mg/g). The body weight of mice significantly decreased during DSS treatment after 7 days compared to the normal mice (Figure. 25 (b)). Although treatment with E. coli MG1655 was not effective in weight loss, treatment with MG1655-BCD-BUT could contribute to weight loss. In addition, EcN-BCD-BUT group showed a decrease in weight loss compared to EcN group, indicating that the butyric acid is effective in weight gain. A reduction in colon length at day 7 in the DSS group was also observed. Nevertheless, oral treatment with EcN-BCD-BUT led to the restoration of colon length. The cytokine profile in colonic tissues was assessed to determine that EcN could be performed more efficiently due to butyric acid production (Figure 26). Oral administration of MG1655-BCD-BUT, EcN and EcN-BCD-BUT decreased the levels of IL-6 compared to DSS group (Figure 26(a)). Although the butyric acid helped to lower down the IL-6 level in MG1655 strains, IL-6 level in EcN was reduced by other metabolites such as salicylic acid, shikimic acid etc. rather than the butyric acid were

secreted from the EcN. Therefore, groups administered with EcN or EcN-BCD-BUT didn't show the difference in IL-6 level. In addition, EcN-BCD-BUT increased the level of TNF-α compared to EcN (Figure 26(b)). However, MG1655-BCD-BUT was the most effective treatment for myeloperoxidase (MPO) level, implying that butyric acid was effective on MPO (Figure 26(c)).

Next, the ability of engineered *E.coli* to prevent DSS-induced colonic damage was evaluated at the histological level. The colon sections of control mice display an intact epithelium, a well-defined crypt length, and no neutrophil infiltration in the mucosal and submucosal layers (Figure 27(a)). In contrast, colon tissues from DSS-treated mice showed severe inflammatory lesions throughout the mucosa and submucosa (Figure 27(b)). Oral administration of MG1655 did not decrease the damage from DSS. However, MG1655-BCD-BUT showed improvements from DSS damage (Figure 27 (c),(d)). EcN strains, especially EcN-BCD-BUT, ameliorated the histological damage after 7days (Figure 27 (e),(f)). Histological scores show that EcN has a better anti-inflammatory effect and that butyric acid further increases the anti-inflammatory effect (Figure 27(g)).


(a)
ileum
cecum
The concentration of Butyric acid (mg/g)
15
12
9
6
3
0
Control
DSS 2%
MG1655
MG1655-BCD-BUT
Nissle1917
Nissle1917-BCD-BUT


(b)
Weight loss
Weight loss (%)
21
15
9
3
-3
-9
*
Normal
DSS 2%
MG1655
MG1655-BCD-BUT
Nissle 1917
Nissle 1917-BCD-BUT

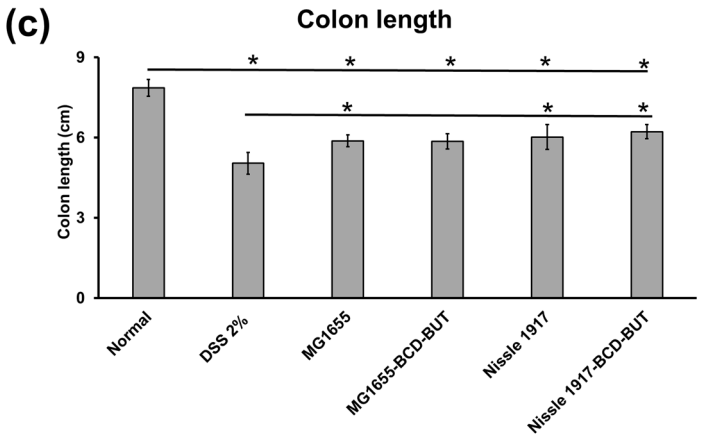
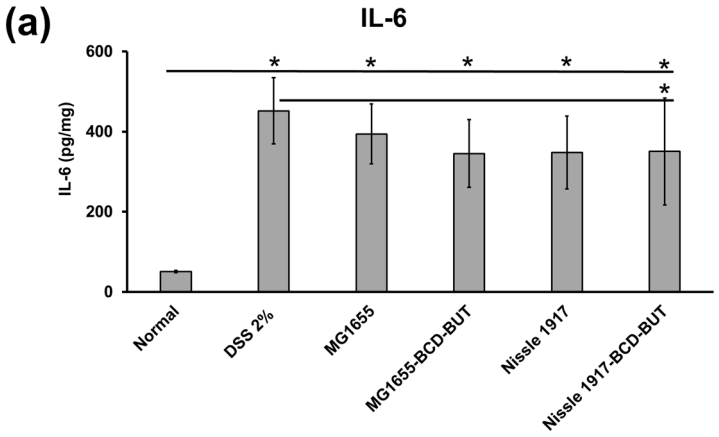
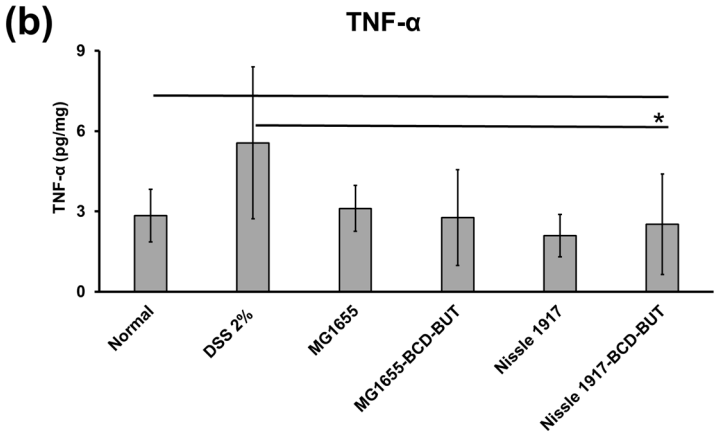

(c)
Colon length
Colon length (cm)
9
6
3
0
*
Normal
DSS 2%
MG1655
MG1655-BCD-BUT
Nissle 1917
Nissle 1917-BCD-BUT

Figure 25. Oral administration of engineered *E.coli* strains improved anti-inflammatory effect; (a) The amount of butyric acid in ileum and cecum (b) Body weight loss, (c) colon length, *: $p < 0.05$


(a)
IL-6
IL-6 (pg/mg)
600
400
200
0
Normal
DSS 2%
MG1655
MG1655-BCD-BUT
Nissle 1917
Nissle 1917-BCD-BUT


(b)
TNF-α
TNF-α (pg/mg)
9
6
3
0
Normal
DSS 2%
MG1655
MG1655-BCD-BUT
Nissle 1917
Nissle 1917-BCD-BUT

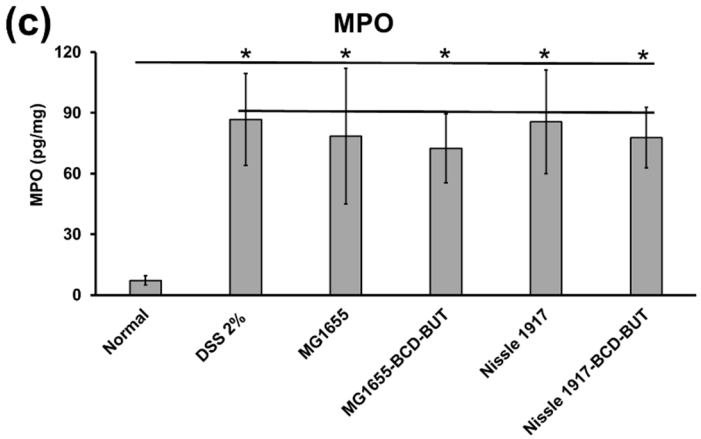
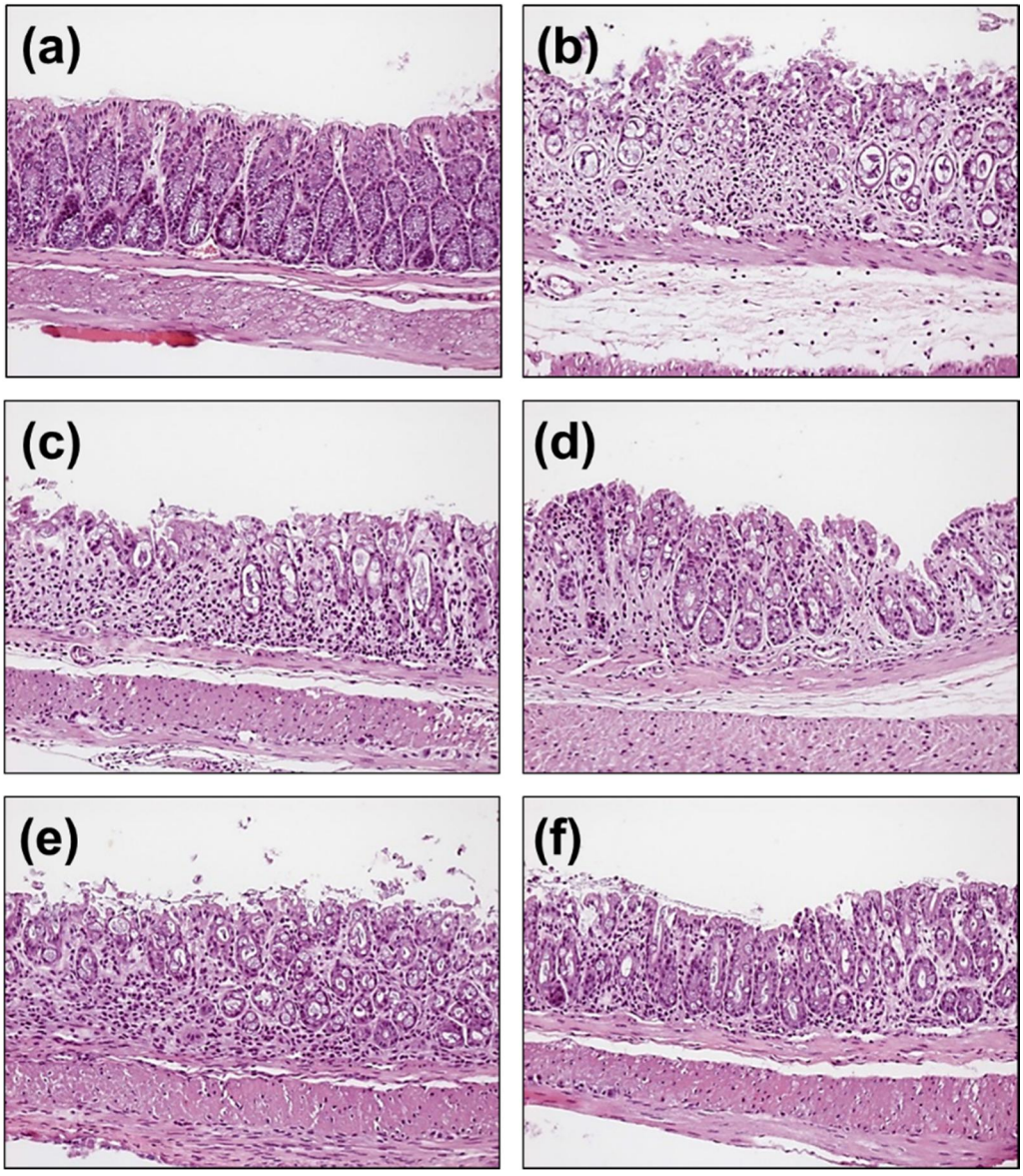

(c)
MPO
MPO (pg/mg)
120
90
60
30
0
Normal
DSS 2%
MG1655
MG1655-BCD-BUT
Nissle 1917
Nissle 1917-BCD-BUT

Figure 26. Effect of engineered *E.coli* on cytokine production by colonic cells; (a) Interlukin-6 (IL-6), (b) Tumor necrosis factor alpha (TNF-α), (c) Myeloperoxidase (MPO) *: $p < 0.05$


(a)
(b)
(c)
(d)
(e)
(f)

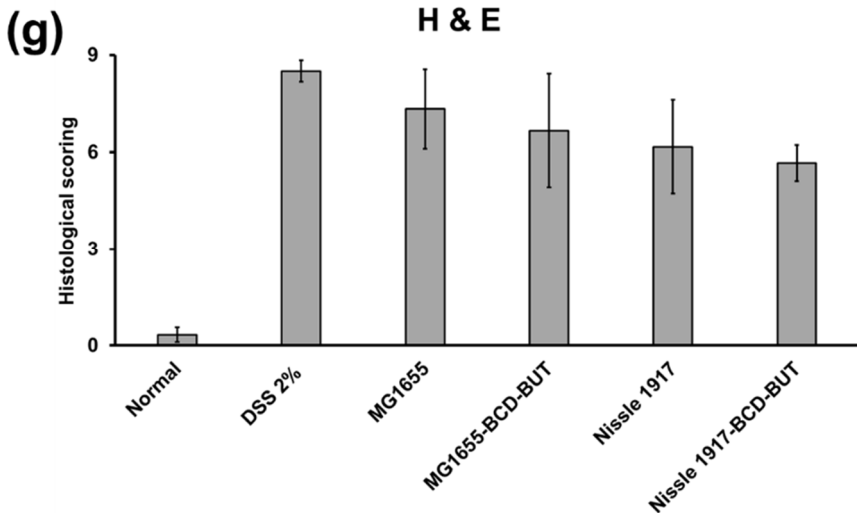


Figure 27. Oral administration of engineered *E.coli* prevented histological damage in the DSS-induced colitis mouse model; (a) Normal, (b) DSS, (c) *E.coli* MG1655, (d) *E.coli* MG1655-BCD-BUT, (e) EcN, (f) ECN-BCD-BUT, (g) Histological scores of colon sections of DSS-colitis mice with or without oral administration of engineered *E.coli.*

4. Conclusion

In this chapter, the effect of butyric acid production of engineered *E.coli* on anti-inflammatory was examined. Overexpression of *F. prausnitzii* BCD could significantly increase the butyric acid production in *E. coli*. In addition, co-expression of BUT with BCD could improve, but not absolutely required for, the butyric acid production. These butyric acid pathway genes were amplified from *E.coli* MG1655 and Nissle1917. The *E.coli* Nissle1917 strains produced more butyric acid than *E.coli* MG1655 strains.

When EcN-BCD-BUT was cultured in M9 with 0.2 % glucose, the butyric acid was produced optimally and a higher concentration of glucose did not induce a significant change. The EcN-BCD-BUT in N-acetylglucosamine present in human large intestine showed a similar level of butyric acid production with glucose and N-acetylgalactosamine. However, inulin was not effective in butyric acid production.

Further engineering was conducted in both types of strains to find out the enzyme that can convert butyric acid from butyryl-CoA in *E.coli* by deleting *scpC* and *tesB* genes. These genetic modifications showed that BUT, *scpC* and *tesB* genes were found to be partially responsible for the conversion process from butyryl-CoA to butyric acid.

In the previous research, a probiotic strain EcN has shown anti-inflammatory activity in DSS-induced colitis model (89). Our results suggest that the anti-inflammatory effect differs by different anti-inflammatory effectors induced by various metabolites produced by EcN and genetically engineered EcN-BCD-BUT. Yet, metabolites of EcN need further studies (Figure 28).

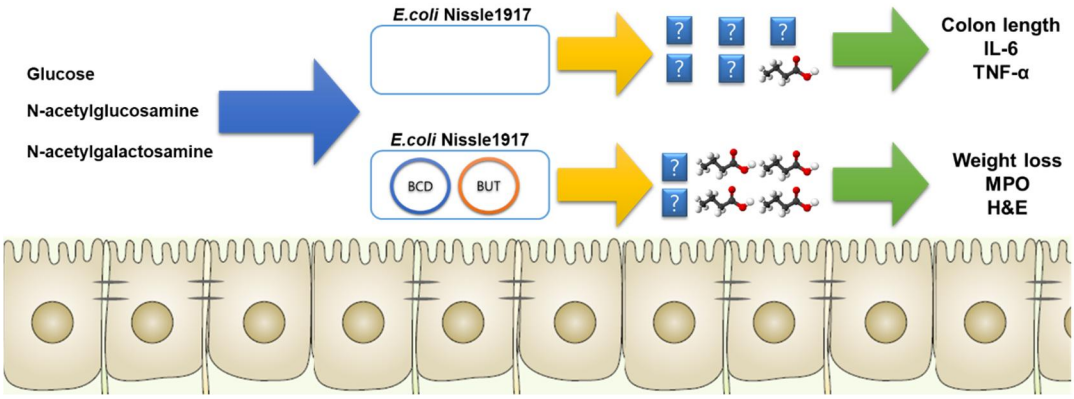


Figure 28. The assumed anti-inflammatory effects of metabolites produced by EcN and engineered EcN in the gut. When wild-type Nissle1917 strain and Nissle1917 carrying a plasmid with *bcd* and *but* genes are injected in the human large intestine, these bacteria will grow by consuming glucose, NAG and NAGA and generate various metabolites. It was confirmed that butyric acid could be produced in a greater amount by the engineered strain in comparison to the wild-type strain, possibly explaining the reduction in weight loss, and the level of MPO and H&E.

Chapter IV

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국문 초록

인간 장내 미생물 중 가장 높은 점유율을 차지하고 있는 대표 마이크로비오타의 한 종류인 *F. prausnitzii*는 장내 건강과 밀접한 관련이 있고 이는 *F. prausnitzii*의 대사 산물에 의하여 항 염증 효과 때문인 것으로 알려져 있다. 대표적으로 알려진 대사산물로는 뷰티르산이 있으며 이는 인간의 장 건강에 유익한 효과를 주는 것으로 알려져 있다. 하지만 이렇게 유용한 미생물임에도 불구 하고 *F. prausnitzii*는 극혐기성 박테리아로서 산소에 극도로 민감하여 이 미생물의 생리적 특성과 관련된 연구는 거의 발표되지 않았다. 본 연구에서는 *F. prausnitzii*의 생리학적 특성을 조사하기 위해 다양한 종류의 당에서의 성장을 시험 한 결과, 글루코스가 존재하는 조건에서 가장 잘 성장하는 것

으로 확인되었다. 글루코스, 프록토스, N-아세틸글루코사민이의 조건에서 *F. prausnitzii*의 뷰티르산 생산은 성장도와 비례하여 글루코스 조건에서 최적 생산량 (355.66 ± 21.4 mg/L)을 보이고 있다. *F. prausnitzii*에서 뷰티릭산 생산에 관여하는 효소인 Butyryl-CoA dehydrogenase (BCD)와 Butyryl-CoA : Acetate CoA-transferase (BUT)를 코딩하는 유전자를 클로닝하여 대장균에서 발현시켜 장내 건강에 미치는 뷰티르산 생산의 효과를 확인 하였다. 두 개의 유전자는 cat 프로모터의 제어하에 개별적으로 또는 함께 플라스미드 pACYC184 및 pBR322에 클로닝하였다. 야생형과 BCD, BUT 각각 혹은 두가지 유전자가 모두 발현하는 대장균에서의 뷰티르산 생산 결과에 의하면 뷰티르산의 생산을 위해서는 BCD 유전자는 필수적으로 필요하였지만 BUT의 경우에는 필수적이지 않은 것으로 나타났다. 당에 의한 뷰티릭산 생산량을 비교하기 위하여 글루코스, 프록토스, N-아세틸글루코사민, N-아세틸갈락토사민 그리고 이눌린 조건에서 실험한 결과 뷰티릭산 생산을 위한 최적 조건은 글루코스을 주입하였을 때 였으며 인간 장내에 존재하는 N-아세틸글루코사민도 뷰티릭산 생산에 사용될 수 있음을 확인하였다. 뷰티르산 생성을 통한 항염증 효과를 확인하기 위해서 DSS-유도 대장염 모델 마우스에 이들 균주를 투여하여 시험 하였고 *E. coli* Nissle 1917-BCD-BUT를 경구 투여하였을 때 대장염에 의해 유발된 장 손상을 방지하는 것을 확인하였다. .

주요어 : *Faecalibacterium prausnitzii*, 당, 뷰티릭산, 대장균, Butyric acid, *E.coli*, DSS 유도 대장염 모델

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