SYNTHETIC BIOLOGY APPROACH FOR THE DESIGN OF BUTYRATE PRODUCING BACTERIA FOR COLON CANCER THERAPY

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SYNTHETIC BIOLOGY APPROACH FOR THE DESIGN OF BUTYRATE PRODUCING BACTERIA FOR COLON CANCER THERAPY

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ABSTRACT

SYNTHETIC BIOLOGY APPROACH FOR THE DESIGN OF BUTYRATE PRODUCING BACTERIA FOR COLON CANCER THERAPY

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Master of Science, Biology
Supervisor: Prof. Dr. Sreeparna Banerjee

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Butyric acid is a short chain fatty acid (SCFA) that is generated in the colon by butyrate-producing bacteria. Butyrate, along with other SCFAs, serve as substrates for energy production in the gut epithelium. Additionally, these compounds are known to reduce inflammation and enhance differentiation of mammalian cells. Several species of bacteria are known to produce butyrate in the gut through different biochemical pathways. In this study we have aimed to generate a butyrate producing commensal *E. coli* K12 strain that is known to be non butyrogenic. For this, we have used a strain in which fermentation related pathways have been deleted (ΔadhE, Δldha, ΔackA-pta, ΔfrdC) while enzymes for the reversal of β-oxidation (AtoB, FadB, FabI) and butyrate production [Butyryl-CoA: Acetate-CoA transferase (ButCoAT)] have been overexpressed. The *E. coli* K12 strain engineered with these alterations was capable of synthesizing mM amounts of butyrate.

Caco-2 colon cancer cell line was incubated with a 1:1(v/v) mixture of conditioned medium generated from the engineered *E. coli* K12 strain and culture medium. A strong activation of the Mitogen Activated Protein Kinase (MAPK) family protein p38 and its downstream target MAPKAPK2 (MK2) was observed in cells treated with the conditioned medium compared to controls. The MAPK pathway orchestrates several

regulatory mechanisms in epithelial cells. Our study suggests that engineered bacteria can be used to regulate gene expression in the gut.

Keywords: ButCoAT, E. coli, MAPK, colon

ÖZ

KOLON KANSERİ TEDAVİSİNDE BÜTİRAT ÜRETEN BAKTERİ TASARIMINA SENTETİK BİYOLOJİ YAKLAŞIMI

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Bütirik asit, kısa zincirli yağ asidi olup kolonda bulunan bütirat üreten bakteriler tarafından üretilebilir. Bütirat, diğer kısa zincirli yağ asitleriyle birlikte bağırsak epitelinde enerji üretimi için sübstrat olarak kullanılmaktadır. Ek olarak, bu bileşiklerin inflamasyonu azalttığı ve memeli hücrelerinin farklılaşmasını arttırdığı bilinmektedir. Bağırsakta bütirat üreten birçok mikro-organizmanın varlığı ve üretim için kullandıkları yolaklar bilinmektedir. Bu çalışmada, bütirojenik olmayan kommensal *E. coli* K12 suşu ile bütirat üretimi yapmayı hedefledik. Üretim için, fermantasyonla ilişkili yolakların genomdan silindiği (ΔadhE, Δldha, ΔackA-pta, ΔfrdC), tersinir β-oksidasyon (AtoB, FadB, FabI) ve bütirat üretimi için gereken [Bütiril-KoA: Asetat KoA transferaz (ButCoAT)] enzimlerinin aşırı ifadelendiği suş kullandık. Bu modifikasyonlarla tasarlanan *E. coli* K12 suşu mM miktarlarında bütirat üretebilmektedir.

Caco-2 kolon kanseri hücre hattı, tasarlanan *E. coli* K12 suşunun büyütüldüğü koşullandırılmış besiyeri ile kültür besiyerinin hacimce 1:1 oranında karışımı ile inkübe edildi. Kontrol grupları ile karşılaştırıldığında, koşullandırılmış besiyeri ile muamele edilen hücrelerde Mitojenle Aktive Edilen Protein Kinaz (MAPK) yolağı proteini olan p38 ve sonraki basamağında yer alan MAPKAPK2 (MK2)'nin güçlü aktivasyonu gözlenmiştir. MAPK yolağı, epitel hücrelerinde çeşitli düzenleyici

mekanizmalarda görev almaktadır. Çalışmamız, tasarladığımız bakterinin bağırsakta gen ifadelenmesini düzenlemek için kullanılabileceğini göstermektedir.

Anahtar Kelimeler: ButCoAT, E. coli, MAPK, kolon

To my devoted family

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CHAPTER 1

INTRODUCTION

1.1. Microbiota

Microbiota is the compilation of all taxa consisting of microbial elements such as bacteria, archaea, fungi and protists. Therefore, ‘the gut microbiota’ or ‘the oral microbiota’ refer to all microbes in the intestinal tract and oral cavity, respectively (Knight et al. 2017).

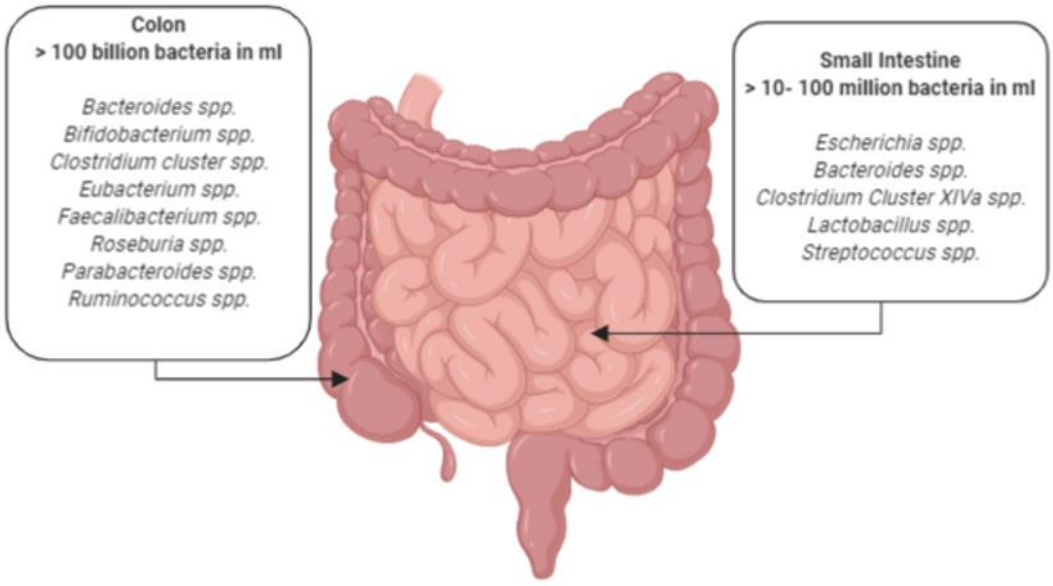


Figure 1. Density of bacterial species through the gastrointestinal cavity based of 16S RNA sequencing analysis (Rivière et al. 2016).

Microbiome, on the other hand, refers to the microorganisms themselves and is comprised of protozoa, archaea, eukaryotes, viruses and predominantly bacteria that

inhabit symbiotically on and within the human body. Oral, genital, gastrointestinal cavity and respiratory tract can be listed as examples of organs where the microbiome occupies the most (Lloyd-Price, Abu-Ali, and Huttenhower 2016). Further, approximately a ratio of 1:1 of microbial cells to human cells has been estimated in the colon, which is the organ most dense with microbes (Sender, Fuchs, and Milo 2016) (Figure 1).

Contributions of the microbiome and microbiome-host interaction were ill-defined through the past decades due to limitations in sequencing technology, inherent complexity and heterogeneity of the human microbiome, non-cultivable microbes and lack of population-wide descriptions of the composition of the microbiota.

With the advancement in sequencing technologies and large-scale sequencing, the Human Microbiome Project and Metagenomics of the Human Intestinal Tract (Meta-HIT) funded by The United States National Institutes of Health (NIH) and European Commission was initiated, which enabled population-based sequencing and facilitated research on the microbiome. The Whole Genome Shotgun (WGS) method ensures that reference genome mapping, meta-genomic assembly, metabolic reconstruction and gene cataloging are more convenient. Analyses with 16S ribosomal RNA sequencing, on the other hand, provide taxonomic characterization of microbiome based on the extraction of DNA and the amplification of 16S ribosomal RNA (Kho and Lal 2018). 16S rRNA sequencing has become the most useful technique to highlight diversity and abundance of the microbiome. The 16S rRNA gene sequences can be exploited with polymerase chain reaction (PCR) and metagenomics sequencing to characterize different microbial strains (Rinninella et al. 2019).

1.2. Prebiotics

Prebiotics, according to a definition by the International Scientific Association for Probiotics and Prebiotics (ISAPP), are fermentable elements in the diet that bring about a variety of changes in the microbiota composition or microbial activity for homeostasis. Thus, the use of prebiotics is often associated with recuperative effects on the host when administered (Gibson, et al., 2004). Prebiotics are found in several foods groups that are rich in carbohydrates (Gibson et al. 2010) and polyphenols (Bindels et al. 2015). Compounds should comply with the following in order to be classified as prebiotics:

1. Can be absorbed by gastrointestinal cells, hydrolyzed by the hosts digestive system and are resistant to gastric acidity.
2. Can be fermented by the gut microbiota.
3. Plays a role in stimulating growth or triggering activity of intestinal bacteria to stabilize homeostasis (Gibson et al. 2004).

Carbohydrates such as inulin-type fructans (ITF), galacto-oligosaccharides, isomalto oligosaccharides, lactulose, xylose and arabinose are counted as prebiotics (Rivière et al. 2016). ITF, for example, is used by colon bacteria, such as *E. rectale*, *F. prausnitzii*, *Roseburia* spp. Oligo-fructose is another prebiotic that was shown to change the abundance of more than a hundred bacterial taxa in mice. Defining a specific food group as a prebiotic is laden in controversy. Thus, fibers were also counted as prebiotics but not all fibers have shown prebiotic features such as the ability to be fermented.

1.3. Probiotics

Probiotics, according to ISAPP, are defined as a sufficient dosage of living microorganisms also has recuperative properties on the host when administered (Hill et al. 2014). Selected species of microorganisms are found in foods or supplements, especially dairy products. For example, consumption of the *Bifidobacterium* spp. has a positive effect on the digestive system, improves lactose intolerance, inhibits antibiotic related diarrhea and has anti-inflammatory effects. Although fecal microbiota transplantations (FMT) have the potential to cure some infections, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), Crohn's disease and ulcerative colitis (Rivière et al. 2016), according to ISAPP, FMTs are not probiotic treatments as they also contain undesired strains (Hill et al. 2014). Probiotic strains can maintain homeostasis in healthy individuals by stimulating healthy microbiota and also they can prohibit a variety of disease states related with dysbiosis. *Bifidobacterium, Lactobacillus*, and *Saccharomyces* are well-known as probiotics and are mostly used and studied for improving human health by remodeling existing microbiota. Consumption of probiotics can encourage interactions between the microbiota and the host by overexpressing genes involved in chemotaxis, adhesion, motility of *Bifidobacterium spp., Eubacterium spp., Roseburia spp.* (Rivière et al. 2016).

General characteristics of probiotics can be listed as being non-pathogenic/non-toxic, consisting of large numbers of living cells, having a beneficial effect on the host, sustaining metabolism in the gut and maintaining its viability when preserved and consumed (Glenn, et al., 2010). Other characteristics include competition for prebiotics and nutrients, reduction of the pH of the gastrointestinal tract, induction of secretion of antimicrobial factors, production of intraluminal solutes, improvement in the intestinal barrier function by modulating tight junction proteins, and regulation of immune responses (Brugman et al. 2018).

1.4. Microbial activity, metabolites and metabolism

Microbial activities in the gut can affect distant organs; even the brain (Fellows et al. 2018), by stimulating endocrine cells and triggering them to produce important hormones. Recent studies show that there are variety of microbial metabolites that can influence host metabolism by interacting with specific host membranes or nuclear receptors. Among the hundreds of metabolites produced by microbial activities, indoles, TMAO, neurotransmitters and short-chain fatty acids (SCFAs) are the most studied. SCFAs such as acetate, propionate and butyrate have attracted attention in recent years as they can be recognized by G-protein-coupled receptors which triggers the secretion of peptides involved in many metabolic reactions such as glucose metabolism or food intake (Figure 2).

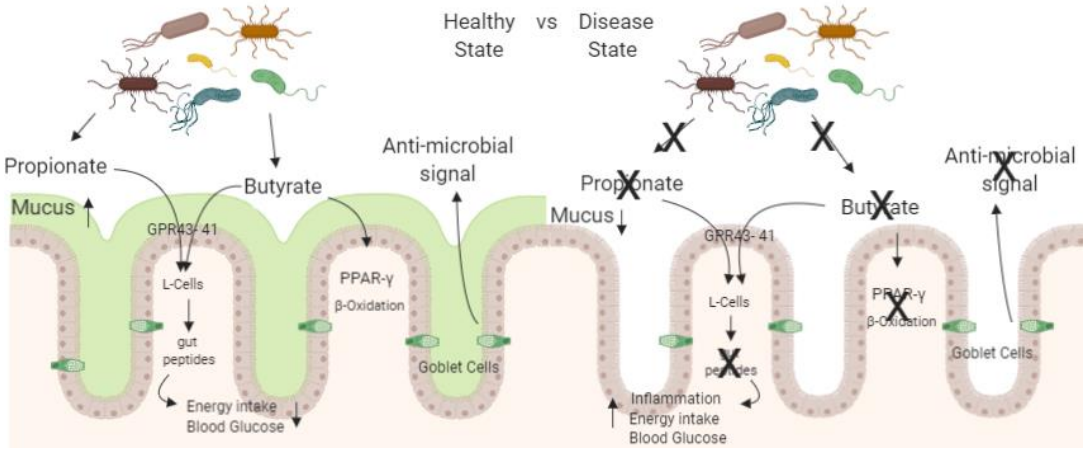


Figure 2. Differences of microbiome and host interaction between healthy and disease states (Cani 2018).

Acetate (2-carbon) can inhibit the growth of pathogens by decreasing the alkalinity of the colon, has anti-inflammatory effects and is utilized by bacterial species as a co-substrate to yield butyrate. Propionate (3-carbon) can reduce proliferation and induce

apoptosis of cancer cells, has anti-inflammatory effects and can act as an immune regulator. Butyrate (4-carbon), on the other hand, can reduce proliferation and induce apoptosis of colorectal cancer cells. (Riviere, et al., 2016). Yet, butyrate not only acts as energy source of the colon epithelial cells through β-oxidation, but can also protect the gut barrier function by triggering proliferation of colon cells, which protects the host against pathogenic bacteria. For example, putative facultative anaerobic pathogens such as Salmonella or Escherichia cannot colonize the gut lumen due to the limited oxygen amount since most of the oxygen is absorbed by colon cells to β-oxidize butyrate in the mitochondria (Cani 2018).

1.4.1. Production of SCFA

Non – digestible dietary carbohydrates are fermented by the human gut microbiota, that eventually yields in short-chain fatty acids (SCFAs). The major SCFAs actetate, propionate and butyrate are produced by the gut microbiome at a ratio of 3:1:1 and can reach a concentration range of 50-150 mM in the intestine (Louis et al. 2014). Acetate is most abundant and is produced as a result of reductive acetogenesis by most of the anaerobes (Louis and Flint 2017). For acetate production, two different pathways have been described (Figure 3). Many enteric bacterial strains are capable of producing acetate from pyruvate through acetyl-CoA, while the acetogens produce acetate through the Wood-Ljungdahl pathway in which CO_2 is used for the synthesis of acetyl-CoA and acetate (Louis et al. 2014) (Figure 3).

Propionate is produced by two different sugar fermentation pathways. The first pathway requires six or five carbon sugars to be processed through the succinate pathway (Figure 3). Accumulation of succinate can be the result of both carbohydrate metabolism or amino acid degradation and converges on the pyruvate and Krebs cycle. In the presence of vitamin B_{12}, succinate can be converted to propionate in several steps. The second pathway is the metabolism of deoxy sugars such as fucose and

rhamnose through the propanediol pathway (Figure 3). Metabolism of most monosaccharides and deoxy sugars converges at an intermediate called 1,2-propanediol, which can be further converted to both propanol and propionate depending on the availability of vitamin B_{12} (Louis and Flint 2017).

Butyrate production depends on the condensation of two molecules of acetyl-CoA or formation of crotonyl-CoA that is a product of carbohydrates, lactate and amino acid metabolism. The pathway diverges into two with a stepwise reaction from acetyl-CoA and crotonyl-CoA ends with butyryl-CoA. Butyrate formation from butyryl-CoA is catalyzed by butyryl-CoA:acetate CoA-transferase (ButCoAT) in one step (Transferase pathway) or via phosphotransbutyrylase and butyrate kinase (Kinase pathway) in two steps (Figure 3). (Louis and Flint 2017).

1.5. Butyric Acid Production

The Firmicutes family of bacteria are known for butyrate production in the gut. The *Clostridium* species, belonging to the Firmicutes family, use the kinase pathway (Figure 3) and convert butyryl-CoA to butyrate. *Eubacterium rectale* and *Roseburia* species, for example, are known as major butyrate-producing Firmicutes that commonly have the butyryl-CoA: acetate CoA transferase (ButCoAT) as the main pathway for forming butyrate (Louis and Flint, 2009). Prebiotics such as dietary starch, arabinoxylan and inulin are utilized by these species of bacteria. Fermentation of these prebiotics yield formate and butyrate accompanied by the net utilization of acetate. The Transferase and Kinase pathways resemble the condensation of acetyl-CoA for the formation of butyryl-CoA. Butyrate production can also contribute to production of ketogenic precursors such as acetoacetyl-CoA and 3-hydroxybutyryl-CoA in bacteria (Clomburg et al. 2012).

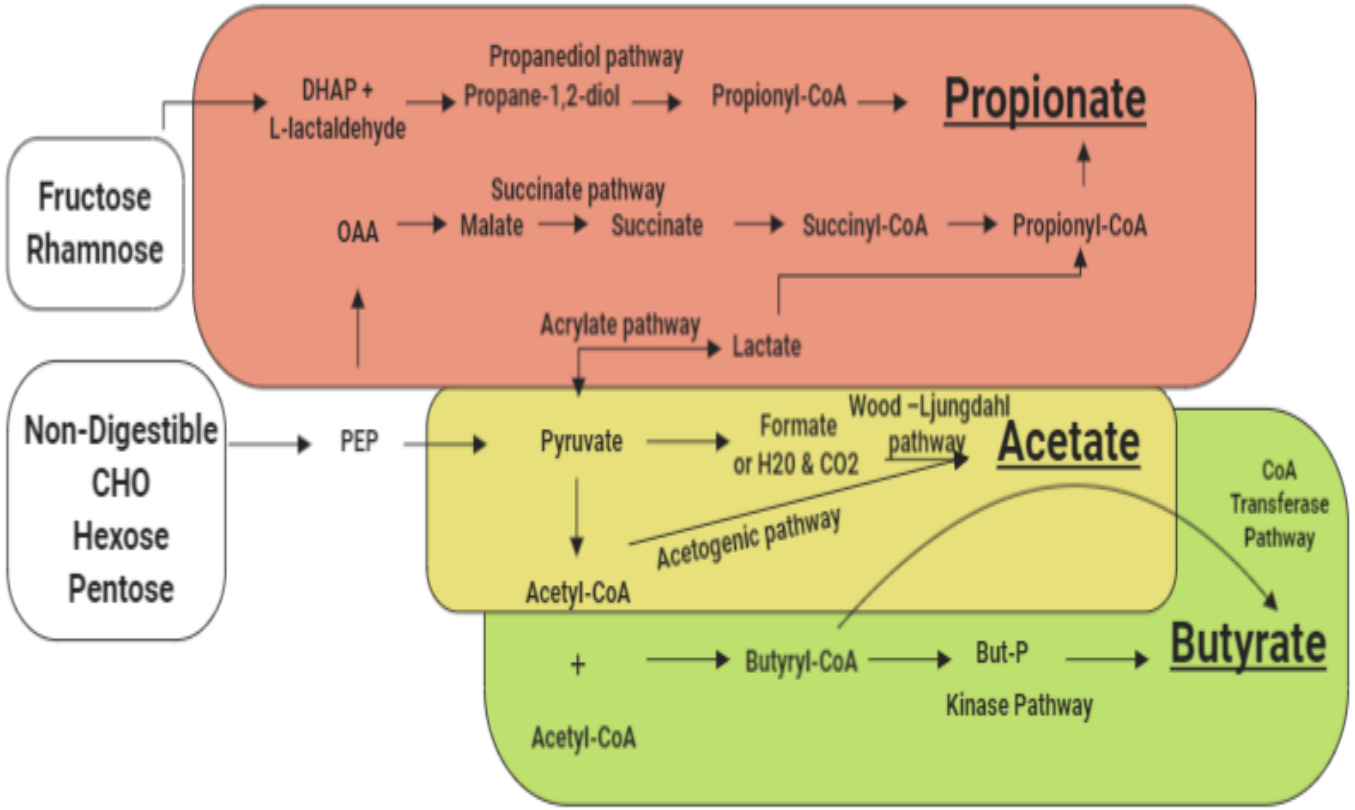


Figure 3. Pathways of acetic acid, propionic acid and butyric acid production in bacteria (Duncan et al. 2002; Louis, Hold, and Flint 2014; Louis et al. 2014; Louis et al. 2017; Louis and Flint 2017)

Thiolase, also known as acetyl-CoA transferase, catalyzes a condensation reaction between acetyl - CoA and acyl-CoA and forms acetoacetyl-CoA, which is further converted to acetoacetate, a precursor for ketone bodies, by the enzyme called *Hydroxymethylglutaryl*-CoA (HMG-CoA) lyase. β-hydroxyacyl-CoA dehydrogenase is the second enzyme involved in the β-oxidation of fatty acids. This enzyme carries out oxidoreductase activity to form 3-hydroxyacyl-CoA. Enoyl-CoA Hydratase/3-hydroxyacyl-CoA dehydratase converts 3-hydroxyacyl-CoA into trans-enoyl-CoA, which can be further reduced to a two carbon longer form of an acyl-CoA by an acyl-CoA dehydrogenase /trans-enoyl-CoA reductase (Clomburg et al. 2012).

1.6. Effects of Butyrate on Colon Epithelial cells and Colon cancer

Ingested dietary fibers are metabolized by colon microbiota to form butyrate and other SCFAs. These SCFAs are taken up by colonocytes through transporters called monocarboxylate transposter-1 (MCPT-1). MCPT-1 was reported to be downregulated in colorectal cancer (Lambert et al. 2002). Butyrate itself or in the form of butyryl-CoA that results from a conversion by acyl- CoA synthetase, enter into mitochondria from the cytoplasm and undergo β-oxidation in healthy colonocytes (Han et al. 2018; Lambert et al. 2002) (Figure 4).

Tumor cells exposed to butyrate, on the other hand, were shown to undergo inhibition of proliferation and induction of apoptosis and differentiation (Hamer et al. 2008). Mechanism of action of butyrate primarily relies on the regulation of gene expression by inhibiting histone de-acetylation and activation of signaling events by binding to its receptors GPR41, GPR43 and GPR109a. It was reported in breast and prostate cancer that binding of butyrate to its receptors induces the intracellular level of Ca^{2+} and it eventually reduces cAMP level. Decreased cAMP level can no longer suppress phosphorylation of mitogen-activated pathway.

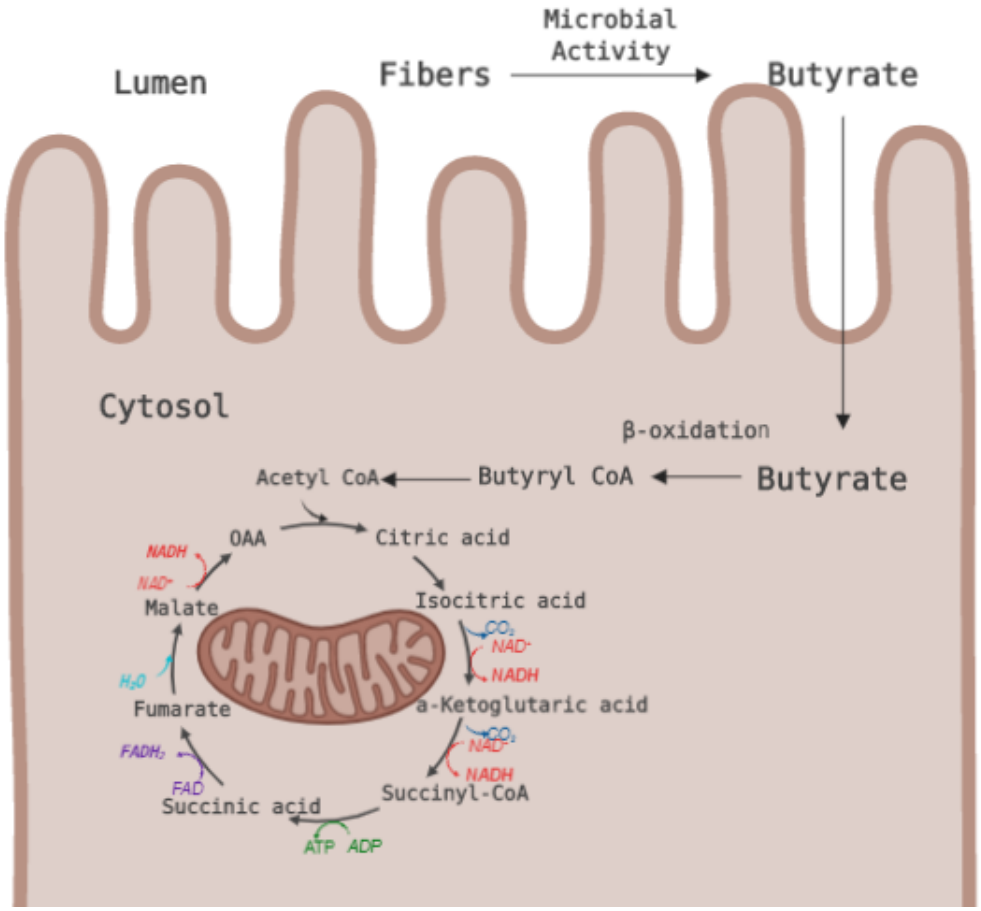


Figure 4. Metabolism of butyrate through β-oxidation in healthy colonocytes (Han et al. 2018).

Phosphorylated p38 MAPK was shown to lead to the activation of caspase-3 dependent apoptosis (Schwab et al. 2006). Mechanism of inhibition of histone de-acetylation (HDAC), cell cycle arrest and apoptosis was described in the literature as starting from Sp1 sites (butyrate response element) on the $p21^{Waf1/Cip1}$ promoter. It is known that two binding sites, Sp3 and Sp1, are associated with this promoter. Zinc finger DNA binding protein 89 (ZBP-89) recruits coactivator and HAT on Sp1 binding sites while Sp3 recruits HDAC and resulted in histone acetylation. Activity of $p21^{Waf1/Cip1}$ promoter is suppressed when chromatin structure is condensed and histones are acetylated. However, inhibition of HDAC by butyrate disrupts the steady

state and increases histone acetylation activity. Hyperactylated histones lead to opening in promoter region of $p21^{Waf1/Cip1}$ and increases gene expression in the breast cancer cell line MCF-7 cells. In epithelial like colon cancer model, Caco-2 cells, butyrate was also shown to enhance phospho- p38 level resulting in cell cycle arrest through elevation of p21 (p53- independent) (Orchel et al. 2003) and caspase-3 mediated apoptosis (Schwab et al. 2006).

1.7. Aims of the thesis and research question

Butyrate together with other SCFAs are known to reduce proliferation, induce apoptosis and reduce motility in colorectal cancer cells (Han et al. 2016). To our knowledge, there are a limited number of mechanistic studies that show the effects of conditioned medium obtained from SCFA-producing bacteria on *in vitro* colorectal cancer models. We have therefore engineered a commensal bacterial strain that is capable of producing butyrate in an inducible manner. Conditioned medium from this bacterial strain was used to determine effects of colon cancer cell viability and alterations in important signaling pathways such as the mitogen activated protein kinase (MAPK) pathway. We observed that even at very low doses, the SCFAs in bacterial conditioned medium showed remarkable alterations in signaling pathways in cancer cells. This suggests that the bacterial model is more realistic in understanding the *in vivo* role of butyrate on signaling pathways in cancer cells.

1.7.1. Rationale for the engineering of the bacterial strain

Exploiting intestinal bacteria for butyrate production is handicapped due to the requirement of strict anaerobic conditions, slow growth and limited genetic tools (Volker et al. 2014). In studies related to bio-fuel production, reversal of the β-

oxidation cycle and elements required for butyrate production have been examined extensively (Wang et al. 2019). Those studies have focused on genomic modifications of *E. coli* strains, specifically K12 MG1655, due to its very well-characterized metabolism and genome. It is known that wild type *E. coli* can utilize carbon sources (glucose, glycerol etc.) and produce ethanol, acetate and lactate under anaerobic conditions (Figure 5). To direct bacterial metabolism towards butyrate production, deletion or blockage of the native fermentation pathway will lead to the preferential production of pyruvate and acetyl-CoA (rather than lactate).

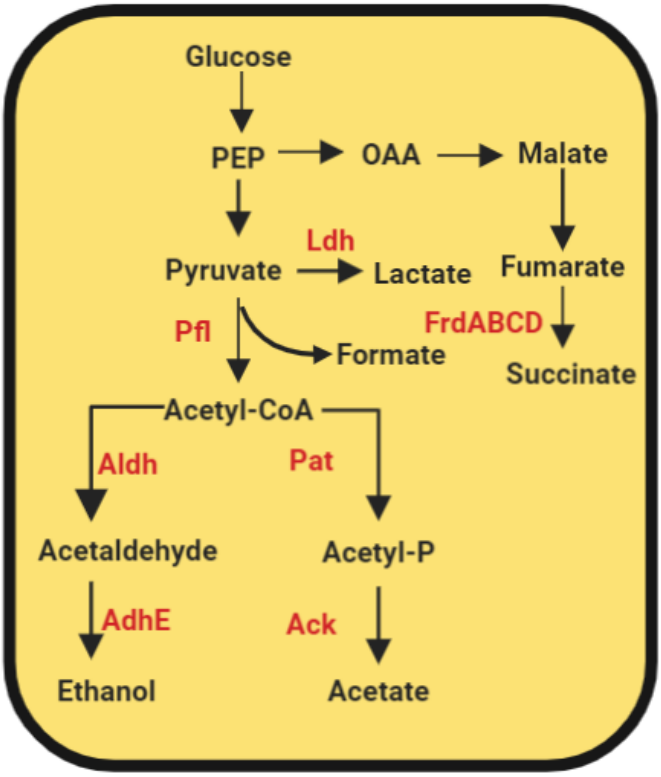


Figure 5. Schema of fermentative products in E. coli (Clomburg et al. 2012).

Additionally, expression of enzymes required for competition with thioesterases would lead to the synthesis of 4-C carboxylic acids through a reverse β-oxidation cycle (Figure 6) (Clomburg et al. 2012).

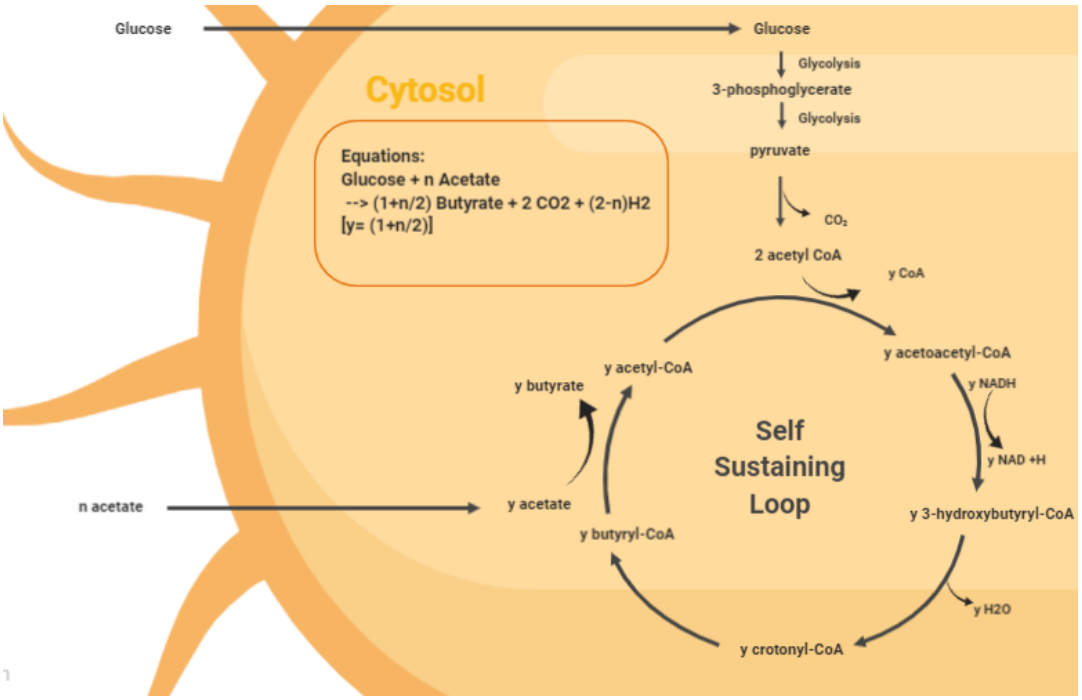


Figure 6. Butyrate production through reversal of the β-oxidation cycle.

1.7.2. Blockade of fermentation pathway

In anaerobic conditions, pyruvate is abundantly synthesized as a result of glycolysis. Pyruvate formate lyase converts the pyruvate to acetyl-CoA and formate. Depending on acidity and the oxidation state of the cell, pyruvate is converted to lactate via lactate dehydrogenase (Ldh), acetyl-CoA is converted either to ethanol via acetaldehyde/alcohol dehydrogenase (AdhE) or to acetate via phosphoacetyltransferase–acetate kinase (Pta-AckA). Phosphoenolpyruvate (PEP), another intermediate in the pathway, is converted to succinate by fumarate reductase (FrdABCD) in anaerobic conditions. All these conversions mentioned above are required to maintain NADH at homeostatic levels and to maintain the correct pH (Figure 5) (Catalanotti et al. 2013). Genomic deletions of *adhE*, *pta*-

ackA, *ldhA*, and member of *frd* was shown to shift the metabolism from the production of ethanol, acetate, lactate and succinate to the production of pyruvate and acetate (Clomburg et al. 2012).

1.7.3. Generation of a self-sustaining metabolic loop for the continuous production of butyrate.

The metabolism of *E. coli* can be programmed for the reversal of β-oxidation cycle metabolites. Among the native thiolases (*atoB, yqef, fadA, fadI*) which can prime the β-oxidation of the fatty acids, AtoB has higher specificity for the condensation of acyl -CoA due to a higher affinity for acetyl-CoA rather than acetoacetyl-CoA. *In vitro* characterization of the enzyme exhibits multiple loops in the cycle. Two enzymes (*fadB* and *fadJ*) are encoded in the genome of *E. coli* that are responsible for both 3-hydroxyacyl-CoA dehydrogenase and Enoyl-CoA hydratase activities that are involved in the subsequent conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA and crotonyl-CoA, respectively. *In vitro* characterization has shown that FadB exhibits multiple turns in the production cycle and better functionality in the reversal of β-oxidation (Clomburg et al. 2012). The last step in the cycle can be catalyzed by native enoyl-acyl carrier protein reductases such as FadE and FabI. Among them, FabI can not only elongate the chain of carboxylic acids even with odd chain priming molecule propionyl-CoA but also more efficiently reduce crotonyl-CoA (Vick et al. 2015). Based on findings of previous studies, we have established the core native elements in our genetic circuit. We focused on the exogenous expression of the enzyme ButCoAT to generate free acetyl-CoA that eventually enters the β-oxidation cycle and increases the yield of butyrate. ButCoAT is likely to be one of the key enzyme for a self-sustaining loop found in the Firmicutes family that catalyzes transferase activity between butyryl-CoA and acetate to yield butyrate and acetyl–CoA (Figure 7). It is also expected

that utilization of acetate present in cytosol would change the ratio of SCFAs secreted by the bacteria.

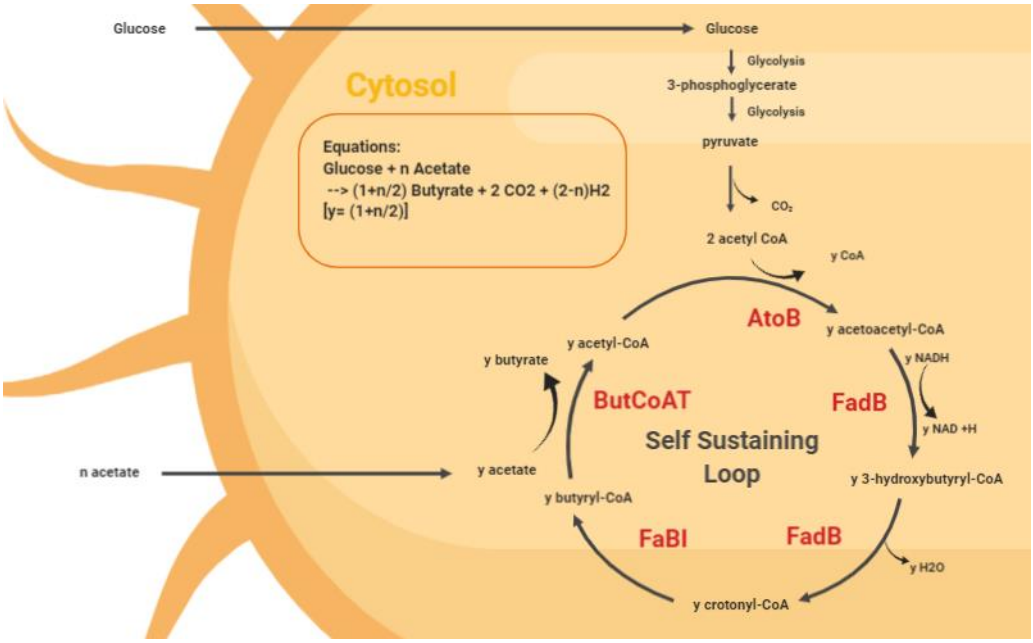


Figure 7. Demonstration of self-sustaining loop of butyrate production.

Reversal β-oxidation cycle enzymes AtoB, FadB, FabI (Clomburg et al. 2012; Vick et al. 2015) and ButCoAT (Sato et al. 2016) can lead to the activation of a self-sustaining loop that can further enhance butyrate production.

CHAPTER 2

MATERIALS & METHODS

2.1. Bacterial Strains

The bacterial strains used in this study are *E. coli* DH5α (ATCC, USA), wild type *E. coli* K12 MG1655 (kindly donated by Prof. Hüseyin Avni Öktem, METU, Ankara, Turkey) and *E. coli* K12 in which the native fermentation pathways were eliminated by genetic deletion of ΔldhA, ΔadhE, ΔackA, Δpta, ΔfrdC (Volker et al. 2014). The fermentation pathway deleted strain was kindly donated by Prof. Thomas Bobik (Department of Biochemistry, Biophysics and Molecular Biology Iowa State University, USA) (Volker et al. 2014).

2.2. Cloning of Genes

The plasmid pSB1C3 is a pUC19 derived high copy number cloning vector that is designed for continuous cloning. The pSB1C3 vector consists of a multiple cloning site bearing the restriction enzyme EcoRI, XbaI, SpeI and PstI. C-terminal cloning in this vector was carried out by using the restriction sites SpeI and PstI on vector and XbaI and PstI on insert. By using the Gibson cloning technique, an insert bearing XbaI and PstI sticky ends was then ligated on SpeI and PstI sides, respectively. When XbaI-SpeI sides are ligated properly, 'TACTAG' pattern is formed and that site cannot again be digested with the same enzymes. The XbaI site, downstream of EcoRI, on vector and SpeI site, upstream of PstI, on the insert are preserved and can be used for future cloning experiments (Figure 8).

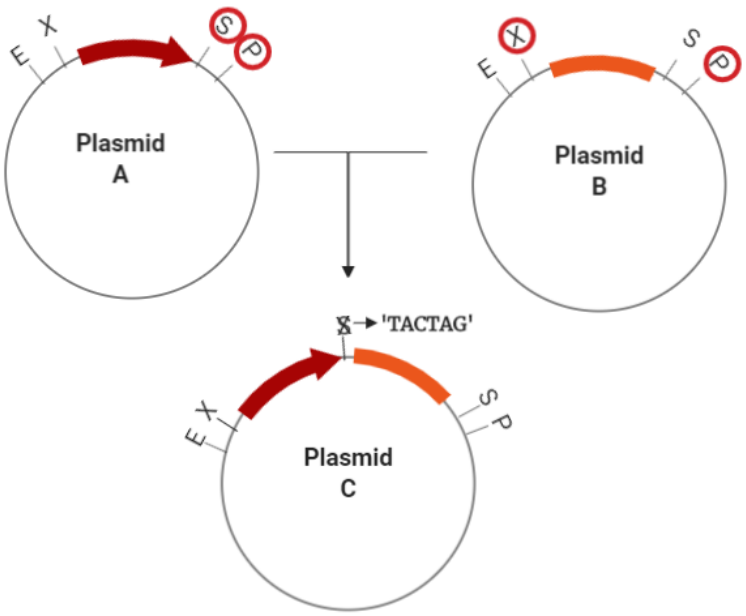


Figure 8. Illustration of the cloning strategy used in the current study. (E: EcoRI, X: XbaI, S: SpeI, P: PstI.)

The elements required for overexpression of selected genes and their detection after overexpression were selected from an International Genetically Modified Organisms (iGEM) catalog. Sequences of the arabinose responsive pBAD promoter (http://parts.igem.org/Part:BBa_K206000), ribosome binding side (http://parts.igem.org/Part:BBa_B0034), and a 10x Histidine tag with a terminator sequence (http://parts.igem.org/Part:BBa_K844000) were used as template vectors or extensions at the flanking ends of primers.

Sequences of *AtoB*, *FadB*, and *FabI* (GenBank: U00096.2, NCBI) from the *E. coli* strain K12 MG1655, were amplified from the genomic DNA by a nested-PCR technique using primers listed in Table 1. The sequence of Butyryl-CoA:acetate CoA-transferase (ButCoAT, Protein ID: ROSINTL182_07121) from *Roseburia intestinalis L1-82* strain cloned into a pUC-57 vector at specific restriction cut sites was obtained from Genscript (Piscataway, USA). After each cloning, the products were transformed and amplified in the *E. coli* DH5alpha strain and confirmed by PCR and Sanger

sequencing. All cloning experiments were performed by obeying the strategy described above.

Table 1. List of the primers used in this study

Gene Name	**1st PCR (5'-3')**	**2nd PCR (5'-3')**	**Tm (°C)**
AtoB Forward	AAAGAGGAGAAA ATGAAAAATTGTG TC	TTTTCTAGAAAAGA GGAGAAAATGAAAA ATTG	58
AtoB Reverse	TTATTAATGGTGA TGATGGTGGTGAT TCAACCG	TTTCTGCAGACTAGT TTATTAATGGTGATG ATGGTGGTG	59
FadB Forward	TCTAGACCATGGA AAGAGGAGAAAA TGCTT	TTTGAATTCTCTAGA CCATGGAAAGAGG	61
FadB R	TCGAGTTATTAAT GGTGATGATGGTG GTGAGCCGT	TTTCTGCAGACTAGT GAATTCCTCGAGTTA TTAATGG	61
FabI Forward	TCTAGACTCGAGA AAGAGGAGAAAA TGGGT	TTTCCATGGGAATTC TCTAGACTCGAGAA AGAGG	62
FabI Reverse	TTATTAATGGTGA TGATGGTGGTGTT TCAGTT	TTTACTAGTTTATTA ATGGTGATGATGGT G	61
ButCoAT Forward	ACCTCTTACGTGC CCGATCA	-	55
ButCoaT Reverse	AAGCCTGCATAAC GCGAAGT	-	54
pBAD Forward	TTAGCGGATCCTA CCTGACG	-	57
pSB1C3 Reverse	AAGCCTGCATAAC GCGAAGT	-	57

2.3. Culture conditions

The growth media that were used in this study are LB (Sigma Aldrich), M9 minimal medium [containing **(**per liter) 64 g Na_2HPO_4. $7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, 5.0 g NH_4Cl, 0.493 g $MgSO_4.7H_2O$, 0.0147 g $CaCl_2.2H2O$, 1 g yeast extract and 20% v/v glycerol as the carbon source] and Terrific Broth [TB; containing (per liter) 12 g tryptone, 24 g yeast extract, 100 ml 0.17 M KH_2PO_4, 100 ml 0.72 M K_2HPO_4 and 20%

v/v glycerol]. Chloramphenicol was used at a concentration of 20 mg/L as the selection antibiotic. Where indicated, the culture medium also contained 5mM calcium pantothenate as a micronutrient and 0.01% L-Arabinose as the inducing agent for gene expression.

Regular bacterial growth for plasmid isolation or stock preparation and primary cultures were prepared in 5ml antibiotic containing LB by inoculating a single colony from a freshly streaked agar plate. The cultures were incubated for 14-16 hours at 37°C with shaking at 180 rpm. For long term storage, the bacteria were spun down and stored at -80 °C in 40% glycerol.

To induce the expression of relevant genes, the bacteria were cultured in three steps: primary culture, main culture and induction culture. The primary culture consisted of 25µg/mL chloramphenicol (Sigma-Aldrich) and single bacteria colony grown in 5 mL LB. For the main culture, 1% of the inoculum from the primary culture was taken into 5ml fresh LB and incubated until the OD_{600} value reached 0.5-0.6 (approximately 3-4h). For the induction culture, 0.01% L-arabinose was then added to the main culture and incubated for 24h at 37°C with shaking at 180 rpm. At the end of the induction period, the cells were harvested by centrifugation at 5000 x *g* for 10 min.

For growth of cells in the fermentation medium, small changes were made to the protocol above in order to ensure maximum induction and therefore expression of the genes of interest prior to culture under fermenting conditions. When the OD_{600} of the main culture (grown under aerobic conditions) reached a value of 0.5-0.6, 0.01% L-arabinose was added to the culture and incubated for 3-6 hours in a shaker incubator. The cells were then pelleted by centrifugation at 5000 x *g* for 10 min and 1 ml aliquot was removed to test for the induction of expression by western blot. The pelleted cells were re-suspended in TB containing calcium pantothenate, inducer (L-arabinose) and antibiotic at the amounts indicated above. To ensure micro-aerobic or anaerobic conditions, the growth vessel was purged of air by flushing with premixed a gas

containing 5% CO_2 and 95% N_2 and tightly sealed with a rubber stopper. The culture was then incubated for 24 h at 37°C with shaking at 180 rpm.

2.4. Nested PCR and Confirmational PCR

Nested Polymerase Chain Reaction was carried out in a final volume of 25 µl containing 100 µg of genomic DNA and reaction mixture containing 0.5 µM of each primer, 0.5 µM dNTP mix, 0.5 µl Q5 Polymerase (NEB, USA), 10 µl Q5, reaction buffer, µl 10 High GC Enhancer and 25 µl was completed with nuclease free water. Reaction was performed in a thermal cycler (Applied Biosystems, USA). PCR conditions were initial denaturation at 98 °C for 30 seconds; denaturation at 98 °C for 10 seconds; annealing at optimized temperature for 30 seconds; extension at 72°C for 30 seconds/kb and final extension at 72 °C for 2 minutes. PCR products were run on a 1.2% agarose gel containing 5µl ethidium bromide solution (Applichem, Germany) at 150 V and images were taken under UV light. Extracted PCR product was used as template in following reaction to elongate the insert with desired extensions such as restriction enzyme cut sites and expression elements.

Confirmational PCR was carried out in a reaction volume of 20 µl containing 0.5 µM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, proper buffer and 1 U of Taq Polymerase (Thermo Fisher Scientific, USA). Final volume was completed with nuclease free water containing picked colonies for colony PCR or denatured plasmid DNA (100 ng) at 95 °C for 5 min. Reaction was carried out in thermal cycler machine (Applied Biosystems, USA) according to manufacturer's instructions. PCR products were run on a 1% agarose gel containing 5µl ethidium bromide solution (Applichem, Germany) at 150 V and images were taken under UV light.

2.5. Protein Purification from Bacterial Pellet

Bacterial cells were pelleted after induction of gene expression by centrifugation 14000 rpm for 1 min and the supernatant was discarded. The cells were resuspended in TEG buffer (25 mM Tris-Cl, 10 mM EDTA, 50 mM Glucose, pH 8.0) to a final concentration of 100mg cells/ml. 1:10 volume of TEG containing 0.5 mg/ml lysozyme was then mixed with the cell suspension and vortexed gently. Samples were taken into water bath at 37°C for 30 min. The lysed bacterial suspension was mixed with a sample loading dye at a ratio of 1:3 and incubated at 100°C for 10 min (Alexander, et al., 2004). The sample loading dye was prepared by mixing 4.4 ml dH_2O, 17.5% v/v glycerol, 0.005% v/v bromophenol blue, 2 ml of 20% SDS solution, 1.1 ml of 4X running buffer (containing 90.85 g Tris-Base, 20 ml of 10% SDS up to 500 ml dH_2O, pH 8.8).

2.6. Enzymatic Activity of ButCoAT Expressed in *E. coli* K12 MG 1655

2.6.1. Isolation of the Crude Enzyme

Total protein was extracted from *E. coli* K12 MG1655 transformed with the *ButCoAT* expressing vector or the empty vector and grown in LB broth. Enzyme activity was determined in both L-arabinose induced and un-induced samples. To isolate the crude extract, vector transformed bacteria was harvested and the cell pellet was washed 2X with Phosphate buffered saline (PBS). The pellet was re-suspended in PBS containing 0.1 mM Tosyl-L-lysyl-chloromethane hydrochloride (TLCK), 0.2 mM phenylmethane sulfonyl fluoride (PMSF) and 0.1 mM leupeptin (as protease inhibitors), and then lysed by ultrasonication on ice. The lysate was centrifuged at 30,000 x *g* for 10 min and supernatant was collected as the crude protein extract. The

protein concentration was calculated using the BCA Protein Assay Kit (Thermo Scientific) and the ButCoAT enzyme activity was determined using a colorimetric assay as described below (Sato, et al., 2016).

2.6.2. Colorimetric Assay

A reaction mixture (final volume 40 µl) was prepared in a 96-well plate by combining 1mM butyryl-CoA (Sigma) with 40mM potassium phosphate buffer (pH 8.0) and 200 mM sodium acetate. The reaction mixture was pre-warmed to 37ºC and the reaction was started by the addition of 25 mg/ml of the crude enzyme. The mixture was incubated at 37ºC for 5 min, after which 10 µl of 4.5% trichloroacetic acid (TCA) was added to terminate the reaction followed by neutralization with 25 µl of 400 mM potassium phosphate buffer (pH 8.0). This reaction leads to an increase in the production of acetyl-CoA and butyric acid. To be able to colorimetrically determine the amount of butyric acid specifically, a second reaction was carried out where a mixture (25 µl final volume) containing 4 mM oxaloacetate, 4 mM 5,50-dithiobis-2-nitrobenzoicacid (Sigma) and 36.8 mg/ml citrate synthase (Sigma) in a 96 well plate. Citrate synthase reduces the Ellman's reagent (DTNB) to TNB which can be measured colorimetrically at 412 or 450 nm. This mixture was incubated at 37ºC for 30 min, and the samples were analyzed by spectrophotometry using MultiSkan GO Microplate Spectrometer (Thermo Scientific) at 450 nm (Sato, et al., 2016).

2.7. High Performance Liquid Chromatography (HPLC) and Gas Chromatography and Mass Spectrometry (GC-MS) for the detection of SCFAs

2.7.1. HPLC for the detection of SCFA

HPLC was used to detect and quantify acetic acid, propionic acid and butyric acid. Conditioned media obtained from bacteria with different overexpressions and control were filtered through a 0.20-µm syringe filter. The samples (10µL) were then injected to a1260 Infinity Series HPLC system (Agilent) equipped with an Aminex HPX87H anion exchange column (Bio-Rad) and refractive index (RI) detector. 4 mM H_2SO_4 was used in mobile phase at a rate of 0.2 mL/min. Temperature of the column was kept at 65°C and concentrations of SCFAs were determined by calibration curve established. These experiments were carried out at the Molecular Biology and Biotechnology Central Laboratory of Middle East Technical University.

2.7.2. Extraction and Derivatization of SCFAs for GC-MS

SCFAs were derivatized and extracted from bacterial conditioned medium prior to GC-MS analysis as a requirement for a carboxylic acid column. 300 µl of conditioned medium from different bacterial strains were taken into 2 ml eppendorf tubes and mixed with 5µg/ml heptanoic acid (internal standard). 500µl of propanol-pyridine-methylchloro formate (3:2:1, v/v/v) was mixed with the conditioned medium and ultrasonicated for 1 min. The derivatized SCFAs were extracted by the addition of 500 µl GC grade hexane and vortexing for 1 min. The tube was centrifuged at 2000 x *g* for 5 min and the organic phase (upper layer) was collected in a fresh glass tube containing 10 mg anhydrous sodium sulfate (dessicant). A second aliquot of 500 µl of hexane was mixed with the derivatized solution and the organic phase was again collected.

To quantify the SCFAs, a standard curve was generated. For this, standards (butyrate, propionate and acetate) were prepared at a range of concentrations of 0.1, 1 and 10 mM in bacterial medium and derivatized as above.

2.7.3. GC-MS Protocol

GC-MS was carried out with a ZB-FFAP column (length, 30m; diameter, 0.32 mm; film, 0.50 µm; Phonemenex Inc., USA) to detect and quantify SCFAs in the bacterial conditioned medium. These experiments were carried out at UNAM, Bilkent University. Injection port temperature was set at 150 ºC. Helium was used as a carrier gas and the oven temperature was held at 80 ºC for 1 min. The temperature was then elevated to 135 ºC at a rate of 10 ºC/min and kept at that temperature for 5 min. The final temperature was adjusted to 230 ºC; the temperature was increased at a rate of 30 ºC/min and held for 6 min. Ionization temperature was set at 230 ºC for mass spectrometry. 4 µl of sample was injected in a splitless mode.

2.8. Cell Culture

Caco-2 cells (ŞAP Enstitüsü, Ankara) was cultured according to ATCC protocols. Briefly, the cells were cultured in Eagle's Minimum Essential medium (EMEM) containing 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1mM non-essential amino acids, 20% fetal bovine serum and 1 % penicillin and streptomycin at 37ºC in a humidified incubator with 5 % CO_2. To prevent confluency dependent spontaneous differentiation of Caco-2 cells, the cells were grown to 50-70 % confluency maximum and passaged using 0.25 % Trypsin-EDTA. Cells were routinely checked for mycoplasma contamination using a PCR assay. To prevent

contamination, the culture medium was supplemented with Plasmocin (Invivogen) used at a concentration of 2.5 µg/ml.

2.9. Proliferation Assay

In order to determine whether the conditioned medium (CM) obtained from bacteria overexpressing various genes in the SCFA synthesis pathway affected cell viability, an MTT assay was carried out on Caco-2 cells incubated with a 1:1 ratio of the conditioned medium and complete EMEM. The cells were seeded equally in each well of three different 96-well plates and allowed to attach and grow until the confluency reached around 70%. The growth medium was then replaced with the filter sterilized conditioned/EMEM mixture (0.2 µm filter, GVS Life Sciences, USA) from experimental and control bacteria and incubated for 6, 12 and 18 h. MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was prepared in PBS as described in the manufacturer's instructions (Thermo Fisher Scientific, USA) and added to the 96-well plate. 1%SDS dissolved in distilled water with 0.01M HCl was then added to each well after 4 hours of MTT treatment. Plates were incubated to allow the formazan crystals to dissolve completely in following 16 h. Colorimetric changes in each wells were detected at 570 nm with a microplate reader (Thermo Fisher Scientific, USA).

2.10. Treatments

Caco-2 cells were seeded in 6-well plates and propagated until the confluency reached 70%. Conditioned medium obtained from experimental or control bacterial groups were filtered through 0.2 µm filter (GVS Life Sciences) and mixed with complete EMEM at a ratio of 1:1. The cells were incubated with the conditioned

medium/EMEM mix for 6h, washed with PBS and scraped with a lysis solution (MPER Mammalian Protein Extraction Reagent (Thermo Scientific) containing PhosStop (Roche, Germany) and proteinase inhibitor cocktail was prepared according to manufacturer's instructions) on ice. Harvested cells were vortexed briefly three times and incubated on ice for 15 min. Total protein lysates are obtained after centrifugation at 14000 x *g* for 10 min. As a control, cells incubated in complete EMEM (without conditioned medium), or a 1:1 mixture of EMEM and Terrific Broth were used.

2.11. Western Blot

Total protein lysates were loaded on 12 % SDS-PAGE gels according to standard protocols. The proteins were transferred to PVDF membranes (Roche) at 115 V for 75 min using a Bio-Rad Mini-TransBlot system. Following protein transfer, the membranes were blocked in 5 % skimmed milk (Applichem) for 1 h at room temperature with mild shaking. The membranes were incubated with pre-optimized concentrations of the primary antibodies (Table 2) overnight at 4ºC followed by 3 washes in 0.1 % TBS-Tween (TBS-T) and incubation with the appropriate horse radish peroxidase (HRP) conjugated secondary antibody for 1h at room temperature. The membranes were again washed 3 times in 0.1 % TBS-T and incubated with a chemiluminescent substrate (Clarity ECL, Bio Rad) and imaged with the Bio-Rad Chemi Doc MP system. Where necessary, the membrane was stripped at 65ºC in a stripping buffer (100 mM β-mercaptoethanol, 2 % SDS, 62.5 mM Tris-HCl, pH 6.8) followed by stringent washes in 0.1 % TBS-T.

Table 2. List of antibodies used in this study

Description	**Origin**	**Brand**	**Dilutions**
p-p38	Rabbit	Cell Signaling	1:1000
Total p38	Rabbit	Santa Cruz	1:500
p-MK2	Rabbit	Santa Cruz	1:500
Total MK2	Rabbit	Santa Cruz	1:500
p-Chk2	Rabbit	Santa Cruz	1:200
Total Chk2	Rabbit	Santa Cruz	1:200
β-actin	Mouse	Santa Cruz	1:4000

2.12. Statistical analyses and graphing

All experiments were repeated 2-4 times as biological replicates with at least 3 technical replicates. All graphs were generated using GrpahPad Prism 6.0 (GraphPad, CA, USA). Statistical significance was considered at the level of $p<0.05$.

CHAPTER 3

RESULTS

In this thesis we have aimed to generate a bacterial strain that can synthesize and secrete high amounts of the short chain fatty acid butyrate. Our first strategy was to overexpress a transferase called Butyryl-CoA:acetate CoA-transferase (ButCoAT) (EC 2.8.3.8) that converts acetyl-CoA into butyrate (Sato, et al., 2016) in the commensal bacterial strain *E. coli* K12 that lacks this enzyme.

3.1. Overexpression of ButCoAT in *E. coli* K12 Commensal Bacteria to Enhance Butyrate Expression

3.1.1. Cloning of ButCoAT

The coding sequence of ButCoAT was obtained from *Roseburia intestinalis* that naturally produces butyrate by fermentation. The cloning strategy for the control vector and the ButCoAT containing vector is shown in Figure 9. The ButCoAT sequence was amplified from *Roseburia intestinalis* using primers containing an N-terminal ribosome binding site (RBS) and a C-terminal 10x- His tag by nested PCR. The amplified sequence was approximately 1.4 kb and was cloned into the pSB1C3 vector (pUC-19 derived, size 2kb). A control plasmid was generated by inserting an RBS and terminator sequence to downstream of the pBAD promoter.

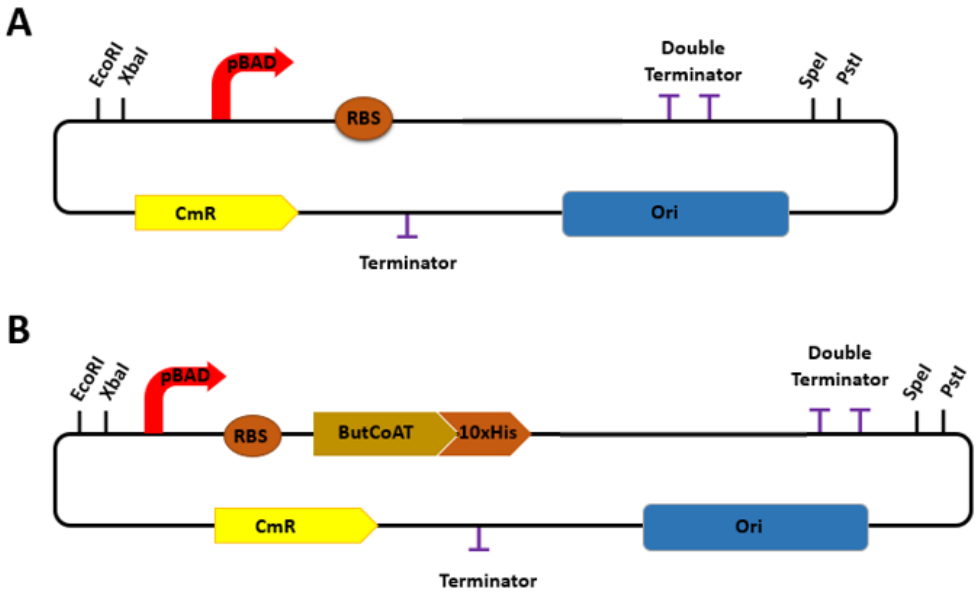


Figure 9. Schema of designed circuit for empty vector (A) and ButCoAT vector (B), showing the order of expression elements in pSB1C3 vector.

To confirm the cloning, the vector was digested with the restriction enzymes XbaI and PstI and the presence of the insert and vector with the correct sizes was seen on an agarose gel (Figure 10). The arabinose inducible promoter pBAD (1.3 kb) was used as template which is already cloned into the pSB1C3, upstream of the ButCoAT sequence. The presence of the pBAD promoter can be seen in Figure 10.

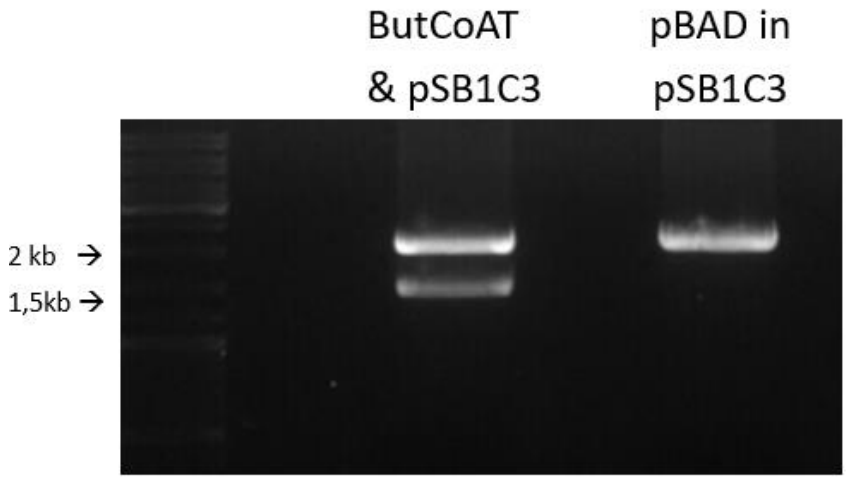


Figure 10. Image of the vector (pSB1C3 containing pBAD) restriction digested with XbaI and PstI and insert (ButCoAT) on 1% agarose gel.

3.1.2. Western blot showing the inducible production of ButCoAT

The vector containing His-tagged ButCoAT sequence was overexpressed in *E. coli* K12 MG1655 under the control of the arabinose inducible pBAD promoter. The cells were lysed and total protein was extracted as described in Materials and Methods. Western Blot using a Histidine antibody showed the induction of ButCoAT with a band at 50 kD. A faint band was observed in the protein extracted from both GFP containing plasmid (negative control) and the un-induced ButCoAT plasmid containing bacteria. Un-induced ButCoAT plasmid containing bacteria has shown minor leakage of the pBAD promoter (Figure 11).

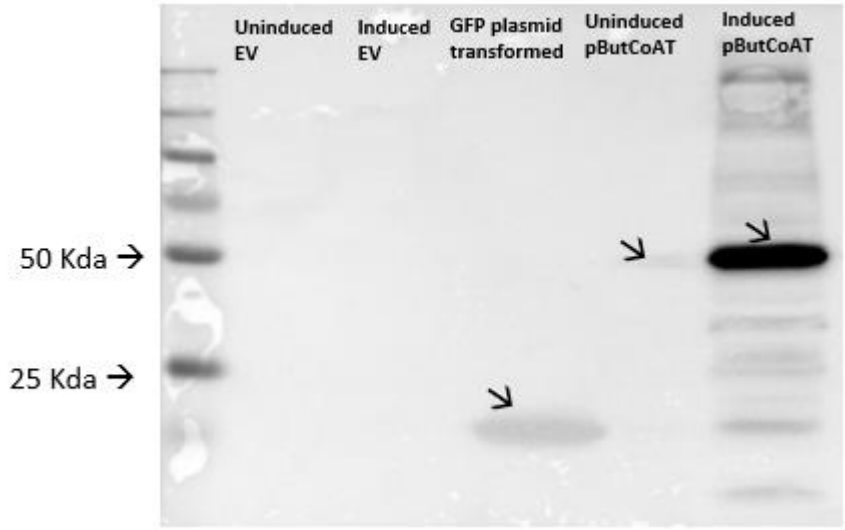


Figure 11. Western Blot analysis showing the expression of ButCoAT.

Protein extracts from pSB1C3 cloned with BuTCoAT or the empty vector transformed *E. Coli* K12 MG1655 were loaded. Lanes 1 and 2 correspond to negative controls. Lane 3: The faint band at 25kD corresponds to GFP expression in a GFP control vector transformed bacteria. Lane 4: The faint band at 50 kD corresponds to ButCoAT expressed. Lane 5: The intense band shows the presence of His-tagged ButCoAT after induction with L-Arabinose.

3.1.3. Functional characterization of ButCoAT for Butyrate Production in vitro

ButCoAT was synthesized as codon-biased for *E. coli* in order to ensure functional enzyme production. Enzyme activity in the overexpressed cells was tested by a citrate synthase assay (Sato et al. 2016). The rationale for this assay is as follows (Scheme 1) acetyl CoA, generated during the enzymatic synthesis of butyrate in the presence of ButCoAT, reacts with oxaloacetic acid in the presence of citrate synthase. In this reaction, citrate and CoA-SH are produced. Co- incubation with DTNB (5,5'-Dithiobis-(2-Nitrobenzoic Acid)) results in a reaction with the thiol group of CoA-SH and the formation of the chromophore 5-merapto-2-nitrobenzoic acid that has an absorption maximum at 412 nm.

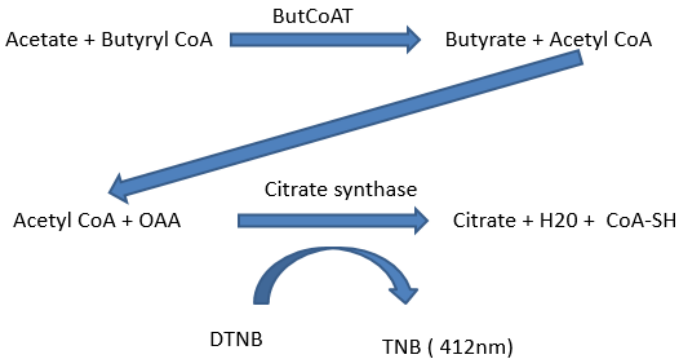


Figure 12. Demonstration of the Citrate Synthase Assay.

Crude protein was extracted from induced ButCoAT expressing or empty vector transformed bacteria and incubated with the substrate ensuring that the pH was alkaline (pH 8.0. Measurement of absorbance of the chromophore at 412 nm indicated that higher enzymatic activity in the ButCoAT expressing cells compared to the control cells suggesting that the ButCoAT expressed in these cells were enzymatically active (Figure 13). The high enzymatic activity observed in the uninduced bacteria could be due to 2 reasons: the pBAD promoter is leaky and leads to gene expression even in the absence of induction, or the assay is non-specific and can be activated by other enzymes as well. We are more in favor of the first explanation, since the control non transformed bacteria do not show any enzymatic activity.

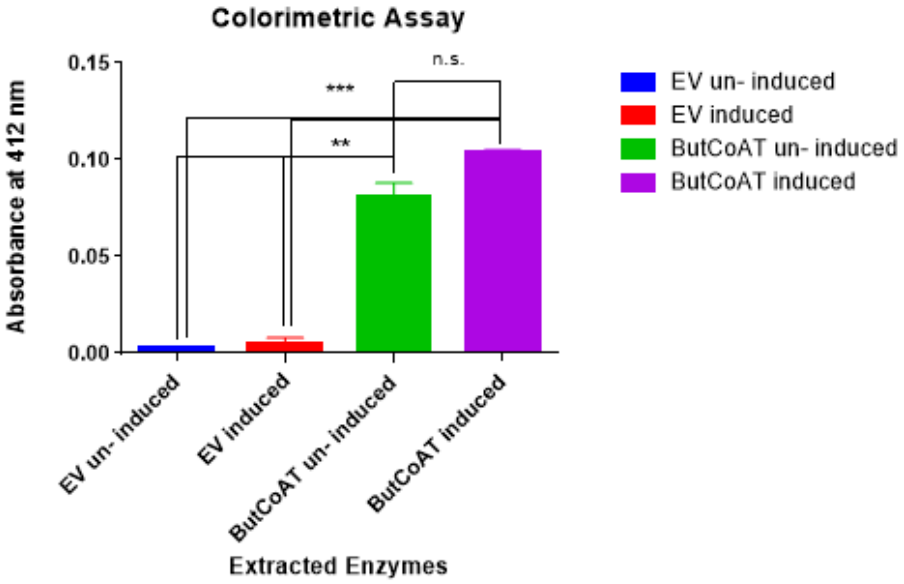


Figure 13. ButCoAT enzyme activity determined by the citrate synthase assay.

The reactions mixtures, containing 10µg/ml crude enzyme extracts, 1 mM butyryl-CoA and 200mM Na:Acetate were incubated at 37°C for 5 min. After CoA transferase activity is completed, change in TNB concentration as a result of citrate synthase was measured at 412 nm. Data obtained from each group were compared, represent mean ± SEM (n=3), **p<0.05; ***p<0.005.

3.1.4. HPLC to verify Butyrate production

High Performance Liquid Chromatography (HPLC) technique was used to detect SCFAs released into media by bacteria overexpressing ButCoAT. It was expected that the conditioned medium would have high levels of butyrate compared to the control cells, as well as detectable levels of the other SCFAs propionate and acetate. The retention time of SCFAs were determined as approximately 14.7 min for acetic acid, 17.3 min for propionic acid and 21 min for butyric acid. Conditioned media were then injected to HPLC column and identified with the help of standards. We observed that butyric acid was not generated in these bacteria within the detection limits of the HPLC protocol used. However, acetic acid and propionic acid were detected (Table 3).

Table 3**.** SCFAs detected in HPLC from condition medium obtained from wild-type *E. coli* K12 containing both EV and ButCoAT plasmids grown in aerated TB**.**

Conditioned Medium Obtained by	Acetic Acid(mg/ml)	Propionic Acid(mg/ml)	Butyric Acid(mg/ml)
Empty Vector	0.721±0.009	0.038±0.006	Not Detected
ButCoAT Vector	0.640±0.016	0.037±0.001	Not Detected

3.2. Strain Engineering for Butyrate Production

3.2.1. Strain Selection and Plasmid Design

The lack of any detectable butyrate production in the ButCoAT overexpressing strain prompted us to examine the intermediaries in the metabolic pathway leading to the production of butyrate. We decided to use the *E. coli* strain K12 MG1655 (ΔldhA, ΔadhE, ΔackA, Δpta, ΔfrdC), referred to from now as 4KO, which has genomic deletions that eliminate native fermentation products to direct the carbon atoms towards the synthesis of butyrate and not lactate (please see Aims of the study for more detailed explanations of the rationale). Additionally, the enzymes AtoB; FadB; and FabI that direct the pathway for production of butyrate (Clomburg et al. 2012) were chosen for overexpression along with ButCoAT in order to increase the amount of substrate available for the final conversion of acetate and butyryl-CoA to butyrate.

The genes *AtoB, FadB* and *FabI* were cloned using primers whose flanking ends were designed to have an N-terminal ribosome binding site (RBS) and C-terminal histidine tag and termination sequences (GenBank: U00096.2, NCBI). The RBS sequence with histidine tag and termination sequence were sequentially cloned downstream of pBAD promoter in pSB1C3 plasmid to a generate empty vector (Figure 14A). The coding sequences for *AtoB, FadB* and *FabI* were cloned in the order shown in Figure 14B.

AtoB and *FadB* were individually cloned into both the pSB1C3 and pBAD vectors. Additionally, these genes were sequentially cloned downstream of the pBAD vector. His-tagged ButCoAT was then cloned upstream of *FabI* and the two plasmids were sub-cloned into an operon to generate new plasmid that was named pAFFB.

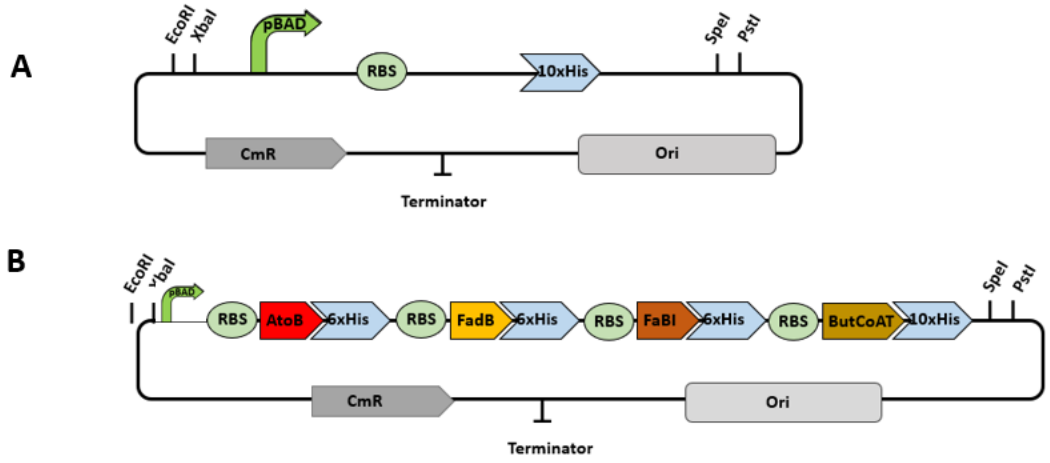


Figure 14. Design of the vector for overexpression of AtoB, FadB, FabI and ButCoAT.

Empty vector containing pBAD promoter, RBS and 10X-His tag (A) & pAFFB vector designed as cloning β-oxidation enzymes with ButCoAT at downstream of pBAD for polycistronic expression (B).

To generate this construct shown above, nested PCR reactions were carried out to amplify the genes *AtoB*, *FadB* and *FabI* from *E. coli* K12 (Figure 15). The agarose gel picture shows the successful amplification of these genes.

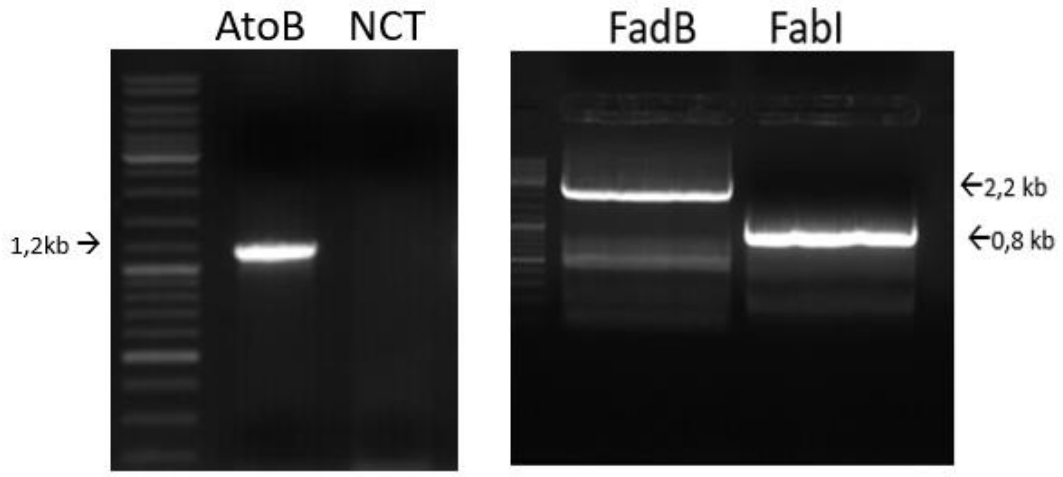


Figure 15. Amplification of AtoB, FadB and FabI from the pAFFB plasmid by PCR.

PCR was carried out from the plasmid using primers indicated in Table 1 (Materials and Methods). Amplification of gene of interests was performed by nested PCR, annealing temperatures were 53°C for AtoB, 62°C for FadB, 54°C for FabI in 35 cycle. NTC: No template control.

Due to the presence of PstI cut sites in the *FabI* sequence, the coding regions of the enzymes AtoB, FadB and ButCoAT were separately cloned in both pBAD plasmid and pSB1C3 vector with the use of XbaI and PstI. FabI was only cloned into pSB1C3 vector with the use of EcoRI and SpeI. To complete overexpression plasmid containing β-oxidation enzymes and CoA transferase, the strategy described in the Material & Method section was used. First, FadB was cloned downstream of pBAD-AtoB and FabI was cloned upstream of ButCoAT. pBAD-AtoB-FadB was then cloned upstream of FabI-ButCoAT to generate completed plasmid. PCR reactions were carried out using the generated plasmid to confirm the presence of all coding regions in a sequential manner (Figure 16) followed by sequencing.

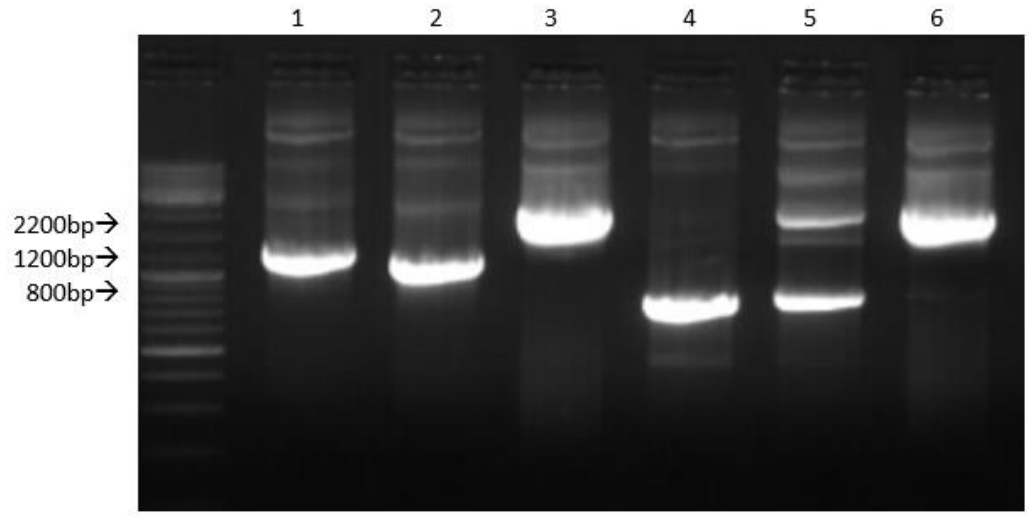


Figure 16. PCR amplification to confirm the presence of all the inserts in the pAFFB plasmid.

Primer pairs were selected as pBAD Forward & AtoB Reverse (Lane 1); AtoB Forward & AtoB Reverse (Lane 2); FadB Forward & FadB Reverse (Lane 3); FabI Forward & FabI Reverse (Lane 4); ButCoAT Forward & ButCoAT Reverse (Lane 5); FabI Forward & ButCoAT Reverse (Lane 6). For primer sequences please see Table1. Confirmational PCR was performed on plasmid DNA obtained from final cloning set in 35 cycle. The correct size of the bands show that all elements were on the plasmid in the correct orientation.

3.2.2. Western blot showing the inducible production of four enzymes

The control and pAFFB vectors were transformed into both wild type *E. coli* K12 MG1655 and the *E. coli* 4KO strain. Proteins were extracted from the two strains after induction in TB and a Western Blot was carried out to confirm the expression of the four enzymes (Figure 17 – 18A). Additionally, to optimize the culture conditions, the 4KO strain was grown in the presence of glucose or glycerol as the carbon source. It was observed that the induction of expression in the presence of glucose was considerably weaker when compared to the expression in the presence of glycerol (Figure 18B) indicating the repression of the pBAD promoter in thepresence of glucose (Guzman et al. 1995). All future experiments were carried out using glycerol as the carbon source.

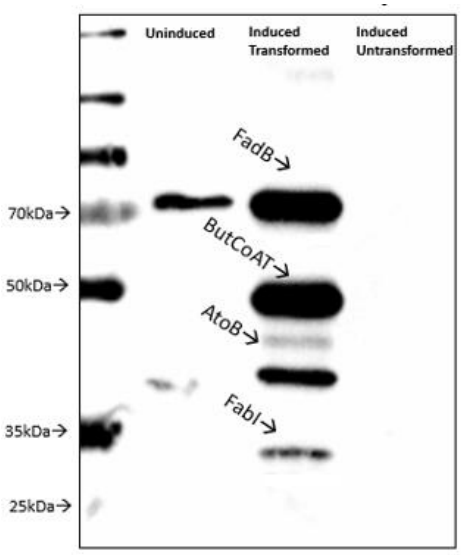


Figure 17. Western Blot analysis showing the expression of FadB, ButCoAT, AtoB and FabI in an inducible manner in wild type E. Coli K12 MG1655.

Western blot was carried out with protein extracts from pSB1C3 transformed and untransformed wild type *E. Coli* K12 MG1655 using a His antibody. Lanes 1 (uninduced) and 3 (untransformed) correspond to negative controls. Lane 2: Intense bands at 27kD, 35kD, 49kD, 79kD show the presence of His-tagged FabI, AtoB, FadB and ButCoAT, respectively.

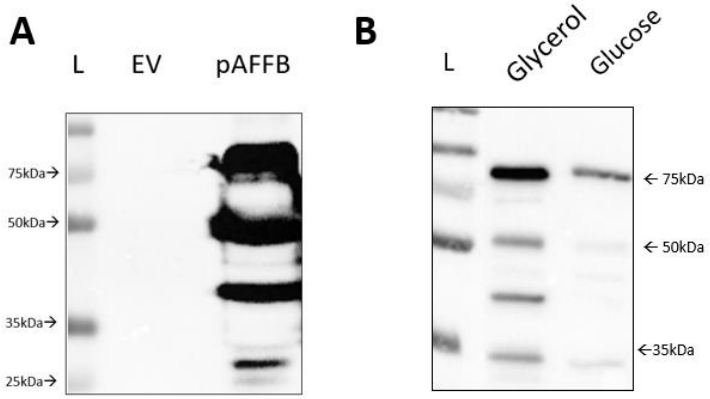


Figure 18. Western Blot analysis showing the expression of FadB, ButCoAT, AtoB and FabI in an inducible manner in E. coli 4KO strain transformed with pAFFB.

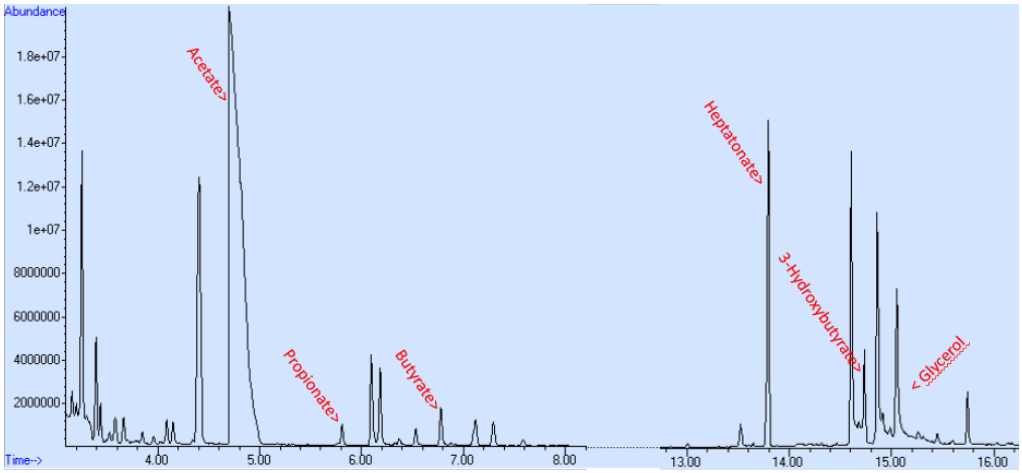
A. Protein extracts from Empty Vector and pAFFB transformed into *E. coli* K12 MG1655 with genomic deletions (ΔldhA, ΔadhE, ΔackA, Δpta, ΔfrdC) were loaded. Lanes 1 (EV) correspond to negative controls. Lane 2: intense bands at 27kD, 35kD, 49kD, 79kD show the presence of His-tagged FabI, AtoB, FadB and ButCoAT, respectively. **B.** Bacteria were grown in the presence of glucose or glycerol as the carbon source. Higher induction in expression was observed when the bacteria were grown in glycerol.

3.2.3. GC-MS to determine the synthesis of SCFAs

In vitro characterization of ButCoAT provided evidence of the ability of enzyme to catalyze transferase reaction (Figure 12). The enzymes AtoB, FadB and FabI were selected to reverse the β-oxidation cycle. Deletion of fermentative pathways (ΔldhA, ΔadhE, ΔackA, Δpta, ΔfrdC) was expected to shift the metabolism from lactate, succinate, ethanol to acetate and pyruvate (Clomburg et al. 2012).

To detect SCFAs production, the bacteria strains *E. coli* K12 MG1655 and *E. coli* 4KO were transformed with the pAFFB plasmid and grown in Terrific Broth (TB) anaerobically (for details on anaerobic growth conditions please see Section 2.3 of Materials and Methods). Conditioned medium from these strains were collected and processed for GC-MS. For the accurate detection and quantification of SCFAs standard solutions of acetate, propionate and butyrate were prepared in the concentrations 0.1, 1 and 10 mM in TB, derivatized and extracted in hexane. The retention times for the three SCFAs, internal standard (heptanoic acid) and 3-hydroxybutyrate, a metabolic intermediate that is also generated in the butyrate pathway, were determined as 4.8 min for acetic acid; 5.8 min for propionic acid; 6.77 min for butyric acid; 12.78 min for heptanoic acid; 14.68 min for 3-Hydroxybutyrate and 15.2 min for glycerol, respectively (Figure 19).

A (EV transformed bacteria)



B (pAFFB transformed bacteria)

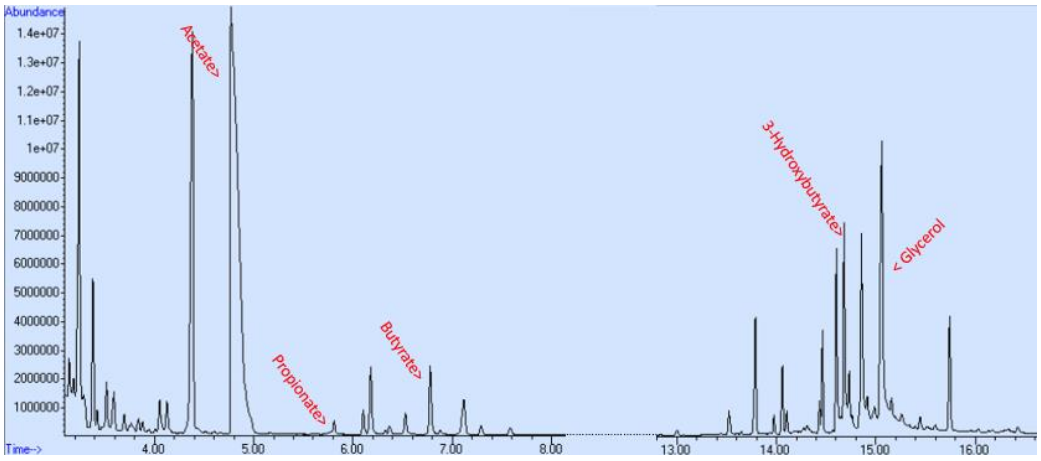


Figure 19. GC-MS analysis for SCFAs.

Acetic acid, propionic acid and butyric acid were quantified in conditioned medium obtained from *E. coli* K12 MG1655(ΔldhA, ΔadhE, ΔackA, Δpta, ΔfrdC) with empty vector (A) and pAFFB (B). 3-hydroxybutyrate were located from GC-MS library and absorbance values obtained from mass spectrometry for empty vector (C) and pAFFB (D) were approximately $4x10^6$; $7.5x10^6$, respectively. Quantifications of butyric acid (BA), propionic acid (PA) and acetic acid (AA) in mM were obtained from conditioned medium of empty vector in comparison with standard curve drawn with mixture of 0.1mM, 1mM and 10mM SCFAs. Continued next page.

C (SCFA amounts in EV transformed bacteria)

Compound	R.T.	QIon	Response	Conc	Units		Dev(Min)
Target Compounds							Qvalue
1) BA	6.777	60	1429767	0.58	UMOL		98
2) PA	5.751	74	1202	0.00	UMOL	#	1
3) AA	4.770	45	23358456	17.27	UMOL		100

D (SCFA amounts in pAFFB transformed bacteria)

Compound	R.T.	QIon	Response	Conc	Units		Dev(Min)
Target Compounds							Qvalue
1) BA	6.777	60	1043395	0.42	UMOL		99
2) PA	5.808	74	234144	0.29	UMOL	#	53
3) AA	4.697	45	47517542	35.14	UMOL		99

Figure 19. Continued.

For the identification of other metabolites, a GC-MS library was used. We did not detect any propionate and butyrate in the conditioned media obtained from wild-type *E. coli* K12 MG1655. This was expected since this strain does not express any ButCoAT. The conditioned medium obtained from the *E. coli* 4KO overexpressing the pAFFB plasmid showed three times higher amount of butyrate compared to the control empty vector transformed bacteria (Figure 20). A slight decrease in the amount of acetate was seen, which is expected since the acetate can be converted to butyrate in the pAFFB overexpressing cells. Propionate levels, on the other hand, were not changed significantly.

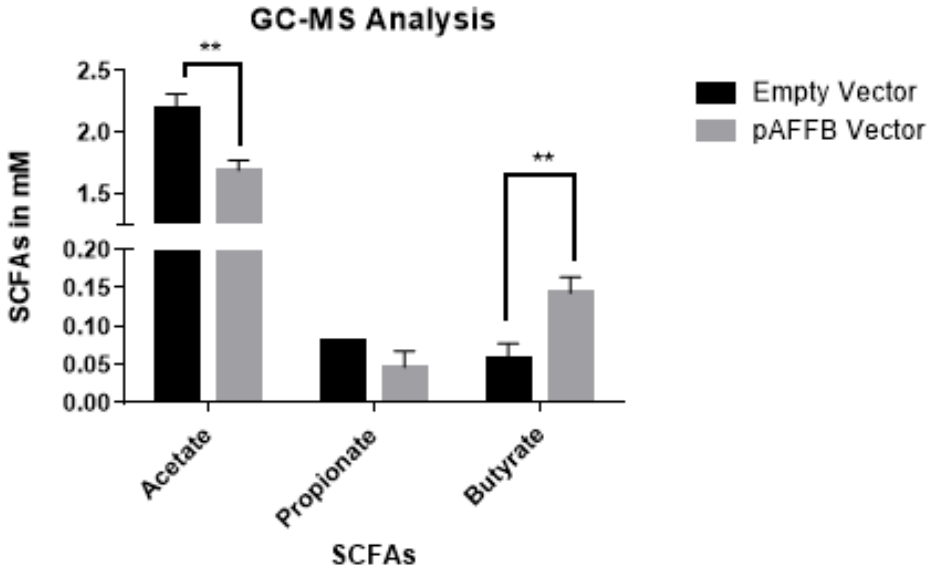


Figure 20. Quantification of SCFAs in conditioned medium after anaerobic cultivation of E. coli 4KO overexpressing the pAFFB plasmid in TB.

Acetic acid, propionic acid, butyric acid in conditioned medium were detected and quantified separately. Data are presented as mean ± SEM (n=3). **p<0.005, compared with empty vector. Student's t- test was used for statistical analysis.

3.2.4. Effects of Pantothenic acid on Butyrate Production

Starting from acetyl-CoA to butyryl-CoA, the β-oxidation cycle is rich in carboxylic acids conjugated with Coenzyme A. Therefore, any supplement that can increase CoA production is likely to be essential in the production of more butyrate. Pantothenic acid is an important precursor of CoA biosynthesis (Lopez Martinez, Tsuchiya, and Gout 2014). Therefore, we examined whether there was any change in butyrate production as a result of supplementation of the fermentation medium with 5 and 10mM of the Ca salt of pantothenic acid (Ca: pantothenate). As seen in Figure 21,

there was a significant increase in butyrate production when Ca:pantothenate was used in the medium.

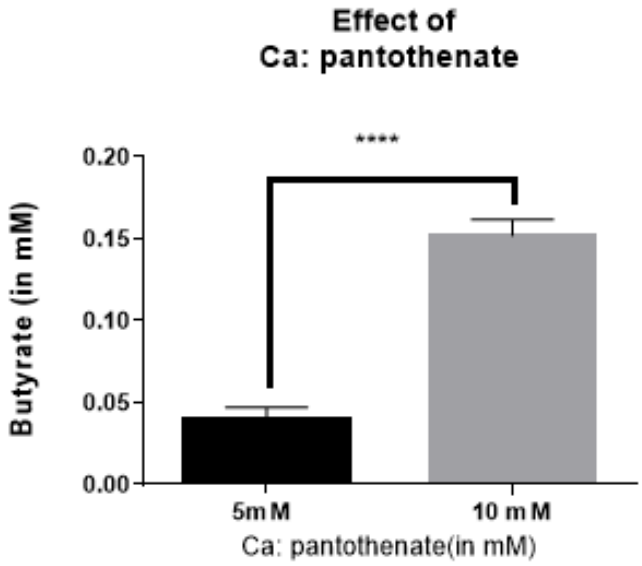


*Figure 21. Change in butyrate amount in fermentation culture by addition of different concentration of Ca: pantothenate. 5 and 10 mM Ca: pantothenate were used in the culture medium. Data are presented as mean ± SEM (n=2). ****p<0.0001, Student's t-test was used as statistical analysis.*

3.3. Effect of conditioned medium from engineered strains on epithelial cell characteristics

Caco-2 cells were used as model colonic epithelial cells to determine whether incubation with the conditioned medium from engineered bacterial strains affected signaling pathways in the epithelial cells.

3.3.1. Viability of Caco-2 cells treated with bacterial conditioned medium

The viability of Caco-2 cells was determined in the presence of bacterial conditioned medium with known SCFA concentrations (4KO strain transformed with pAFFB: 0.58 mM butyrate; 0.0 mM propionate and 17.27 mM acetate) at different time points. Cells were also incubated with conditioned medium from 4KO strain transformed with the empty vector (0.42 mM butyrate; 0.29 mM propionate; 35.14 mM acetate), wild-type *E. coli* K12 (0.0 mM butyrate; 0.0 mM propionate and 0.06 mM acetate) and a simulated mixture of SCFAs (as obtained in the pAFFB transformed conditioned medium) in TB (Figure 22).

It was observed that the EV transformed 4KO *E. coli* strain (EMEM+EV Cond Medium 1:1) could reduce the viability of Caco-2 cells in a temporal manner with nearly 50% of cell death after 18 h of treatment. Of note, the 4KO *E. coli* strain that was transformed with the pAFFB plasmid showed higher cell viability compared to the EV transformed strain. This can be interpreted as an effect of the higher butyrate production on maintaining cell viability and functionality of the gut. However, the viability of Caco-2 cells in the presence of conditioned medium from EV or pAFFB transformed bacteria was lower than the viability of the wild type *E. coli* strain that had negligible levels of SCFA. Maximal cell death was observed with the Caco-2 cells incubated with standard SCFAs dissolved in TB for 18h. The concentration of different standard SCFAs was set at the ratio obtained from GC-MS data of the pAFFB transformed bacterial conditioned medium. This indicates that incubation of epithelial cells with SCFA can lead to cell death. It should also be kept in mind that the conditioned medium from bacteria has many other undefined components that may also have contributed to the viability of Caco-2 cells.

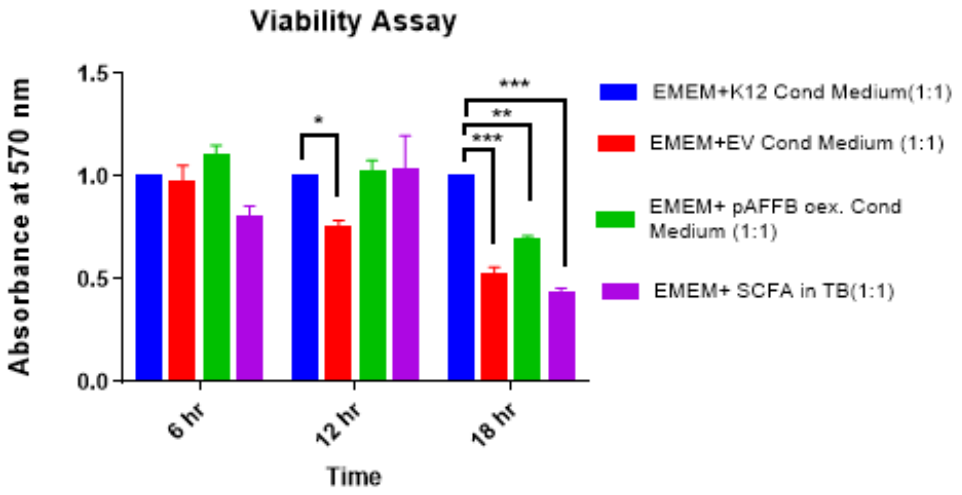


Figure 22. Effects of conditioned medium obtained from bacteria on proliferation of Caco2 cells by MTT assay.

Conditioned media was mixed with complete EMEM at a ratio of 1:1 and cells were incubated for 6, 12 and 18h. The conditioned media were used from the following strains: wild type *E. coli* K12 (blue bar), ΔldhA, ΔadhE, ΔackA, Δpta, ΔfrdC knock out *E. coli* K12 strain (red bar), ΔldhA, ΔadhE, ΔackA, Δpta, ΔfrdC knock out *E. coli* strain overexpressing pAFFB (green bar). The purple bars refer to Caco-2 cells treated with a simulated mixture of SCFAs. The viability of Caco-2 cells was measured using an MTT assay. Cells incubated with complete EMEM only were used as a control. Data are represented by using One-way ANOVA as statistical test with mean ± SEM (n=3). *p<0.05, **p<0.01, ***p<0.0001.

3.3.2. Signaling pathways affected in Caco-2 cells incubated with conditioned medium

To understand whether the conditioned medium from the engineered strains could lead to alterations in signaling pathways in epithelial cells, the phosphorylation and activation of several kinases were determined. The cells were treated the following conditioned media mixed with a 1:1 ratio with EMEM: wild-type *E. coli* K12, *E. coli* 4KO transformed with EV and *E. coli* 4KO transformed with pAFFB. The control groups consisted of cells incubated in complete EMEM alone and 1:1 ratio of EMEM and Terrific Broth. Additionally, Caco-2 cells were treated with a simulated mixture of SCFA (at the ratio obtained from GC-MS as shown in Figure 19) in TB and EMEM

at a ratio of 1:1. The cells were treated for 6h and then processed for protein isolation and Western blot.

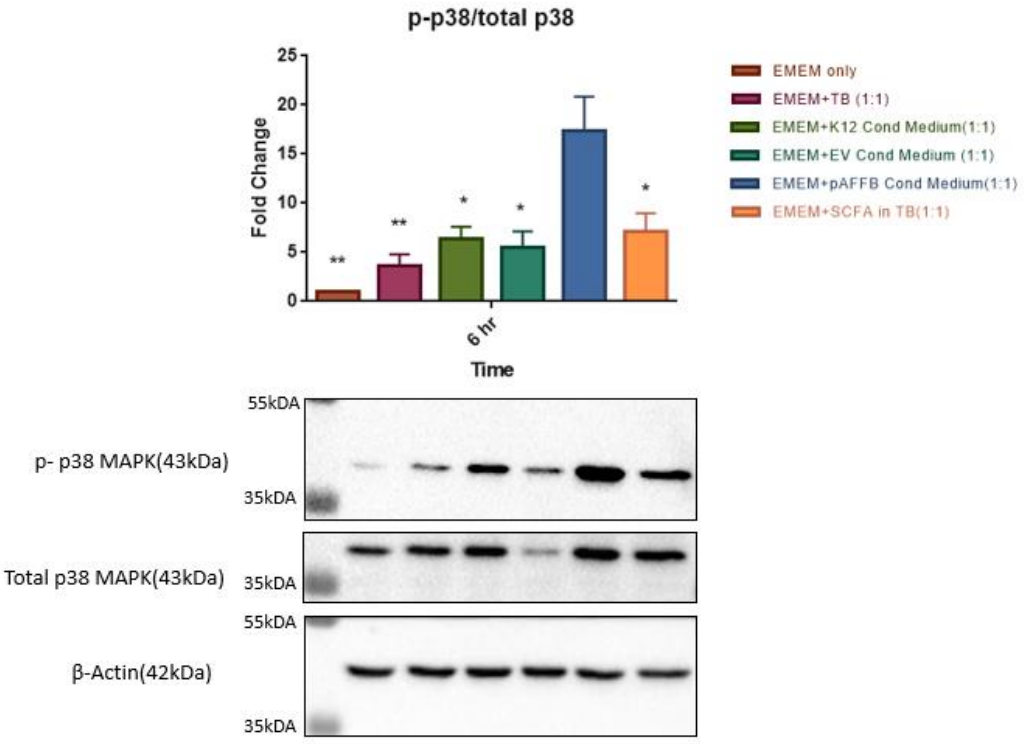


Figure 23. Effects of conditioned medium obtained from bacterial fermentation on p-38 and p-p38 MAPK protein in Caco-2 cells.

Whole cell lysate (20µg protein) from Caco-2 cells treated with conditioned medium from wild type *E. coli* K12 (green bar), ΔldhA, ΔadhE, ΔackA, Δpta, ΔfrdC knock out *E. coli* K12 strain (cyan bar), 4KO *E. coli* strain overexpressing pAFFB (blue bar) were processed for western blot. The proteins were loaded according to the order shown in the bar diagram. β-actin was used as a loading control. Fold change in phosphorylation of p38 is shown with respect to total p38. Three independent biological replicates were carried out. One-way ANOVA was carried with as a statistical test. *$p<0.05$, **$p<0.05$.

The Mitogen-activated Protein Kinase (MAPK) pathway in mammalian cells regulates the expression of many genes that regulated functions such as apoptosis, differentiation and proliferation (Schwab et al. 2006). Western blot indicated that the

levels of phosphorylated p38 significantly increased after 6 h of incubation of Caco-2 cells with conditioned medium from the pAFFB overexpressed strain (in the 4KO background) compared to the control (Figure 23). This change was approximately 3-times higher than simulated SCFAs treatment. Interestingly, levels of both phosphorylated and total p38 proteins were very low in Caco-2 cells treated with the conditioned medium from the 4KO strain transformed with the empty vector.

Upon phosphorylation, p38 can phosphorylate many substrates such as kinases and transcription factors. MAPKAPK2 (MK2) is an important target of p38 that is translocated to the nucleus upon phosphorylation for further signaling events that can regulate stress response, DNA damage response and RNA stability (Schieven 2005). We observed that concomitant with the increase in phosphorylation of p38 (Figure 22) an 8-fold increase in phosphorylation of MK2 was observed in the Caco-2 cells treated with conditioned medium from the 4KO *E. coli* K12 strain transformed with pAFFB compared to the control (Figure 24). Although not as robust as the conditioned medium, treatment of Caco-2 cells with the simulated SCFA mix also resulted in increased phosphorylation of p38 and MK2 suggesting that the increased phosphorylation events observed with conditioned media were, at least in part, mediated by SCFAs.

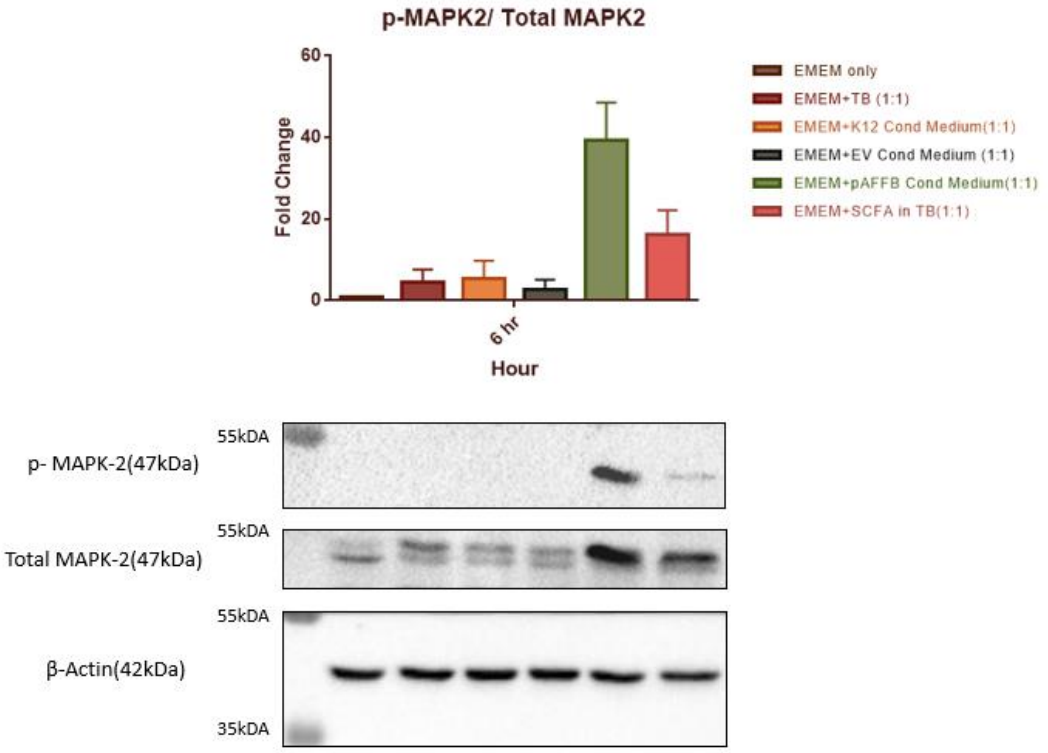


Figure 24. Protein levels of phosphorylated and total MK2 in Caco2 cells treated with conditioned medium.

Whole cell lysate (20µg protein) from Caco-2 cells treated with conditioned medium from wild type *E. coli* K12 (orange bar), 4KO *E. coli* K12 strain (blue bar), 4KO *E. coli* K12 strain transformed with pAFFB (green bar) were processed for western blot. The proteins were loaded according to the order shown in the bar diagram. β-actin was used as a loading control. Fold change in phosphorylation of MK2 is shown with respect to β-actin. Three independent biological replicates were carried out.

Chk2 is a DNA damage sensing protein that can lead to the activation of cell cycle checkpoints, cell cycle arrest and eventually apoptosis (Kato et al. 2004). Once Chk2 is activated by phosphorylation, it can phosphorylate downstream molecules such as p53 and Cdc25 that regulate cell-cycle progression and apoptosis pathways (Kato et al. 2004). Ovarian cancer cells treated with butyrate alone were reported to activate proteins involved in cell cycle progression via relaxation of the $p21^{WAF1/Cip1}$ promoter through the HDAC inhibitory activity of butyrate (Seifrtová et al. 2017). We observed that 6 h of incubation of Caco-2 cells with conditioned medium from the engineered

bacterial strains did not lead to any significant change in phosphorylated and total Chk2 levels (Figure 25).

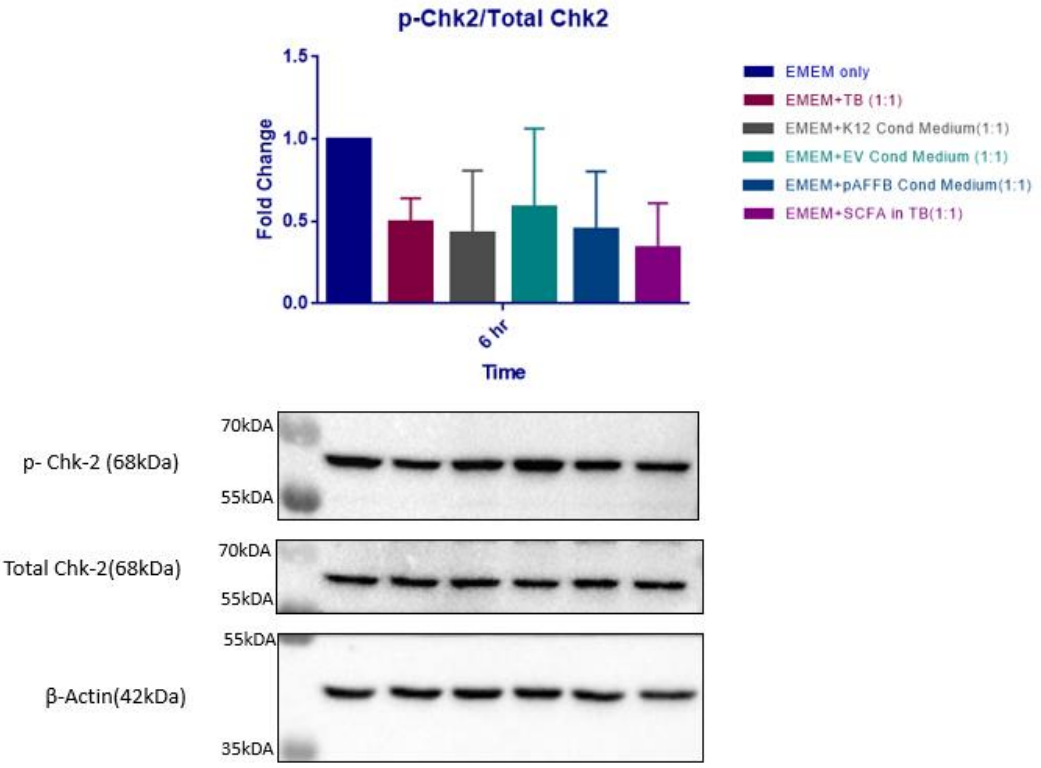


Figure 25. Effects of bacterial conditioned medium on Chk2 phosphorylation in Caco-2 cells.

Whole cell lysate (20µg protein) from Caco-2 cells treated with conditioned medium from wild type *E. coli* K12 (grey bar), 4KO *E. coli* K12 strain (cyan bar), 4KO *E. coli* strain transformed with pAFFB (blue bar) were processed for western blot. The proteins were loaded according to the order shown in the bar diagram. β-actin was used as a loading control. Fold change in phosphorylation of Chk2 is shown with respect to total Chk2. Three independent biological replicates were carried out. One-way ANOVA was carried with as a statistical test. No significant differences were found.

CHAPTER 4

DISCUSSION

SCFAs are produced by the gut microbiota and they are essential metabolites that can be utilized by colonocytes as an energy source. SCFAs also contribute to the maintenance of barrier function and are known to have anti-inflammatory properties (Rivière et al. 2016). To study effects of SFCAs obtained from gut bacteria such as *Clostridia spp*. or *Bifidobacterium spp.* is challenging due to their slow growth, requirement of obligatory anaerobic conditions and restricted genetic tools available for engineering (Volker et al. 2014). Butyrate, along with other SCFAs such as acetate and propionate secreted into the conditioned medium from *L. reuteri* strain, was shown to have anti-proliferative effects when incubated with colon cancer cells. However, the concentrations of SCFAs varied from batch to batch and strain to strain (Malhotra M 2015). In the literature, generation of butyric acid by engineered *E. coli* is mostly dominated by the fields of bio-fuel and bacterial biochemistry. In recent studies, butyrate production was successfully engineered in *E. coli* synthetically and in a more restrained way by cloning enzymes of reverse β-oxidation cycle and CoA transferases and deleting native fermentation enzymes from the genome. These studies were also designed primarily as a means for bio-fuel production (Clomburg et al. 2012; Volker et al. 2014). Using similar strategies, we have engineered *E. coli* K12, a commensal gut bacterial strain to produce higher amounts of SCFAs. We aimed to examine whether the secretome of the engineered strain could alter cell proliferation and signaling pathways in colon epithelial cells.

ButCoAT is best known for catalyzing the terminal reaction in the synthesis of acetyl-CoA and butyrate from butyryl-CoA and acetate in butyrate-producing bacteria in the gut (Sato et al. 2016). To generate butyric acid, we have started with overexpression of ButCoAT enzyme from *Roseburia Intestinalis spp.* and expected that endogenous

enzymes for β-oxidation cycle together with overexpressed ButCoAT can result in butyrate production. However, we could not detect butyric acid in the conditioned medium with this approach. This can be as a consequence of two factors: 1. The presence of native fermentation enzymes that reduce the amount of priming elements of the β-oxidation cycle. 2. The endogenous thioesterase activity competed with enzymes of β-oxidation which simultaneously convert intermediates conjugated with CoA to relevant carboxylic acid counterparts. In the literature, overexpression of enzymes of the butyric acid pathway in wild-type *E. coli* has either resulted in no yield of butyrate or yield of levels of butyrate at levels no higher than 0.07 g/L (Baek et al. 2013; Clomburg et al. 2012). We changed our strategy to ensure higher production of butyrate. Studies for the generation of bio-fuel were consulted and inhibition of native fermentation enzymes using an *E. coli* strain with deletions ΔldhA, ΔadhE, ΔackA, Δpta, ΔfrdC was identified as a strategy to shift the pathway to generate more pyruvate and acetyl-CoA and less lactate. Additionally, overexpression of native β-oxidation enzymes (AtoB, FadB and FabI) and a CoA transferase (ButCoAT) was carried out to increase the yield of butyrate. In this way, acetyl-CoA generated as a result of activity of CoA transferase was again incorporated into β-oxidation cycle. Thus, the production of butyrate could be cycled in a self -sustaining loop.

Our first challenge in an inducible system was the leaky expression with the arabinose induced pBAD promoter. Bacterial metabolism may increase the amount of endogenous sugars such as rhamnose, xylose or ribose that eventually lead to leakage in expression(http://parts.igem.org/Part:BBa_K206000:Characterization). Therefore, we designed an empty vector consisting of the same expression elements but not an operon.

Overexpression of the native β-oxidation enzymes along with ButCoAT using the pAFFB construct in the *E. coli* K12 ΔldhA, ΔadhE, ΔackA, Δpta, ΔfrdC (4KO) background resulted in major alterations in the production of butyrate when compared to wild type *E. coli* K12 strain. However, the *E. coli* K12 4KO strain transformed with the pAFFB construct did not show very dramatic changes in the levels of SCFAs. This

was a surprise to us since we expected the butyrate levels to be much higher in the pAFFB tranformed strains. Of note, the utilization of acetate as a substrate by CoA transferase was reflected in these engineered bacteria since the concentration of acetic acid was decreased in the bacteria transformed with pAFFB compared to the control. The lack of a major increase in butyrate production in the pAFFB tranformed strain can be interpreted as activation of other metabolic pathways in the *E. coli* K12 4KO strain that could result in high yield of precursor of ketone bodies. Intermediates through β-oxidation such as acetoacetyl-CoA, 3-hydroxybutyryl-CoA are precursors of ketone bodies. We have detected presence of high amounts of 3-hydroxybutyrate in the conditioned medium obtained from *E. coli* K12 4KO strain transformed wit pAFFB (please see GC-MS results, Figure 18)

A number optimization steps carried out in this study suggested the importance of many variables (such as the carbon source and presence of micronutrients) to ensure maximal butyrate production. For example, from acetyl-CoA to butyryl–CoA, all intermediates generated are carboxylic acids that are conjugated with CoA groups. This increases a demand for CoA that can only be supplied by pantothenic acid (a precursor of vitamin B_5). We have observed that supplementation of the anaerobic bacterial culture medium with increasing amounts of pantothenic acid led to a corresponding increase in SCFAs amount secreted into the conditioned medium. The presence of a carbon source is essential for production of precursors required for fermentation (Clomburg et al. 2012). The pBAD promoter was observed to be repressed in the presence of glucose (Guzman et al. 1995). To overcome this, the carbon source for fermentation was changed from glucose to glycerol, which resulted in much higher expression of the proteins of interest.

Overall, our data indicate that higher amounts of butyrate and other SCFAs could be generated in bacterial strains where native fermentation pathway enzymes were deleted (*E. coli* K12 ΔldhA, ΔadhE, ΔackA, Δpta, ΔfrdC strain) compared to the wild type strain. Moreover, overexpression of β-oxidation enzymes resulted in a modest but statistically significant increase in the amount of butyrate. This change was

sufficient to result in remarkable alterations in the phosphorylation and activation of a number of kinases in colon epithelial cells that were incubated with the conditioned medium from these bacteria.

We first examined whether the viability of Caco-2 was changed in the presence of conditioned medium from bacteria since bacterial components such as lipopolysaccharide, CpG DNA and flagellin are sensed by pattern recognition receptors that are expressed in gut epithelial cells and may lead to death of epithelial cells due to inflammation (Louis et al. 2014; Malhotra M 2015). The secretome of the *E. coli* K12 4KO strain resulted in a significant time dependent cell death compared to the secretome of wild type *E. coli*. This may have resulted from significant alterations in the secretory phenotype of the bacteria where lactate and ethanol production were abrogated. The resultant secretome was rich in components that could activate various cell death processes in epithelial cells. Of note, when the 4KO strain was transformed with the pAFFB vector, a significant recovery in cell numbers was seen. This can be interpreted as an increased viability of cells due to reduced activation of inflammatory pathways, which may eventually lead to the survival of the gut epithelial cells and thereby maintain the gut barrier formation. Indeed, it has been reported that butyrate can increase gut barrier function of Caco-2 cells (Lopez Martinez et al. 2014). However, further studies are needed to confirm this hypothesis.

Gut epithelial cells have the ability to sense bacterial invasion through the expression of Toll-like receptors in their basolateral surface. These TLRs can sense the presence of inflammatory molecules such as LPS, flagellin and CpG. LPS, for example, can be sensed by TLR4 and can activate the p38 MAPK signaling pathway (Wang et al. 2018). Caco-2 cells were specifically reported to have a diminutive response to LPS due to impaired expression of TLR4 (Furrie et al. 2005). It is therefore likely that the contribution of TLR4 mediated signaling in the responses observed in Caco-2 cells incubated with different conditioned medium is low. However, other inflammatory signaling pathways may contribute to cell death or activation of signaling pathways. Of note, we have stringently maintained identical growth conditions of the different

bacterial strains with respect to duration of culture, volume of growth medium used and optical density of bacteria (indicating extent of bacterial growth) to normalize the basal bacterial secretome.

The conditioned medium from bacteria containing the highest amount of SCFAs was used to incubate Caco-2 cells and determine the activation of signaling pathways. We observed that although the conditioned medium had much lower concentration of butyrate compared to the doses of sodium butyrate used for treatments in literature (Chen, Zhao, and Vitetta 2019; Schwab et al. 2006), we observed a dramatic increase in the phosphorylation of p38 and MK2 within just 6h of incubation. Mitogen-activated protein kinases (MAPKs) are known to orchestrate pathways involved in differentiation, apoptosis and proliferation of colon epithelial cells (Schwab et al. 2006). MAPKs also play a critical role in responding to stress and barrier dysfuntion. Therefore, it is likely that the engineered strains are likely to activate several crucial signaling pathways in epithelial cells. The increase in phosphorylated levels of p38 and MK2 in the presence of the simulated SCFA mix suggested that this activation of the p38 pathway was at least in part contributed by the presence of SCFAs. This induction of phosphorylation of kinases was specific for the p38 pathway since the phosphorylation of Chk2 did not change in the Caco-2 cells treated with the different conditioned media. Interestingly, the total amount of SCFA obtained from the *E. coli* K12 4KO strain transformed with EV was higher than the total amount of SCFA obtained from the same strain transformed with pAFFB. Thus, subtle changes in the ratio of different SCFAs (rather than the total amount of SCFA) secreted from bacteria are likely to alter signaling pathways in gut epithelial cells significantly.

CHAPTER 5

CONCLUSIONS AND FUTURE STUDIES

In this study, we examined aimed to engineer a bacteria strain that could increase the production of the SCFA butyrate. We also aimed to examine whether the alterations in SCFA concentrations in the secretome of bacteria could change signaling pathways in colon epithelial cells. Our major findings have been listed below.

1. Butyryl-CoA:AcetateCoA transferase (ButCoAT) can be functionally expressed in *E. coli.*
2. Co-expression ButCoAT with reverse β-oxidation cycle enzymes can induce the production of butyrate in a non-butyrogenic *E. coli* strain.
3. Optimization of SCFAs secretion by bacteria required changes in a number of parameters such as the culture medium, presence of oxygen, trace elements such as vitamin B5 and glucose as well as induction variables such as time of induction and amount of inducers.
4. A number of kinases important for cell proliferation and stress response were differentially activated in cells incubated with conditioned medium from SCFA producing bacteria.

Several questions remain to be answered in this study:

- Effects of the ketone body precursors that are produced due to the catalytic activity of reverse β-oxidation cycle enzymes. Recent studies indicate that ketogenic diets may increase metabolism and protect from metabolic syndrome. Indeed, recently, the ketone body 3-hydroxybutyrate was shown to influence self-renewal and lineage decisions in intestinal stem cells (Cavaleri and Bashar 2018). This ketone body was also produced by the engineered

bacterial strain generated in the current study. It remains to be seen whether bacterial ketone bodies can similarly influence cell fate decisions in the gut.

- SCFAs can alter the expression profile of various genes related to inflammation, proliferation, barrier function and differentiation in the gut. Many of these genes are regulated through the histone deacetylase inhibitory activity of SCFAs. However, whether this is the only mechanism for regulation remains to be examined.

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APPENDICES

MATERIALS USED FOR WESTERN BLOT

10X TRANSFER BUFFER

0.25 M Tris

1.92 M Glycine

pH 8.3 in 1L dH2O

1X TRANSFER BUFFER

100 ml 10X Transfer Buffer

200 ml Methanol

700 ml dH2O

SDS-PAGE RUNNING BUFFER

25 mM Tris

190 mM Glycine

0.1% SDS

10% SEPARATING GEL MIX

4.1 ml dH2O

2.5 ml 4X Separating Buffer (10% SDS, 1.5M Tris, pH 8.8)

3.33 ml Acrylamide/Bis Solution (SERVA, Germany)

100 µl 10% Ammonium Persulfate (APS)

10 µl TEMED

4% STACKING GEL MIX

3.1 ml dH2O

1.25 ml Stacking Buffer (10% SDS, 1.5M Tris, pH 6.8)

650 µl Acrylamide/Bis Solution (SERVA)

50 µl 10% APS

5 µl TEMED

MILD STRIPPING BUFFER

15 g GLYCINE

1 g SDS

10 ml Tween-20

pH 2.2 in 1L dH2O

TBS-T

50mM Tris-HCl pH 7.4

150mM NaCl

0.1% Tween-20 is added to 1X TBS before use

6X SAMPLE LOADING DYE

12% SDS

30% β-Mercaptoethanol

30% Glycerol

0.012% Bromophenol Blue

0.375 M Tris-HCl pH 6.8