Butyrate, A Major Bacterial-derived Metabolite: Understanding of Butyrate Metabolism in Cancerous Colonocytes

Anna Han

University of Tennessee, Knoxville, ahan3@vols.utk.edu

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I am submitting herewith a dissertation written by Anna Han entitled "Butyrate, A Major Bacterial-derived Metabolite: Understanding of Butyrate Metabolism in Cancerous Colonocytes." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutritional Sciences.

Dallas R. Donohoe, Major Professor

We have read this dissertation and recommend its acceptance:

Jay Whelan, Zhao Ling, Todd Reynolds

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)
Butyrate, A Major Bacterial-derived Metabolite: Understanding of Butyrate Metabolism in Cancerous Colonocytes

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The University of Tennessee, Knoxville

Anna Han
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ABSTRACT

The prohibitory effects of dietary fiber in colorectal cancer have been identified, although the detailed mechanism of this beneficial effect remain unclear. Butyrate, a fermentation metabolite of fiber, has anti-cancer properties by inhibiting cell proliferation and increasing cell apoptosis in cancer cells. A major mechanism, whereby butyrate exerts anti-cancer effects in colorectal cancer, is its role as an HDAC inhibitor. Moreover, it has been suggested that the metabolic fate of butyrate is significantly related to its role as an HDAC inhibitor. Therefore, understanding butyrate metabolism in cancerous colonocytes sheds important light on how butyrate has its selective and inhibitory effects toward colorectal cancer.

This dissertation reports (1) colorectal cancer cells exhibit reduced ability to oxidize butyrate; (2) the mechanisms of butyrate oxidation are carnitine-dependent and carnitine-independent in colorectal cancer cells; (3) the Warburg effect, inactivation of pyruvate dehydrogenase (PDH), is a critical event to repress the carnitine-dependent butyrate oxidation in colorectal cancer cells. Also, this dissertation further describes that (1) butyrate suppresses its own oxidation by regulating short-chain acyl dehydrogenase (SCAD) levels in colorectal cancer cells; (2) butyrate acts as an HDAC inhibitor and (3) selectively inhibits HDAC 1 in order to suppress SCAD expression in colorectal cancer cells.

These findings bridge the important relationship between butyrate metabolism and its epigenetic role in order to explain its inhibitory effects in colorectal cancer cells. Also, the results raise a key question (Why is butyrate
regulation in its own oxidation in colorectal cancer cells?) for future studies that may discover other mechanisms of the preventive effects of butyrate in colorectal cancer.
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INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed and mortal cancer in the United States (American Cancer Society, 2016). The approximate economic burden of CRC including drug therapy is up to $40,000 per month (Latremouille-Viau et al., 2016; Meropol & Schulman, 2007). Most risk factors of CRC are highly associated with modifiable lifestyles, such as diet, alcohol consumption, smoking, physical activity and obesity (Johnson et al., 2013). Of these risk factors, dietary factors play a significant role in CRC incidence, and the modifications of food patterns might decrease CRC risk up to 70% (Haggar & Boushey, 2009).

The protective function of dietary fiber has been emphasized through many epidemiological, clinical and experimental studies, even though it is controversial. A diet high in dietary fiber (DF) has a beneficial impact on colon health and is proposed to decrease the likelihood of developing (Blackwood, Salter, Dettmar, & Chaplin, 2000; Liu, 2003; Mudgil & Barak, 2013; Vargas & Thompson, 2012). Currently, the critical roles of the gut microbiota and its derived metabolites toward colonic health and CRC development has been the main focus in CRC research (Holmes, Li, Athanasiou, Ashrafian, & Nicholson, 2011; Louis, Hold, & Flint, 2014). DF escapes digestion in the small intestine and is metabolized in the colon by gut microbiota (Gropper & Smith, 2012; Tungland & Meyer, 2002). Through fermentation, DF gives rise to short chain fatty acids (SCFAs) and gases as end-products that have various advantageous roles for colonic and host health (Blackwood et al., 2000; Macfarlane & Macfarlane, 2012).
Among the microbial-derived metabolites, butyrate plays an important role in colonic health and has anti-cancer effects (Havenaar, 2011). Primarily, butyrate is the preferred energy substrate for colonocytes and also functions as a histone deacetylase (HDAC) inhibitor (Hamer et al., 2008). As an HDAC inhibitor, butyrate causes epigenetic modifications of genes involved in cell proliferation, apoptosis and differentiation and reduces growth of tumor cells (Leonel & Alvarez-Leite, 2012; Tan et al., 2014). However, the obvious opposing roles of butyrate in cell proliferation in normal versus cancerous colonocytes has been observed and referred to as the “butyrate paradox” (Hague, Singh, & Paraskeva, 1997; Leonel & Alvarez-Leite, 2012). Recent evidence indicates that the metabolic transformation of cancerous colonocytes to utilize more glucose over butyrate (the Warburg effect) results in a change of butyrate’s metabolic fate and function (Bultman, 2014; Donohoe, Curry, & Bultman, 2013). Also, a more complicated role of butyrate in histone acetylation has been described (Donohoe et al., 2012). If butyrate is oxidized as an energy source, it is involved in histone acetylation as a cofactor for histone acetyltransferase (HAT). However, if butyrate is accumulated in the nucleus due to the Warburg effect, it acts as an HDAC inhibitor.

Recent evidence has also shown that reduced gene expression that related to mitochondrial metabolism in the tumor tissue from CRC patients might bring the change of butyrate metabolism in cancerous colonocytes (Birkenkamp-Demtroder et al., 2002). For example, short-chain acyl-CoA dehydrogenase (SCAD), an enzyme functioning associated with the first step of butyrate
oxidation is decreased in CRC. This may allude to a reduced ability in cancerous colonocytes to oxidize butyrate, which would also impact the molecule’s epigenetic roles. Therefore, a better understanding of mechanisms that regulate butyrate oxidation in cancerous colonocytes is necessary to unravel how butyrate exerts its selective and inhibitory effects toward colorectal cancer. However, there has been less understanding regarding the mechanism of butyrate oxidation in cancerous colonocytes and the link between SCAD and butyrate oxidation in cancerous colonocytes.

Thus, this dissertation begins with general information about CRC, the role of dietary fiber in CRC, and overall knowledge about butyrate. Chapter II focuses on the mechanisms of butyrate oxidation in cancerous colonocytes by emphasizing carnitine-dependent oxidation mechanisms. Chapter III focuses on butyrate’s function in its own metabolism in cancerous colonocytes by describing the role of SCAD.
CHAPTER I

LITERATURE REVIEW
1.1 Colorectal cancer

Colorectal cancer (CRC) is one of the most common and deadly cancers worldwide, along with lung, breast and prostate cancer (Arnold et al., 2015; Siegel, Miller, & Jemal, 2016). In 2016, the American Cancer Society expects 16% new CRC cases and deaths in the United States (Siegel et al., 2016). The causes of CRC are most likely associated with an individual’s lifestyle, which includes diet, alcohol consumption, physical activity and smoking (Arnold et al., 2015; Arnold et al., 2016; Patel & De, 2016). Therefore, an understanding of the pathology and risk factors associated with CRC are critical components in the fight to decrease CRC incidence and mortality. Section 1 will provide comprehensive background about CRC (including a general introduction, molecular approach of carcinogenesis and risk factors).

1.1.1. General Introduction: Definition, Stages and Diagnosis of CRC

Colorectal cancer is the development of malignant cells in the colon and/or rectum (NCI, 2014). The TNM classification of malignant tumors (TNM) classifies CRC into four stages (Edge SB et al., 2010): Stage I - cancer has dispersed to submucosa and slightly invaded the muscle layer of the colon wall; Stage II - cancer has spread throughout the colon wall close to organs but not to lymph nodes; Stage III – cancer has dispersed to regional lymph nodes, but not to other normal organs; Stage IV- cancer has spread through blood and lymph circulation to distant organs.
Currently, various screening methods are used to diagnose and prevent CRC, and these methods can be used differently based on the CRC stages (Burt et al., 2013; Kuipers, Rösch, & Bretthauer, 2013). For early detection, fecal occult blood test (FOBTs) and fecal immunochemical test (FITs) can be applied, and a positive result from either test will lead to further screening, such as a sigmoidoscopy and colonoscopy. Another current tool for CRC diagnosis, involves the analysis of molecular biomarkers in a fecal samples, reflecting the mechanisms of malignant cells (Imperiale et al., 2014).

1.1.2. Molecular Development of CRC: Vogelgram of CRC

Colorectal cancer serves as an important disease model for understanding tumor progression and metastasis since gradual changes in phenotype are associated with known genetic alterations (Yeatman & Chambers, 2003). Therefore, insight into how molecular and metabolic alterations occur during the development of the disease may uncover novel treatment strategies or aid in CRC prevention.

In the large intestine, a monolayer of epithelial cells (i.e. stem cells, proliferating cells, and differentiated cells) makes up the basic colonic structural unit called the crypt. Stem and proliferating cells cover the bottom of the crypts, while differentiated cells are found in the upper two-thirds (Riccardo Fodde, Smits, & Clevers, 2001). CRC originates at this epithelium-layer of the mucosal crypts. Aberrant crypt foci (ACF) are the initial sign of colorectal neoplasia. Due to its ability to encircle other crypts, including normal and/or dysplasia cells, ACF
promotes the formation of a polyp (Riccardo Fodde et al., 2001). The adenomatous (dysplastic) polyp is a tumor that protrudes into the lumen of the colon causing an aberrant inter- and intracellular structure, but most ACF or adenomas never become cancerous (Riccardo Fodde et al., 2001). Additional genetic alterations in oncogenes and tumor-suppressor genes are required to develop neoplasia at the level of ACF and drive its further development into what would be considered cancerous (Markowitz & Bertagnolli, 2009).

The Vogelgram is a model of tumor progression to CRC that provides a better understanding of the molecular and genetic phenomenon, which occur in CRC development. This model addresses the relationship between acquired genetic mutations and the progression of CRC (Bellacosa, 2003). The mutations occur in genes involved in cell proliferation, differentiation and survival that lead to phenotypic changes needed to convert regular epithelium into an adenoma and eventually a carcinoma (Bellacosa, 2003). The Vogelgram representation of CRC is composed of four main genetic mutations as well as associated signaling pathways to yield each event in CRC development and includes adenomatous polyposis coli (APC), K-Ras, SMAD 2 and 4, and TP53 (Figure 1) (Bellacosa, 2003; R Fodde, 2002; Riccardo Fodde et al., 2001; Goel & Boland, 2012; Markowitz & Bertagnolli, 2009; Perše & Cerar, 2014). First, the adenomatous polyposis coli (APC) is a tumor-suppressor gene, and its mutation is the most common event in the initiation of CRC. APC is necessary to activate the β-catenin/Wnt signaling pathway which removes extra intracellular β-catenin and inhibit its movement into nucleus; hence, without functional APC, β-catenin/Wnt
signaling pathway is inappropriately activated. Thus, β-catenin is not degraded and moves to the nucleus, where it drives transcription of genes that promote the cell cycle, such as DNA-binding proteins of the T-cell factor (TCF) family resulting in gene transcription. The second major mutation occurs in K-Ras, which is an oncogene that, when mutated, plays a role in the promotion by activating mitogen-activated protein kinases (MAPK) pathway to increase cell proliferation and decrease apoptosis. The synergetic action between abnormal APC and K-Ras is required to form large adenomas in CRC. SMAD 2 and 4 are pivotal tumor-suppressor genes belonging to the growth-suppressing transforming growth factor β (TGFβ) pathways, which results in slowing cell growth. Therefore, mutations in these two genes play a role in the malignant transformation and expansion of CRC. Lastly, TP53 is another critical tumor-suppressor gene in CRC development. Inactivation of p53 leads to the loss of checkpoint for DNA damage during the cell cycle after G phase, increasing proliferation and reducing apoptosis and promoting malignant tumorigenesis. The most important thing to understand about the development and progression of CRC is that it is not a mutation in any single gene, but a combination of all of them that drives the disease.

1.1.3. Risk Factors of CRC

The risk factors of CRC include demographic factors (age and gender), medical conditions (family history, individual history with inflammatory bowel disease (IBD) and metabolic diseases), lifestyle-related factors (physical activity,
smoking and alcohol consumption) and dietary factors (Figure 2). Behavioral and environmental modification in lifestyle-related factors such as diet has been suggested to influence CRC incidence.

1.1.3.1. Age

According to the National Cancer Institute (NCI), incidence of CRC increases after the age of 40 (45-54 years: 12.0%) as compared to those under 40 years old (20-24 years: 1.1%, 35-44 years: 3.8%). The likelihood of CRC occurrence rises aggressively after the age of 50 (55-84 years: 71.1%, +84 years: 12.1%) (Horner et al., 2009). Even though the age of 50+ is an apparent risk factor for CRC, current incidence and mortality has been decreasing in recent years in these populations due to the early detection by routine colonoscopy (Zauber et al., 2012). This trend holds true for other countries that have adopted colonoscopy as a primary screening tool (Kaminski et al., 2010; Patel & De, 2016).

Conversely, an increasing trend for CRC is being observed in the younger population (20-49 years) (Ahnen et al., 2014; O'Connell, Maggard, Liu, & Etzioni, 2003). The incidence of CRC has been rising, and becoming one of the most commonly diagnosed cancers in this age group (Fairley et al., 2006). This upward pattern among younger generation may be due to the fact that they are not generally recommended for screenings of CRC despite their high risk from consuming a Westernized-diet (Patel & De, 2016; Yusof, Isa, & Shah, 2012).
1.1.3.2. Gender

The impact of gender on CRC incidence and mortality has not been clearly established. However, considering gender as a risk factor along with age, excess body weight and obesity, there have been higher positive associations with males in CRC incidence compared to females. Many studies have reported that men have higher rates of CRC incidence and mortality (age-adjusted) compared to women (Brenner, Hoffmeister, Arndt, & Haug, 2007; Brenner, Hoffmeister, & Haug, 2008; Matanoski, Tao, Almon, Adade, & Davies-Cole, 2006). In addition, the correlation between gender (age-adjusted) and advanced neoplasia in the colon presents that men have a greater risk for advanced colorectal neoplasia across all age groups than women (Nguyen, Bent, Chen, & Terdiman, 2009). Epidemiological studies also report that the risk of CRC in overweight or population with high body mass index (BMI) was significantly higher in males compared to females (Brändstedt et al., 2012; Johnson et al., 2013). The reason for the gender differences is still unclear, but the differences in genetic background, hormones and fat distribution between male and female have been suggested as rationales for gender-dependent differences (EGiovannucci, 2002; McMichael & Potter, 1980; Press et al., 2008). In our studies, HCT116 colorectal cancer cells represent an in vitro model that is routinely used. These cells were derived from a 53-old male with a colorectal carcinoma.
1.1.3.3. Family history

Approximately 20% of CRC cases come from the patients who have a previous family history of this disease (Fund & Research, 2007; Haggar & Boushey, 2009). This signifies the important genetic component associated with CRC development. The susceptibility of CRC is increased up to 8 times in a first-degree relative (i.e. parents, siblings, or offspring) (Haggar & Boushey, 2009; Johns & Houlston, 2001; Johnson et al., 2013). Moreover, sibling risks are higher than parent-offspring risks (Carstensen, Soll-Johanning, Villadsen, Søndergaard, & Lynge, 1996; Maire et al., 1984). Since early onset of CRC is mainly derived from genetic alterations, the reasonable explanation for this strong correlation between family history and CRC can be attributed to gene inheritance patterns (Haggar & Boushey, 2009). In conjunction with genetic inheritance, communal environments among family members also contributes to CRC (Slattery, 2000).

1.3.3.4. Individual History: Inflammatory Bowel Disease

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn’s disease, is considered as a predisposing risk factor for CRC (Haggar & Boushey, 2009). UC is a chronic disease characterized by inflammation and colonic/rectal ulcers and Crohn’s disease is also associated with inflammations; however, it can occur in any part of the gastrointestinal tract (GI) (NDDIC, 2013; NIDDK, 2014). The overall risk of developing CRC in IBD patients relies on the duration, age of onset, and the severity of the disease (Haggar & Boushey, 2009; Johnson et al., 2013; Mellemkjær et al., 2000). Therefore, patients who are
diagnosed with IBD are recommended to have regular CRC screenings (Haggar & Boushey, 2009; Munkholm, 2003). For example, an early finding of IBD with colonoscopy screening reduces CRC incidence (Eaden, Abrams, Ekbom, Jackson, & Mayberry, 2000). In addition, treatment with 5-aminosalicylic acid (5-ASA) decreases CRC incidence among IBD patients (Munkholm, 2003). Thus, early detection and treatment of IBD can reduce its progression to CRC. It is important to recognize that IBD does not cause CRC (i.e. not an initiator), but rather is a promotor becoming the chronic through evaluated inflammatory environment.

1.3.3.5. Metabolic Disease: Obesity and Diabetes

Obesity is a critical risk factor that increases the prevalence of other metabolic diseases such as type II diabetes, hypertension and cardiovascular disease (Eckel, Grundy, & Zimmet, 2005). In addition, there is ample evidence that indicates that obesity is strongly correlated with the pathology of certain cancers, and a risk factor for colon cancer (Calle & Kaaks, 2004). According to epidemiological studies, a greater body mass index (BMI) and waist circumference (WC) are positively linked with CRC (Ma et al., 2013; Patel & De, 2016). For instance, individuals who have a BMI less than 25 (BMI ≤ 25kg/m²) have a lower likelihood of CRC development compared to those with BMI greater than 30 (BMI ≥ 30kg/m²) (Huxley et al., 2009). Although the role of obesity in CRC incidence is not fully understood, abnormal metabolic changes including insulin resistance, leptin resistance and chronic inflammation have been
identified as underlying factors that promote the positive association between obesity and CRC (Ma et al., 2013).

Along with obesity, diabetes is a metabolic disease that has been correlated with CRC development (Flood, Strayer, Schairer, & Schatzkin, 2010; Huxley et al., 2009). For example, people with diabetes have a 19% higher risk of developing CRC compared to non-diabetics (He et al., 2010). Although the mechanistic link between diabetes and CRC is unclear, hyperinsulinemia is considered a direct causal factor due to the role insulin and insulin growth factor-1 (IGF-1) have in tumor cell proliferation (Deng, Gui, Zhao, Wang, & Shen, 2012; Edward Giovannucci et al., 2010). It is difficult to establish the separate contributions from obesity and diabetes as risk factors for CRC due to their direct influence on each other.

1.3.3.6. Lifestyle-related Factors: Physical activity, Smoking and Alcohol Consumption

Lifestyle-related factors that impact the development of CRC include physical activity, smoking and alcohol consumption (Haggar & Boushey, 2009). Since these factors are modifiable, any knowledge toward how each contributes in CRC development is pivotal in prevention.

First, participating in physical activity reduces the likelihood of developing CRC (Haggar & Boushey, 2009; Johnson et al., 2013). When participating in regular physical activity, the risk of CRC is decreased by 24% (Wolin, Yan, Colditz, & Lee, 2009). Several physiological mechanisms on how regular physical
activity attenuates CRC incidence have been suggested, but are still not clearly understood (Haggar & Boushey, 2009; Harriss et al., 2009; Wu, Paganini-Hill, Ross, & Henderson, 1987). These mechanisms include improvement in body weight or BMI, improvement in insulin sensitivity, elevation of metabolic rate (includes increase in metabolic efficiency) and enhanced GI tract motility. Moreover, the frequency and intensity of physical activity to prevent CRC development is important (Haggar & Boushey, 2009). For example, the American Cancer Society (ACS) suggests at least 150 min of physical activity per week in order to reduce CRC (Grimmett, Simon, Lawson, & Wardle, 2015; Kushi et al., 2012).

Smoking has been known as the primary risk factor for lung cancer, but many studies also established a positive association between smoking and CRC (Haggar & Boushey, 2009; Johnson et al., 2013; Patel & De, 2016). A meta-analysis reports that 20% of smokers have an elevated risk for CRC in comparison with people who have never smoked (Tsoi et al., 2009). In addition, the frequency of smoking plays a role in CRC risk. Johnson et al. (2013) found an 11% increased risk of CRC from smokers with 17 cigarettes/month and 21% increased risk in CRC from smokers with 34 cigarettes/month compared to non-smokers (Johnson et al., 2013). The effects of smoking on CRC may be explained by the carcinogens produced from cigarettes which accelerate tumor formation and growth (Botteri, Iodice, Raimondi, Maisonneuve, & Lowenfels, 2008).
Like smoking, alcohol consumption is associated with an increased incidence of CRC. The frequency and quantity of alcohol consumption are also significantly related to CRC development (Haggar & Boushey, 2009; Wu et al., 1987). For example, the relative risk (RR) for CRC when consuming 10, 50, and 100 g/day alcohol consumption was 1.07, 1.38 and 1.82, respectively (Moskal, Norat, Ferrari, & Riboli, 2007). Heavy alcohol consumption may result in carcinogen formation in colon, failure in DNA repair and inefficient absorption of nutrients in the colon, making cells vulnerable to tumor formation and CRC (Fund & Research, 2007; Haggar & Boushey, 2009).

In summary, regular physical activity, smoking cessation, and limiting alcohol consumption are strongly suggested to prevent CRC development and reduce mortality.

1.3.3.7. Dietary Factors

Dietary factors strongly impact CRC development because dietary factors can act as pro- and/or anti- cancer factors at any point in the multiple stages of CRC pathology (Vargas & Thompson, 2012). Thus, dietary factors can be utilized successfully to decrease the CRC incidence and progression (Haggar & Boushey, 2009; Vargas & Thompson, 2012).

Convincing dietary factors include red and processed meat, dietary fibers and whole grains. High consumption of red and processed meat is a high risk factor for CRC (Chan et al., 2011; Edward Giovannucci et al., 1994). Chan et al. (2001) observed that the risk of CRC elevated by 29% for every 100 g/day of red
meat intake and 21% for every 50 g/day of processed meat consumption (Chan et al., 2011). In addition to the amounts of consumption, the frequency of red and processed meat intake also increase the risk of CRC development by 13% and 9% for five servings per week respectively (Johnson et al., 2013). Moreover, the cooking temperature and time of red and processed meat is also partially involved in the risk of CRC incidence (de Verdier, Hagman, Peters, Steineck, & Övervik, 1991). The potential mechanisms involved in CRC development have been found to involve the natural (heme) and artificial (nitrite-preserve) containing DNA-damaging N-nitroso compounds that occur in both red meat and processed meat (Babbs, 1990; Cross, Pollock, & Bingham, 2003; Santarelli, Pierre, & Corpet, 2008). The N-nitroso compounds have a direct cytotoxic and genotoxic impacts on the epithelium layer of colon and increase lipid peroxidation to promote colorectal tumorigenesis (Bastide et al., 2015; Bastide, Pierre, & Corpet, 2011).

There is ample evidence that demonstrates low consumption of dietary fiber increases the risk of CRC (Aune, Chan, et al., 2011; Park et al., 2005). For instance, low fiber intake (<10 g/day) increases the risk of CRC by 18% compared to higher intake (10-15 g/day) (Park et al., 2005). In addition, in reference to dietary fiber components, whole grains have a negative association with CRC incidence (Aune, Chan, et al., 2011; Jacobs Jr, Marquart, Slavin, & Kushi, 1998; Schatzkin et al., 2007). A meta-analysis using case-control publications found that high consumption of whole grains reduced the risk of CRC incidence by 20% (Jacobs Jr et al., 1998). The next section (1.2 The role of
dietary fibers in CRC) will discuss the relationship between dietary fibers and CRC in more detail, which will include the epidemiological findings and its underlying mechanisms that maybe involved in CRC prevention.

Limited, but suggestive dietary factors that impact CRC development include high-fat diets and low intake of fruits and vegetables. Various studies report that consuming a diet high in overall/total fat, saturated fat and animal fat increase CRC development (T. T. Fung et al., 2010; Graham, Dayal, Swanson, Mittelman, & Wilkinson, 1978). However, in other studies, there was no association between fat intake and CRC (Alexander, Cushing, Lowe, Sceurman, & Roberts, 2009; Edward Giovannucci et al., 1994). Importantly, because of the inconsistent findings in epidemiological studies, it is difficult to define the contribution of dietary fat content toward CRC incidence. However, a high intake of fat increases the total energy intake and the likelihood of obesity that is significantly associated with CRC (GRAHAM et al., 1988; Ma et al., 2013). It has been demonstrated that saturated fatty acids increase inflammation, change bile acid metabolism, and colon environments, which leads to an elevated risk for CRC development (Mathias & Dupont, 1979; Van Eldere, Celis, De Pauw, Lesaffre, & Eyssen, 1996). Therefore, lower consumption of saturated fat and animal fat is suggested to prevent CRC incidence.

Overall epidemiological studies regarding the relationship between fruit and vegetable intake and CRC incidence suggests there is an inverse correlation (Johnson et al., 2013; Patel & De, 2016). High intake of fruits and vegetables decreased the risk of CRC (Aune, Lau, et al., 2011). These findings were
controversial since other studies found no association between fruits and vegetables intake and CRC (van Duijnhoven et al., 2009). Although the beneficial effects of fruits and vegetables consumption on CRC incidence are still not clearly established, many experimental studies found that bioactive compounds and antioxidants in fruits and vegetables have anti-inflammatory, anti-cancer and tumor-suppressive effects (Liu, 2003; Steinmetz & Potter, 1996; Vanamala et al., 2006). Therefore, the intake of fruits and vegetables is recommended to reduce the incidence and mortality of CRC.

In conclusion, dietary factors play an important role in CRC incidence. In addition, because dietary factors can be manipulated, it is important to implement public awareness and intervention. Therefore, based on the findings from the epidemiological and clinical studies, Figure 3 describes dietary recommendations in order to prevent CRC incidence.

1.2 Role of Dietary Fiber in CRC

Although advancements in technology related to detecting CRC have lowered CRC incidence, it is still the third deadliest cancer in the United States (Kahi, Imperiale, Juliar, & Rex, 2009; Siegel et al., 2016). As described in section 1, dietary factors are important in decreasing CRC incidence, slowing progression and aiding in prevention (Vargas & Thompson, 2012). Although controversial, the importance of dietary fiber (DF) in CRC incidence has been established through many epidemiological studies (Baena & Salinas, 2015; Park et al., 2005; Romaneiro & Parekh, 2012; Trock, Lanza, & Greenwald, 1990).
However, special attention has been devoted to the relationship between CRC, DF, and the functions of the gut microbiota (Davis & Milner, 2009; Sears & Garrett, 2014; Zackular et al., 2013). For example, the metabolism of DF through the gut microbiota is highly associated with colonic health, the development of CRC, and the general health of an individual (Cani & Delzenne, 2009; Sekirov, Russell, Antunes, & Finlay, 2010). Section 2 will present a general introduction of DFs as well as a summary of previous studies regarding the role of DFs in CRC incidence. In addition, the general information for gut microbiota and its roles in CRC will be described.

1.2.1. General Introduction: Definition, Classification and Physiological roles of DF

It is difficult to describe a single, simple definition of DF because there is no standard definition and it varies between organizations (Table 1). According to current definitions, the term ‘dietary fiber’ compromises a broad spectrum of information on DF from the sources (plants and animal substances), chemical properties, and physiological effects to origin (natural and synthesized). These diverse definitions of DF can be problematic due to their inability to provide unified guidelines for DF in food labeling, references of nutrients values, and health claims (Cummings, Mann, Nishida, & Vorster, 2009). In 2008, the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) addresses ‘an agreed definition’ of DFs in order to impose a more clear standard (Table 1). (Alimentarius, 2008).
Dietary fiber come from natural and/or artificial sources. As listed in Table 2, naturally-occurring DF includes indigestible-polysaccharides (non-starch polysaccharides, cellulose, hemicellulose, pectin, β-glucans, gums and mucilages), resistant oligosaccharides (inulin and fructo-oligosaccharides), analogous carbohydrates (resistant -starch and -dextrin), lignin, constituents of plants (suberin, waxes and cutin), algal materials, and animal substances (chitin, chitosan, collagen and chondroitin). Artificial DF include resistant oligosaccharides (fructo-oligosaccharides and galacto-oligosaccharides) and analogous carbohydrates (resistant -starch and -dextrin, modified cellulose and polydextrose) (Gropper & Smith, 2012; Mudgil & Barak, 2013; Tungland & Meyer, 2002). The general classifications of DF rely on their origins and/or physicochemical properties (Table 3) (Blackwood, Salter, Dettmar, & Chaplin, 2000; Joanne Slavin, 2013; J. L. Slavin & Lloyd, 2012; Tungland & Meyer, 2002). However, some studies classify DF based on their physiological effects and their physicochemical properties (J. L. Slavin & Lloyd, 2012). Physicochemical properties of DF include solubility, viscosity and fermentability, and each of these properties is commonly associated with each other (Gropper & Smith, 2012; Raninen, Lappi, Mykkänen, & Poutanen, 2011; Tungland & Meyer, 2002). For example, soluble fiber is more likely to have high viscosity and fermentability; whereas insoluble fiber has relatively low viscosity and poor and/or partial fermentability (Table 3).

Major physiological effects of DF can be described as: improvements of blood lipid and glucose levels, laxation, overall improvement of colonic health
and decreased CRC development (Anderson et al., 2009; Blackwood et al., 2000; Gropper & Smith, 2012; Kendall, Esfahani, & Jenkins, 2010; Mudgil & Barak, 2013; Raninen et al., 2011; J. L. Slavin & Lloyd, 2012; Tungland & Meyer, 2002). Figure 4 shows the physiological effects of DF and their underlying mechanisms. In addition, many studies demonstrate that DF have a beneficial impact on cardiovascular disease, diabetes, and body weight, and appetite (Anderson et al., 2009; Kristensen & Jensen, 2011; Joanne Slavin, 2013; J. L. Slavin, 2005).

According to the Institute of Medicine (IOM), an adequate intake (AI) of DF is 14 g of fiber/1000 kcal from age one and above (J. L. Slavin & Lloyd, 2012; Stipanuk & Caudill, 2013). The conversion of AI is based on median energy intake and is equal to 25 g/day for women and 38 g/day for men ages 19-50 years (Stipanuk & Caudill, 2013). However, the current average DF consumption of Americans is ~15 g per day, which fails to meet the AI levels (JL Slavin, 2008). Therefore, food choices is pivotal in order to meet the recommended levels of DF and reduce the risk of chronic diseases such as CRC. Foods rich in fiber that help meet the AI levels include whole grains, legumes, fruits and vegetables (Stipanuk & Caudill, 2013).

1.2.2. A Summary of Previous Studies: The Roles of DF in CRC

Although DF have diverse physiological roles, there has been a large focus on the effects of DF on general colonic health, which may aid in the decrease in CRC incidence (Figure 4). In the last decade, epidemiological
studies have indicated that high intakes of DF prevent CRC, but some studies have suggested that there is no and/or weak association between DF intake and CRC risk (Anderson, Smith, & Gustafson, 1994; Baena & Salinas, 2015; Howe et al., 1992; Park et al., 2005). Moreover, the correlation between the intake of DF and CRC depends on which variables are applied such as age and/or sources of DF. Table 4 summarizes these epidemiological studies regarding the intake of DF and its impact on CRC incidence (Aune, Chan, et al., 2011; Bingham et al., 2003; Dahm et al., 2010; Fuchs et al., 1999; Howe et al., 1992; Mai et al., 2003; Michels et al., 2000; Park et al., 2005; Terry et al., 2001; Trock et al., 1990).

Based on evidence regarding an inverse relationship between DF and CRC, studies are categorized as protective effects'; 'no and/or weak association'; or 'changeable depending on variables'.

Discordant associations from previous publications may be due to several reasons. For example, limitations of epidemiological studies include: potential bias and confounders, such as the measurement of DF consumption; interactions with other nutrients; and potential influences from lifestyle factors such as smoking, physical activity, and body weight (Baron, 2005). Other conflicting results may be due to study design, such as population size and the follow-up period (Baron, 2005; Lawlor & Ness, 2003; Romaneiro & Parekh, 2012). In addition, multiple definitions and classification of DF across studies complicate the findings (Potter, 1990). Overall, the differences in DF sources in these studies make it difficult to generate consistent associations between DF and CRC risk (Potter, 1990; Romaneiro & Parekh, 2012; Terry et al., 2001). Other
variables include genetic background and the community of gut microbiota, which may contribute to conflicting findings from the epidemiological studies (Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012). For example, a change of gut microbiota structure by diet is associated with CRC development (Ahn et al., 2013; Sobhani et al., 2011). Moreover, the differences in gut microbiota between healthy and CRC subjects indicate the important roles of gut microbiota in CRC risk (Clemente, Ursell, Parfrey, & Knight, 2012). Therefore, failure to consider gut microbiota differences in the subjects can cause discordant results.

1.2.3. Gut Microbiota: Definition, Physiological Functions and Importance

Microbiota, microflora and normal flora are all terms to describe the gathering of microorganisms (bacteria, archaea, viruses, and unicellular eukaryotes) living in the human body (Sekirov et al., 2010). The majority of microbiota is composed of bacteria in the human body, and most of them colonize in the gastrointestinal tract (GI), especially the colon. These organisms have been defined as gut microbiota. Through the Human Microbiome Project (National Institutes of Health, 2008), the composition and functions of gut microbiota have been identified and characterized (Gill et al., 2006; Peterson et al., 2009). Approximately 1000 different species of bacteria exist in the colon, and these bacteria produce saccharolytic enzymes that metabolize undigested nutrients (Joanne Slavin, 2013; Tungland & Meyer, 2002). DF such as pectin, guar gum, fructo-oligomers, and resistant starch are the primary substrates for this fermentative metabolism by colonic bacteria (Blaut, 2002; Tungland & Meyer,
These are fermented into various end-products such as short chain fatty acids (SCFAs; acetate, propionate and butyrate) and gases (H₂, CO₂ and CH₄) which impact various physiological mechanisms to maintain host health (Holmes, Li, Athanasiou, Ashrafian, & Nicholson, 2011; Kinross, Darzi, & Nicholson, 2011; Macfarlane & Macfarlane, 2012). For example, the metabolites produced by gut microbiota help maintain epithelial homeostasis, regulate lipid metabolism, promote nutrient digestion and absorption and mediate immune-cell development (Holmes et al., 2011). Figure 5 describes the effects of bacterial-derived metabolites and their underlying mechanisms (Blackwood et al., 2000; Macfarlane & Macfarlane, 2012; Sekirov et al., 2010; Joanne Slavin, 2013).

Current publications support the notion that SCFAs orchestrate the central effects of gut microbiota for host and colonic health. Acetate (C2), propionate (C3) and butyrate (C4) are the major SCFAs produced by bacterial-fermentation in the colon with concentrations ranging from 60 to 150 mM in humans (Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987; Nordgaard & Mortensen, 1994). SCFAs are involved in both host energy metabolism and colonic homeostasis as a major energy source. Acetate and propionate are absorbed and transported to the liver and other tissues for metabolism to ATP (Blaut, 2002). Butyrate is directly absorbed by colonic epithelial cells and utilized as the primary energy source for colonocytes (Donohoe et al., 2011; Hague, Singh, & Paraskeva, 1997; Hamer et al., 2008). Additionally, colonic bacteria can utilize their self-produced SCFAs as energy sources. Therefore, SCFAs produced by gut microbiota are associated with the structural and homeostatic
balance that is an important component toward achieving a healthy colonic environments (Topping & Clifton, 2001). Since SCFAs are weak acids, they lower intestinal pH resulting in a decrease risk of CRC incidence (Hamer et al., 2008; Tungland & Meyer, 2002). Lower intestinal pH leads to diminished carcinogenesis via altered bile acid metabolism, peptide degradation, and formation of toxic compounds in the colon.

Extensive research has been conducted to identify the contributions of specific colonic microbe/microbial groups or microbial-derived molecules to host health and diseases (Kinross et al., 2011; Sekirov et al., 2010). Kinross et al. (2011) mentioned that gut microbiota have been directly associated with multiple diseases including circulatory disease, obesity, IBDs and CRC. Therefore, an interruption of colonic- and gut microbial-homeostasis is significantly related to the development of those diseases (Kinross et al., 2011). Factors that change gut microbiota and its resulting metabolites are the host’s physiological status (age, stress, health condition and genotype), diet (high-fat and DF) and environmental conditions, such as antibiotic therapy (Figure 6) (Blackwood et al., 2000; Joanne Slavin, 2013). Of these factors, the role of diet is the most critical since diet directly influences the composition of gut microbiota and the production of microbial-derived metabolites (Scott, Gratz, Sheridan, Flint, & Duncan, 2013). A high-fat diet decreases total gut microbial levels and increases Gram-negative bacteria (Holmes et al., 2011). In addition, dietary fructose-oligomer and -polymer beneficially alter the bacterial compositions by increasing Bifidobacteria, whereas resistant starch prompts the growth of anaerobic Clostridia (Jaskari et al., 1998;
Distinct DFs have different effects on the composition and quantity of bacterial-derived metabolites. For instance, guar gum generates the highest levels of total SCFAs compared to other DF, while ispaghula produces higher level of butyrate rather than other SCFAs (Blackwood et al., 2000).

In conclusion, gut microbial homeostasis is a significant factor in maintaining a healthy colonic environment, which in turn decreases the risk of CRC. Diet plays a critical role in helping determine gut microbiota and maintaining this colonic homeostasis.

1.2.4. Gut Microbiota and CRC

Accumulating findings emphasize the involvement of gut microbiota in CRC development through physiological mechanisms resulting from microbial-derived metabolites and from an alteration of metabolic composition (Holmes et al., 2011; Kinross et al., 2011).

First, the structure of gut microbiota contributes to the etiology of CRC (Hagland & Søreide, 2015; O'Keefe, 2008). For example, alterations in the diversity and number of gut microbiota provide possible mechanisms to develop CRC (Figure 6). These mechanisms include the production of bacterial-derived carcinogenic metabolites, increases in disadvantageous bacteria (ex. *Streptococcus* spp., *Escherichia Coli*, *Fusobacterium nucleatum*, *Clostridium* and *Bacteroides*) and decreases in beneficial species (ex. *Bactobacillus*) (O'Keefe, 2008; Sobhani et al., 2011). According to Louis et al. (2014), particular
pathogenic bacteria (ex. \textit{Clostridium difficile}, \textit{Fusobacterium} spp., and \textit{Campylobacter} spp.) promote CRC development via pro-inflammatory interactions with mucosal tissues, such as reactive oxygen species (ROS) production and subsequent DNA damage (Louis, Hold, & Flint, 2014). In addition, elevation of sulfur-reducing bacteria (\textit{Desulfovibrio Vulgaris}) accompanied by high meat consumption yields carcinogenic compounds such as hydrogen sulfide, which causes DNA damage, mucosal inflammation, and cell death (Bultman, 2014a; O'Keefe, 2008). Hence, understanding the functions of individual bacteria and the potential carcinogenic metabolites produced by these bacteria during physiological conditions is pivotal in preventing CRC (Sears & Garrett, 2014; Sobhani et al., 2011).

Next, as mentioned in the previous section, gut microbiota plays a significant role in colonic- and microbial- homeostasis through the production of metabolites that reduce CRC development (Figure 5). More than any other bacterial-derived metabolites, butyrate has been highlighted as a tumor-suppressive molecule due to its specialized biochemical role (Davie, 2003; Havenaar, 2011; Tong, Yin, & Giardina, 2004). By acting as a histone deacetylase inhibitor (HDACi), butyrate suppresses cell proliferation and increases apoptosis in CRC \textit{in vivo} and \textit{in vitro} (Donohoe, Collins, et al., 2012; Hague et al., 1993; Holmes et al., 2011; Macfarlane & Macfarlane, 2012). Interestingly, the overall reduction of butyrate-producing bacteria (ex. \textit{Roseburia}, \textit{Fecalibacterium prausnitzii}, and \textit{Eubacterium rectale}) has been observed when comparing the fecal and tissue samples from healthy individuals and CRC.
patients (Balamurugan, Rajendiran, George, Samuel, & Ramakrishna, 2008; Bultman, 2014a; Chen et al., 2013; Wang et al., 2012). In the next section (1.3 Butyrate), a more comprehensive review on butyrate, its functions, and its metabolism in both normal and CRC conditions will be addressed.

A diet intervention, particularly including DF and/or pre/probiotics, improves the community of gut microbiota and SCFAs production leading to the amelioration of tumor size and numbers in CRC progression (Chen et al., 2013; Davis & Milner, 2009; Zackular et al., 2013). Prebiotics and probiotics block the detrimental effects of gut microbiota and the potential carcinogenic mechanisms by introducing beneficial living bacteria strains (probiotics) or substrates (prebiotics) (Bultman, 2014a; Geier, Butler, & Howarth, 2006). In conclusion, targeting certain pathogenic bacteria and improvement of bacterial composition through the probiotic or prebiotics represents a promising preventative strategy toward CRC.

### 1.3 Butyrate

The gut microbiota produces diverse metabolites, resulting from the fermentation of DFs. Of these metabolites, special attention has focused on SCFAs due to their contributions in the maintenance of microbiota structure and colonic health (Figure 5). In particular, butyrate functions as the primary energy source for colonic epithelial cells and acts as an HDACi to regulate cell proliferation, apoptosis and differentiation (Davis & Milner, 2009; Havenaar, 2011). However, butyrate has the ability to impose contrasting effects on
cancerous colonocytes compared to normal colonocytes. This has been referred to as the 'butyrate paradox' (Bultman, 2014a; Leonel & Alvarez-Leite, 2012). Section 3 will provide more information regarding the origination of butyrate, its functions and metabolism, and explanations for the butyrate paradox.

1.3.1. Butyrate: Synthesis and Functions

In the colon, several bacteria species are responsible for synthesizing butyrate and are divided into two main groups according to enzymes in the last steps of their butyrate production pathway (Flint, Duncan, Scott, & Louis, 2015). Butyrate is produced by bacteria employing either butyryl-CoA: acetate-CoA transferases or less frequently, phosphotransbutyrylase and butyrate kinase as final enzymes of the pathway (Figure 7) (Flint, Duncan, Scott, & Louis, 2007; Louis et al., 2014). Eubacterium rectale, Roseburia spp., Coprococcus catus, Anaerostipes spp., Eubacterium hallii and Faecalibacterium prausnitzii are bacterial species that produce butyrate via the CoA-transferase pathway. Coprococcus eutactus and Coprococcus comes produce butyrate via the butyrate kinase pathway. In addition to these two main pathways, butyrate can be generated from other metabolites, this is sometimes referred to as cross-feeding. For instance, Eubacterium hallii and Anaerostipes caccae can convert acetate and lactate into butyrate (Pryde, Duncan, Hold, Stewart, & Flint, 2002).

The production of butyrate is affected by several factors, including an individual’s difference in gut microbiota, intestinal pH, sources of DF, and other bacterial-derived metabolites and environmental factors (biotic therapy) (Flint et
al., 2015; Guilloteau et al., 2010; Pryde et al., 2002). Appropriate intestinal pH is pivotal to maintain butyrate-producing bacteria and butyrate levels in the colon. For example, an in vitro fermentation study found that the population of butyrate-producing bacteria and the butyrate level was higher at pH 5.5, suggesting that lower intestinal pH helps to maintain a beneficial environment for butyrate-producing bacteria (Walker, Duncan, Leitch, Child, & Flint, 2005). Although most fermentable DF play a role in SCFAs productions, resistant starches and fructooligosaccharides are considered the most effective butyrogenic DF (Guilloteau et al., 2010; Topping & Clifton, 2001). Moreover, other metabolites such as acetate and lactate are critical in butyrate’s synthesis due to microbial community interaction and cross-feeding (Pryde et al., 2002).

The approximate butyrate concentration in the human colon ranges from 10 to 30 mM (total SCFAs concentration is 60-150 mM). The majority of the butyrate (95%) is rapidly absorbed into and utilized by colonic epithelial cells resulting in undetectable butyrate levels in portal blood (Louis et al., 2014; Pryde et al., 2002; Rémésy, Demigne, & Morand, 1992). Butyrate is mainly transported into colonocytes via monocarboxylate transport protein 1 (MCT1) (M Astbury & M Corfe, 2012). Once transported into the cell, butyrate undergoes mitochondrial β-oxidation to produce acetyl-CoA, which then enters the TCA cycle and is used for ATP production (Hagland & Søreide, 2015). β-oxidation of butyrate is modulated by several enzymes including short-chain acyl-CoA dehydrogenase (SCAD), enoyl-CoA hydratase, and short-chain hydroxyacyl-CoA dehydrogenase (SCHAD) (M Astbury & M Corfe, 2012). Of these enzymes, SCAD plays a
significant role in butyrate metabolism; hence, SCAD deficiency increases butyrate excretion and decreases catabolism up to 60% (Bhala et al., 1995; M Astbury & M Corfe, 2012). In addition, the deletion and/or mutation of SCAD results in inefficient metabolism of butyrate as an energy source (Augenlicht et al., 1999). Donohoe et al. (2011) observed that colonocytes of germfree mice (lacking bacterial-derived butyrate production) have lower expression of key enzymes necessary for the TCA cycle (Donohoe et al., 2011). Interestingly, the addition of butyrate allows germfree mice colonocytes to recover their defects in mitochondrial respiration and inhibit their autophagy. Moreover, this rescue is due to butyrate’s role as an energy substrate for colonocytes rather than as an HDACi (Donohoe et al., 2011). Later it was reported that the proximal colon of germfree mice showed lower ATP levels when compared to other tissues (Donohoe, Wali, Brylawski, & Bultman, 2012). However, the addition of a butyrate-fortified diets partially rescued the slowed cell cycle and stimulated oxidative metabolism in germfree colonocytes, thus suggesting that butyrate was a major factor in these two processes (Donohoe, Wali, et al., 2012). This evidence also illustrates butyrate’s primary role as an energetic substrate for colonocytes. Butyrate is also a crucial substrate for lipogenesis through the mitochondrial β-oxidation pathway and eventual conversion to cytosolic acetyl-CoA (Rémésy et al., 1992). Specifically, butyrate-derived acetyl-CoA in the mitochondria in combination with oxaloacetate (OAA) is used to generate citrate in the initial step of the TCA cycle. Citrate is then exported into the cytosol, where
it is broken back down into acetyl-CoA and OAA (Bultman, 2014b). The acetyl-CoA provides the initial backbone used in lipogenesis.

Another major function of butyrate is its role as an HDACi which regulates epigenetic modifications by increasing histone acetylation (Havenaar, 2011; Macfarlane & Macfarlane, 2012). HDACs inhibition relaxes the chromatin wrapped around histones, making DNA more accessible to transcription factors and eliciting a subsequent change in gene expression (Encarnacao, Abrantes, Pires, & Botelho, 2015). Butyrate has been deemed as the strongest HDACi among bacterial-derived SCFAs, which allows it to effectively reduce cell proliferation and regulate the expression of specific genes such as cyclin-dependent kinase, p21 and pro-apoptotic proteins, BAX and Fas (Bultman, 2014b; Davie, 2003). Most HDACs, except class III HDAC and class II HDAC 6 and 10, are suppressed by butyrate (Davie, 2003). As an HDACi, butyrate also protects against intestinal inflammation via suppression of NF-γB activation, inhibition of interferon-γ production and/or signaling, and upregulation of PPAR-γ, suppression of oxidative stress via reduction of H₂O₂-induced DNA damage, enhanced antioxidant glutathione activity and increase catalase and glutathione-S-transferase (Canani, Di Costanzo, & Leone, 2012; Hamer et al., 2008; Jacobs, Gaudier, Duynhoven, & Vaughan, 2009; Leonel & Alvarez-Leite, 2012; Scharlau et al., 2009). Butyrate can also exert beneficial effects in the intestinal barrier (Plöger et al., 2012). Due to the multiple effects of butyrate, it has received widespread attention as a general anti-CRC molecule.
Recent advances indicate that butyrate’s role in histone acetylation is more complicated than previously described (Bultman, 2014b; Donohoe, Curry, & Bultman, 2013). According to Donohoe et al. (2012), butyrate is involved in histone acetylation through two distinct mechanisms, ACL-dependent and/or ACL-independent, which relies on butyrate concentrations (Bultman, 2014b; Donohoe, Collins, et al., 2012). This dose-base mechanism is significant with regards to regulation of cell proliferation and/or apoptosis of colonocytes (Figure 8) (Bultman, 2014b; Donohoe, Collins, et al., 2012; Donohoe et al., 2013). For example, an ACL-dependent mechanism is more likely to occur at relatively low doses of butyrate (0.5 mM) where butyrate undergoes mitochondrial β-oxidation, producing acetyl-CoA via ACL. Although cytosolic acetyl-CoA is utilized to synthesize lipids, nuclear acetyl-CoA serves as a cofactor for histone acetyltransferases (HATs) (Wellen et al., 2009). In the nucleus, butyrate modifies histone acetylation, accounting for 75% of gene expression, which is mostly related to cell proliferation (Donohoe, Collins, et al., 2012). In contrast, at high doses (5 mM), butyrate accumulates in the nucleus and mediates histone acetylation as a HDACi (ACL-independent mechanism). The accumulation of butyrate at high concentrations is due to the limited oxidative metabolic ability of these cells (1-2 mM) (Andriamihaja, Chaumontet, Tome, & Blachier, 2009). As an HDACi, butyrate accounts for 75% gene expression (such as FAS and WNT10B) which are associated with apoptosis (Donohoe, Collins, et al., 2012).

Butyrate is believed to exist in a gradient of higher concentration in the upper side of the crypts and lower levels at the base of the crypts due to the
mucous flow (Donohoe, Collins, et al., 2012; Tan et al., 2014). According to the previous findings, the base of the crypts is composed of proliferative cells while the upper part of the crypts is filled with apoptotic cells for luminal exfoliation (Bultman, 2014b; Donohoe, Collins, et al., 2012; Yu, Wang, Wei, & Ni, 2012). The recent findings indicate that butyrate’s regulation of gene expression differences may be due to dose-dependent mechanisms (Donohoe, Collins, et al., 2012). Therefore, butyrate may maintain colonic epithelial cells homeostasis through these interesting mechanisms (ACL-dependent and/or ACL-independent) that regulate gene expression (Figure 8) (Bultman, 2014b; Donohoe, Collins, et al., 2012). Overall, the relationship between the metabolic fate of butyrate (being oxidized and/or accumulated) and its functions (i.e. HDACi) may account for the inconsistent butyrate actions in normal versus cancerous colonocytes (Donohoe et al., 2013; Hague et al., 1997; Leonel & Alvarez-Leite, 2012). The next section (1.3.2. cancer cell metabolism -The Warburg effect) will cover the metabolic characteristics of cancerous cells that may influence butyrate’s metabolism and its functions.

The ability of butyrate to regulate biological mechanisms also comes from interactions with G-protein-coupled receptors (GPCRs) that modulate signaling pathways (Louis et al., 2014). Butyrate can be a ligand for GPR41, GPR43, and GPR109A that are expressed along the entire GI tract (Guilloteau et al., 2010; Jacobs et al., 2009; Tan et al., 2014). GPR 43 and GPR 41 are primarily expressed in the intestinal endocrine L-cell, where it releases intestinal peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) (Kasubuchi, Hasegawa,
Hiramatsu, Ichimura, & Kimura, 2015). PYY suppresses the appetite as an neuroendocrine factor and GLP-1 stimulates the pancreas to secrete insulin (Gropper & Smith, 2012; Stipanuk & Caudill, 2013). Tolhurst et al. (2012) observed that butyrate triggers GLP-1 secretion in mixed colonic cultures in vitro (Tolhurst et al., 2012). In addition, administration of tributyrin, a butyrate prodrug, improves insulin resistance in obese mice (Vinolo et al., 2012). By interacting with GPR109A, butyrate also attenuates colonic inflammation and anti-inflammatory mechanisms via colonic immune cells (Kasubuchi et al., 2015). Thus, butyrate can enhance insulin sensitivity, mediate food intake and inhibit colonic inflammation as a ligand of GPCRs (Kasubuchi et al., 2015; Tan et al., 2014).

As an energy substrate, epigenetic regulator and GPCR ligand, butyrate can be involved in a variety of mechanisms related to colonic and host health (Figure 9). However, the most highlighted role of butyrate is as an HDACi, which is highly associated with its metabolic fate in colonocytes. Therefore, it is important to understand butyrate’s metabolic fate in colonocytes in order to identify its anti-cancer mechanisms against CRC.

1.3.2. Cancer Cell Metabolism -The Warburg Effect

Cancer cell metabolism is often described as involving the Warburg effect, which is characterized by rapid glucose utilization and lactate production (also known as aerobic glycolysis) (Warburg, 1956b). In aerobic glycolysis, most cancer cells rapidly metabolize glucose for ATP generation (~4 ATP/mol
glucose), rather than the more efficient pathway of oxidative phosphorylation (OXPHOS, ~36 ATP/mol glucose) (Cairns, Harris, & Mak, 2011; Vander Heiden, Cantley, & Thompson, 2009). In the 1950s, Otto Warburg hypothesized that mitochondrial dysfunction lead to this metabolic phenomenon in cancer cells; however, it has been demonstrated that most cancer cells maintain their normal OXPHOS ability (Garber, 2004; Rossignol et al., 2004; Warburg, 1956a). It has been suggested that the Warburg effect is the metabolic adaptation of the cancer cells in order to obtain abundant ATP production and biosynthetic advantages (Hsu & Sabatini, 2008; Kaelin Jr & Thompson, 2010).

Cancer cells consume high levels of energy and proliferate rapidly, requiring sufficient amounts of ATP as well as ample cellular components such as nucleotides, fatty acids, membrane lipids, and proteins availability for rapid growth (Hsu & Sabatini, 2008). Although aerobic glycolysis is less efficient regarding ATP production, the abnormal glycolytic rate of cancer cells can counteract this low productive efficiency (Feron, 2009; Vander Heiden et al., 2009). According to previous research, cells using aerobic glycolysis also have a high ratio of ATP/ADP and NADH/NAD exceeds the results from OXPHOS (DeBerardinis, Lum, Hatzivassiliou, & Thompson, 2008; Vander Heiden et al., 2009). In addition, this fast glycolytic rate allows the cancer cells to obtain the required building blocks needed for their rapid cellular expansions (Jones & Thompson, 2009). The TCA cycle is known as a main bioenergetic center as well as biosynthetic hub for the cells by supplying diverse precursors for nucleotides, lipids and protein synthesis (DeBerardinis, Sayed, Ditsworth, & Thompson, 2008;
As shown in figure 10, the high rate of aerobic glycolysis allows cancer cells to obtain biosynthetic intermediates via the TCA cycle (DeBerardinis, Sayed, et al., 2008; Feron, 2009; Jones & Thompson, 2009). The pentose phosphate pathway generates ribose-5-phosphate for nucleotide synthesis, and NADPH (nicotinamide adenine dinucleotide phosphate, reduced) for nucleotides and fatty acids synthesis (Jones & Thompson, 2009; Vander Heiden et al., 2009). In addition, cytosolic citrate is cleaved by ATP-citrate lyase (ACL) to supply acetyl-CoA, which is the lipogenic precursor for cholesterol and fatty acids synthesis and oxaloacetate (OAA) for non-essential amino acids (Feron, 2009; Hsu & Sabatini, 2008).

The mechanisms that permit cancer cells to achieve this metabolic transformation are stimulated by altering signaling pathways; mutations in oncogenes and tumor-suppressor genes; and modifying expressions of enzymes that impact metabolic flux rate and activity (Bensinger & Christofk, 2012; Dang & Semenza, 1999; DeBerardinis, Sayed, et al., 2008; Kim & Dang, 2006; Ward & Thompson, 2012). These molecular and cellular alterations cause the cancer cells to preferably use aerobic glycolysis rather than OXPHOS, resulting in an efficient bioenergetic and biosynthetic system for rapid growth and proliferation (Table 5) (Bensinger & Christofk, 2012; Cairns et al., 2011; DeBerardinis, Lum, et al., 2008; DeBerardinis, Sayed, et al., 2008; Feron, 2009; Hsu & Sabatini, 2008; Ward & Thompson, 2012). The current understanding behind metabolic transformation of cancer cells allows more specific therapeutic approaches against cancer (Tennant, Durán, & Gottlieb, 2010). For example, dichloroacetate
(DCA), an inhibitor of pyruvate dehydrogenase kinase, ameliorates tumor growth in vivo and in vitro studies (Michelakis, Webster, & Mackey, 2008).

Therefore, understanding the metabolic transformation of cancer cells may eventually lead to the development of therapies against CRC. In addition, an anti-CRC therapy like butyrate may be effective, but further research is needed to understand its metabolism and functions in cancerous colonocytes.

1.3.3. Butyrate Metabolism in Colorectal Cancer Cells

As the preferred energy substrate, butyrate increases cell metabolism and proliferation of normal colonocytes. However, in cancerous colonocytes, butyrate inhibits cell proliferation and induces apoptosis. This opposing effect of butyrate on the proliferation in normal versus cancerous colonocytes is known as butyrate paradox (Bultman, 2014a; Leonel & Alvarez-Leite, 2012). Previous publications emphasized that the butyrate paradox may result from the differences in butyrate concentration and exposure time across the studies and the cell’s ability to oxidize butyrate (Hamer et al., 2008; Leonel & Alvarez-Leite, 2012).

Since colonocytes metabolize butyrate as their primary energy source, metabolic transformation in cancerous colonocytes significantly changes butyrate’s metabolic fates (Hague et al., 1997). According to Donohoe et al. (2012), normal colonocytes metabolize butyrate through the mitochondrial oxidative pathway; however, cancerous colonocytes undergo high levels of glycolysis with a reduction in mitochondrial OXPHOS (the Warburg effect) (Figure 11) (Donohoe, Collins, et al., 2012; Donohoe et al., 2013). As a result,
butyrate accumulates in the nucleus and acts as an HDACi to control genes that inhibit cell proliferation and increase apoptosis (Donohoe et al., 2013). Interestingly, inhibition of the Warburg effect and addition of butyrate stimulates proliferation in a way that resembles normal colonocytes (Donohoe, Collins, et al., 2012). However, butyrate-stimulated proliferation was dependent upon low levels (0.5-1 mM), whereas butyrate reduces proliferation and increases apoptosis at high concentrations (2-5 mM) (Donohoe, Collins, et al., 2012). This was consistent with the previous observation that 1-2 mM is the oxidative capacity of these cells (Andriamihaja et al., 2009). Thus, at levels higher than 2 mM, butyrate accumulates in the nucleus instead of being oxidized, and acts as a HDACi both in normal colonocytes and cancerous colonocytes (Donohoe et al., 2013). These 0.5~5 mM concentration range in their studies is physiologically relevant because butyrate’s approximate concentration is ~30 mM in the lumen of colon (Donohoe et al., 2013; M Astbury & M Corfe, 2012). Overall, the metabolic fate of butyrate in colonocytes is significantly involved in its function as an energy source or HDACi.

Previous studies observed altered gene expression of the metabolic enzymes relating to the mitochondrial functions and butyrate β-oxidation in CRC (K. Y. Fung, Cosgrove, Lockett, Head, & Topping, 2012; Kitahara et al., 2001). One study investigated the gene expression in CRC throughout progression and different stages; they found genes involved in metabolism, particularly mitochondrial metabolism, are the most commonly altered (22%) (Birkenkamp-Demtroder et al., 2002). They also found that lipid metabolism related genes are
mainly down-regulated, and expression of SCAD is significantly decreased in all stages of CRC (Birkenkamp-Demtroder et al., 2002). Since SCAD catalyzes the first step of butyrate oxidation, decreased SCAD may influence butyrate metabolism and subsequent change in butyrate roles (M Astbury & M Corfe, 2012; van Maldegem et al., 2006).

In summary, the dysfunction of mitochondrial β-oxidation system and reduced gene expression of related enzymes in CRC may change butyrate metabolism in the colonocytes. Since the metabolic fate of butyrate in colonocytes is highly related to its function, further understanding of butyrate metabolism in cancerous colonocytes is strongly suggested to understand its role as an HDACi and ability to exert anti-cancer effects against CRC.
1.4 References


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Donohoe, Dallas R, Garge, Nikhil, Zhang, Xinxin, Sun, Wei, O’Connell, Thomas M, Bunger, Maureen K, & Bultman, Scott J. (2011). The microbiome and


Kitahara, Osamu, Furukawa, Yoichi, Tanaka, Toshihiro, Kihara, Chikashi, Ono, Kenji, Yanagawa, Renpei, ... Tsunoda, Tatsuhiko. (2001). Alterations of gene expression during colorectal carcinogenesis revealed by cDNA


Trock, Bruce, Lanza, Elaine, & Greenwald, Peter. (1990). Dietary fiber, vegetables, and colon cancer: critical review and meta-analyses of the epidemiologic evidence. *Journal of the National Cancer Institute, 82*(8), 650-661.


1.5 Appendix
Table 1 Definitions of dietary fiber

<table>
<thead>
<tr>
<th>Organization</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hipsley (1953)</td>
<td>Non-digestible constituents making up the plant cell wall.</td>
</tr>
<tr>
<td>FAO/WHO (1995)</td>
<td>The edible plant or animal material not hydrolyzed by the endogenous enzymes of the human digestive tract as determined by the agreed upon method.</td>
</tr>
<tr>
<td>AACC (2000)</td>
<td>The edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation.</td>
</tr>
<tr>
<td>NAS (2002)</td>
<td>Dietary fiber consisting of non-digestible carbohydrates and lignin that are intrinsic and intact in plants. Functional fiber consisting of isolated, non-digestible carbohydrates which have beneficial physiological effects in humans, and Total Fiber as the sum of Dietary fiber and Functional Fiber.</td>
</tr>
<tr>
<td>CAC (2006)</td>
<td>Defined dietary fiber as: carbohydrate polymers with a degree of polymerization not lower than three, which are neither digested nor absorbed in the small intestine. Dietary fiber consists of one or more of: edible carbohydrate polymers naturally occurring in the food as consumed; Carbohydrate polymers obtained from food raw material by physical, enzymatic, or chemical means; Synthetic carbohydrate polymers. (Continued on the next page)</td>
</tr>
</tbody>
</table>
### Table 1 Continued.

<table>
<thead>
<tr>
<th>Organization</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CCNFSDU (2008)</strong></td>
<td>Dietary fiber is carbohydrate polymers with 10 or more monomeric units, which are not hydrolyzed by endogenous enzymes in the small intestine of human beings and belong to following categories: Edible carbohydrate polymers naturally occurring in food as consumed; Carbohydrate polymers, which have been obtained from raw material in food by physical, enzymatic, or chemical means and which have been shown to have physiological effects of benefit to health by generally accepted scientific evidence to competent authorities; Synthetic carbohydrate polymers, which have been shown to have physiological effect of benefit to health by generally accepted scientific evidence to competent authorities.</td>
</tr>
</tbody>
</table>

The definitions of dietary fibers from the different organizations are listed. FAO/WHO; Food and Agriculture Organization of United Nations and World Health Organization, AACC; American Association of Cereal Chemists, NAS; National Academy of Science, CAC; Codex Alimentarius Commission, CCFNSDU; Codex Committee on Nutrition and Foods for Special Dietary Uses.
### Table 2 Dietary fiber in the diet

<table>
<thead>
<tr>
<th>Origin</th>
<th>Source</th>
<th>Fiber element</th>
<th>Sub- fiber element</th>
<th>Foods source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural</td>
<td>Plants</td>
<td>Indigestible - polysaccharides</td>
<td>Non-starch polysaccharides (NSP), Cellulose, Hemicellulose Sugar acids (pectin) β-glucans</td>
<td>fruits, vegetables, cereals, wheat bran, grains, nuts, legumes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resistant oligosaccharides</td>
<td>Frutans (inulin, fructo-oligosaccharides)</td>
<td>burdock, chicory, dandelion root, leeks, onions, asparagus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analogous carbohydrates</td>
<td>Resistant starch Resistant dextrin</td>
<td>carbohydrate-containing foods</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lignin</td>
<td>Lignin</td>
<td>fruits, vegetables, cereal grains</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Components of plants</td>
<td>Suberin, Waxes, Cutin,</td>
<td>fruits, vegetables</td>
</tr>
<tr>
<td>Algal</td>
<td>Indigestible-polysaccharides</td>
<td>Gums Mucilages</td>
<td></td>
<td>seeds plants, seaweed and plants extract</td>
</tr>
<tr>
<td>Animals</td>
<td></td>
<td>Chitin, Chitosan, Collagen, Chondroitin</td>
<td></td>
<td>yeasts, invertebrates</td>
</tr>
<tr>
<td>Artificial</td>
<td>Synthesized</td>
<td>Resistant oligosaccharides</td>
<td>Frutans : Fructooligosaccharides Galactooligosaccharides</td>
<td>fortified foods, carbohydrate-containing foods</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analogous carbohydrates (=carbohydrate polymers)</td>
<td>Resistant starch Resistant dextrin Modified cellulose Polydextrose</td>
<td>fortified foods, carbohydrate-containing foods</td>
</tr>
</tbody>
</table>

Dietary fiber is listing based on the origin (natural- or artificial- occurring). Then, it is categorized depend on the source (plants, algal, animals and/or synthesized). All fiber elements and sub-fiber elements are listed along with food sources.
Table 3 General classification of dietary fiber

<table>
<thead>
<tr>
<th>Physicochemical property</th>
<th>Degree and dietary fiber</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility</td>
<td>High</td>
</tr>
<tr>
<td>Pectin, ß-glucan, Gums,</td>
<td></td>
</tr>
<tr>
<td>Polydextrose, Inulin</td>
<td>Wheat bran, cellulose,</td>
</tr>
<tr>
<td></td>
<td>Some hemicellulose, Lignin</td>
</tr>
<tr>
<td>Viscosity</td>
<td>High</td>
</tr>
<tr>
<td>Pectin, ß-glucan, Gums,</td>
<td></td>
</tr>
<tr>
<td>Mucilages</td>
<td>Cellulose, Hemicellulose, Lignin</td>
</tr>
<tr>
<td>Fermentability</td>
<td>Well</td>
</tr>
<tr>
<td>Pectin, ß-glucan, Gums,</td>
<td></td>
</tr>
<tr>
<td>Inulin, Resistant starch,</td>
<td>Cellulose, Hemicellulose, Lignin</td>
</tr>
<tr>
<td>Non-starch polysaccharides (NSP), Resistant Oligosaccharides, Polydextrose, Chondroitin</td>
<td></td>
</tr>
</tbody>
</table>

Dietary fiber is differentiated regarding their physicochemical properties including solubility, viscosity and fermentability.
Table 4 Summary of epidemiological studies regarding the consumptions of DF and CRC incidence

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Dietary fiber</th>
<th>Conclusion</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trock, Lanza, &amp; Greenwald</td>
<td>meta-analysis (37 observational studies, 15 case-control studies)</td>
<td>fibers, vegetables, grains or fruits</td>
<td>Fiber-rich diets provide the protective effects against CRC</td>
<td>1990</td>
</tr>
<tr>
<td>Howe et al.</td>
<td>combined analysis (13 case-control studies)</td>
<td>fibers</td>
<td>Intake of fiber-rich foods is inversely associated with the risk of CRC</td>
<td>1992</td>
</tr>
<tr>
<td>Bingham et al.</td>
<td>observational study (N=519,978)</td>
<td>fibers</td>
<td>DFs in foods was inversely related to large bowel cancer incidence</td>
<td>2003</td>
</tr>
<tr>
<td>Dahm et al.</td>
<td>prospective case-control study (N=579)</td>
<td>fibers</td>
<td>Consumption of DFs has inverse association with CRC risk</td>
<td>2010</td>
</tr>
<tr>
<td>Fuchs et al.</td>
<td>prospective study (N=88,757)</td>
<td>fibers</td>
<td>There is no supportive evidence on the protective effects of DFs to CRC incidence</td>
<td>1999</td>
</tr>
<tr>
<td>Michels et al.</td>
<td>prospective study &amp; combined analysis (2 cohort studies)</td>
<td>fruits and vegetables</td>
<td>Consumption of fruits and vegetables dose not shown protective effect to CRC</td>
<td>2000</td>
</tr>
<tr>
<td>Romaneiro &amp; Parekh</td>
<td>cohort study (N=45,491)</td>
<td>fibers</td>
<td>Relatively low intake of DFs has weak association with CRC risk</td>
<td>2012</td>
</tr>
<tr>
<td>Terry et al.</td>
<td>cohort study (N=61,463)</td>
<td>fruits and vegetables, cereal fiber</td>
<td>No association between cereal fiber and CRC risk, but low consumption of fruits and vegetables have the greater risk of CRC</td>
<td>2001</td>
</tr>
<tr>
<td>Park et al.</td>
<td>pooled analysis (prospective cohort studies)</td>
<td>fibers</td>
<td>In aged-adjusted cases, DFs inversely related to CRC. But, with accounting with other dietary risk factors, there is no association</td>
<td>2005</td>
</tr>
<tr>
<td>Aune et al.</td>
<td>systematic review &amp; meta-analysis (25 prospective studies)</td>
<td>Fruits and vegetables, legume, cereal fibers, whole grains</td>
<td>High intake DFs, particularly cereal fiber and whole grains has inverse association with CRC, whereas other fibers have weak association</td>
<td>2011</td>
</tr>
</tbody>
</table>

Based on their conclusions, epidemiological studies are divided into the three categories; protective effects, no and/or weak associated, and changeable depending on variables. DF; dietary fibers, CRC; colorectal cancer.
Table 5: The molecular and cellular alterations in cancer cells that leads metabolic transformations

<table>
<thead>
<tr>
<th>Altered mechanism</th>
<th>Detail mechanisms</th>
<th>Results</th>
</tr>
</thead>
</table>
| Activated signaling pathway | PI3K/Akt | • glucose transporter expression ↑  
• glycolytic enzymes expression ↑ (e.g. hexokinase)  
• glucose-carbon flux into biosynthetic pathways ↑ (e.g. direct regulation of ACL activity)  
• lipogenic gene expression ↑ (e.g. SREBP-1, ACL, ACC and FAS)  
• β-oxidation and fatty acids degradation ↓  
• activation of mTOR pathway |
| mTOR | | • protein synthesis and cell growth ↑ |
| Gene expression (activated or loss) | Activation of HIFα | • glucose transporter expression ↑  
• aerobic glycolytic enzymes expression ↑ (e.g. PDK and LDH)  
• promotion of aerobic glycolysis & reduction of OXPHS  
• activation of PI3K/Akt pathway |
| | Activation of c-Myc | • glucose transporter expression ↑  
• aerobic glycolytic enzymes expression ↑ (e.g. PDK and LDHA)  
• promotion of aerobic glycolysis & reduction of OXPHS |
| | Loss of p53 | • glycolysis ↑  
• expression of enzymes related to OXPHS ↓ (e.g. cytochrome C oxidase 2) |
| Expression of enzyme and protein (increased) | LDH | • conversion of pyruvate into lactate ↑ |
| | PDK | • inactivation (=phosphorylation) of PDH ↑  
• import of pyruvate into the TCA cycle ↓ |
| | ACL | • conversion cytosolic citrate into acetyl-CoA and OAA ↑  
• fatty acids and lipid synthesis ↑ |

The altered molecular and cellular metabolism that leads cancer cells to attain high rates of aerobic glycolytic systems and efficient biosynthetic mechanisms. PI3K; phosphatidylinositol 3’ kinase, ACL; ATP-citrate lyase. mTOR; mammalian target of rapamycin, SREBP-1; sterol regulatory element-binding protein-1, ACC; acetyl-CoA carboxylase, FAS; fatty acid synthase, HIFα; hypoxia-inducible factor α, PDK; pyruvate dehydrogenase kinase, LDH(A); lactate dehydrogenase (A), OXPHS; oxidative phosphorylation, PDH; pyruvate dehydrogenase, OAA; oxaloacetate.
Figure 1 Vogelgram of colorectal cancer

Involvement of four main gene’s alteration (bolds) related to CRC development and subsequent signaling pathways from (in) activation of those genes are shown (▲: activated, ▼: inactivated). Consequent events that influence on cell proliferation and apoptosis resulting in formation of aberrant crypt foci, adenoma and carcinoma are described at the bottom (↑: increase, ↓: decrease). At the beginning of CRC development, inactivation of APC gene triggers the adenomatous process. Next, activation of K-Ras is required to promote the growth of adenoma. Mutations in SMAD 2/4 and TP53 give rise to tumor expansion and malignant transformation of CRC. APC denotes adenomatous polyposis coli, MAPK mitogen-activated protein kinases, TGF-β transforming growth factor-β.
Figure 2 Risk factors of colorectal cancer.

The risk factors toward CRC are shown. It includes non-modifiable factors (age, gender, family history and individual history), modifiable factors (lifestyle-related and dietary factors) and semi-modifiable factor (metabolic diseases). The box with each risk factor has a summary of the facts that increase or decrease the susceptibility of CRC development. The Solid line and box represent a positive association with CRC and the dotted line and box represent a negative association with CRC.
To reduce CRC risk, several dietary interventions can be suggested. Less intake of red and processed meat, saturated fat and animal fat decrease CRC development. More intake of dietary fiber, whole grains, and fruits and vegetables reduce CRC incidence. In addition to these dietary factors, moderate energy intake focusing on variety, nutrients density and amounts of diet can be suggested to improve CRC incidence.
Figure 4 The beneficial physiological effects of dietary fiber and its underlying mechanisms.

Based on their physicochemical properties (▲: relatively high, ▼: relatively low), DF is responsible for a variety of physiological effects. These effects are mainly involved in the improvement of blood glucose and lipid metabolism, laxation, overall improvement of colonic status and reduced risk in CRC development (↑: increase, ↓: decrease). CRC; colorectal cancer, SCAFs; short-chain fatty acids.
Figure 5 The beneficial physiological influences from gut microbiota and its underlying mechanisms

Gut microbiota supply various metabolites from DF fermentation. Those bacterial-derived metabolites, SCFAs and gases are involved in the maintenance of colonic health and homeostasis, and host energy metabolism. Also, they are related to CRC incidence through diverse physiological routines (↑: increase, ↓: decrease). DFs; dietary fibers, SCFAs; short-chain fatty acids, CRC; colorectal cancer, E; energy, ETC; electron transport chain.
Figure 6 The influential factors on the composition of gut microbiota and its impacts on the maintenance of colonic health and CRC development.

Several factors can impact on the maintenance and/or change of the gut microbiota composition in terms of diversity, number and their activity. The condition of the host (genetic, age, stress), diseases (IBDs, obesity, diabetes), diet (HFD and DFs), and environmental factors (antibiotic therapy) can alter the composition of gut microbiota. The transformation of gut microbiota is directly related to colonic homeostasis and their outcomes (bacterial-derived metabolites) in respect of the types and amounts. Interruption of microbial homeostasis can change the population of gut microbiota, and produce the potential carcinogenic molecules and toxic compounds leading to CRC incidence. Therefore, the balance of gut microbiota is important in maintaining gut microbiota's homeostasis, colonic conditions, and host health. IBDs; inflammatory bowel disease, HFD; high-fat diet, DFs; dietary fibers, CRC; colorectal cancer.
Figure 7 The pathways of butyrate production and the related butyrate-producing bacteria in the colon.

In the human colon, several bacteria participate in butyrate production and two major pathways are utilized by them. According to enzymes that they utilize at the end of pathway, butyrate-producing bacteria can be categorized into either CoA-transferase pathway or butyrate kinase pathway. The overall butyrate biosynthesis pathway and the involved bacteria are shown. DHAP; dihydroxyacetonephosphate, PEP; phosphoenolpyruvate, DFs; dietary fibers
Figure 8 Two distinct mechanisms of butyrate in the histone acetylation.

(A) Butyrate presents in a gradient through the crypts (upper ~ base) due to the mucosal flow. The gradient concentration of butyrate relates the butyrate mechanisms in histone acetylation. Near the lumen, colonocytes are exposed to high doses of butyrate (5mM). In here, butyrate increases cell apoptosis and decreases proliferation as HDAC inhibitor (ACL-independent mechanism) to exfoliate. Whereas, at the bottom of the crypts, butyrate concentration is relatively lower (0.5mM). At this locations, butyrate is metabolized via mitochondrial β-oxidation and produces acetyl-CoA. As cofactor (acetyl-CoA) for HATs, butyrate regulates histone acetylation leading to an increase in cell proliferation and decrease in cell apoptosis (ACL-dependent mechanism). It may associate with butyrate’s roles in the turnover of colonic epithelial cells to maintain colonic homeostasis. (B) The graph shown above is to explain butyrate’s two distinct mechanisms in histone acetylation depending on its concentration. ACL; ATP-citrate lyase, HATs; histone acetyltransferase, HDACs; histone deacetylase.
As an energetic substrate, epigenetic regulator, and ligand, butyrate plays a role in many mechanisms to maintain colonic- and host health. Butyrate produces ATP for colonic epithelial cells and the host. It also acts on signaling and mechanisms to exert anti-inflammatory and anti-oxidative stress effect. In addition, butyrate is involved in body weight regulation through activation of GPCRs, and controls gene expressions as HDAC inhibitor to show anti-cancer effects. SCFAs; short chain fatty acids, ATP; adenosine triphosphate, GPCR; G-protein-coupled receptor, HDACi; histone deacetylase inhibitor, ROS; reactive oxygen species, PYY; intestinal peptide YY, GLP-1; glucagon-like peptide 1.
Figure 10 The metabolic transformation in cancer cells.

From the high glycolytic metabolism rate, cancer cells can get massive amounts of biosynthetic substrates to meet their rapid growth and cellular expansions. The pentose phosphate pathway generates the nucleotides (ribose-5-phosphate) for RNA and DNA synthesis, and NADPH for lipid synthesis. Cytosolic citrate divides into acetyl-CoA and oxaloacetate that are necessary for fatty acids and amino acids synthesis, respectively. G-6-P; glucose-6-phosphate, 6-G-gluconate; 6-phosphogluconate, PEP; phosphoenolpyruvate, α-KG; α-ketoglutarate, OAA; oxaloacetate, TCA; tricarboxylic acid, AAs; amino acids, NADP; nicotinamide adenine dinucleotide phosphate, RNA; ribonucleic acid, DNA; deoxyribonucleic acid, LDHA; lactate dehydrogenase A, PDH; pyruvate dehydrogenase, ACL; ATP-citrate lyase.
Figure 11 The different metabolisms of butyrate and roles between normal colonocytes and cancerous colonocytes.

Normal colonocytes utilize butyrate as a major energy source because butyrate enters mitochondrial β-oxidation and yields ATP. In contrast, because of the Warburg effect, cancerous colonocytes metabolize glucose over butyrate resulting in butyrate accumulation in the nucleus. In the nucleus, butyrate regulates genes that reduce proliferation and increases apoptosis as an HDAC inhibitor. ACL; ATP-citrate lyase, HATs; histone acetyltransferase, HDAC; histone deacetylase, ATP; adenosine triphosphate, TCA cycle; tricarboxylic acid cycle.
CHAPTER II

CELLULAR METABOLISM AND DOSE REVEAL CARNITINE-DEPENDENT AND -INDEPENDENT MECHANISMS OF BUTYRATE OXIDATION IN COLORECTAL CANCER CELLS
A version of this chapter was originally published by Anna Han, Natalie Bennett, Amber MacDonald, Megan Johnstone, Jay Whelan, and Dallas R. Donohoe entitled “Cellular Metabolism and Dose Reveal Carnitine -Dependent and -Independent Mechanisms of Butyrate Oxidation in Colorectal Cancer Cells” Journal of Cellular Physiology. 9999:1-10 (2015).
2.1 Abstract

Dietary fiber has been suggested to suppress colorectal cancer development, although the mechanisms contributing to this beneficial effect remain elusive. Butyrate, a fermentation product of fiber, has been shown to have anti-proliferative and pro-apoptotic effects on colorectal cancer cells. The metabolic fate of butyrate in the cell is important in determining whether, it acts as an HDAC inhibitor or is consumed as a short-chain fatty acid. Non-cancerous colonocytes utilize butyrate as the primary energy source whereas cancerous colonocytes increase glucose utilization through the Warburg effect. In this study, we show that butyrate oxidation is decreased in cancerous colonocytes compared to non-cancerous colonocytes. We demonstrated that colorectal cancer cells utilize both a carnitine-dependent and carnitine-independent mechanism that contributes to butyrate oxidation. The carnitine-dependent mechanism is contingent on butyrate concentration. Knockdown of CPT1A in colorectal cancer cells abolishes butyrate oxidation. In terms of selectivity, the carnitine-dependent mechanism only regulated butyrate oxidation, as acetate and propionate oxidation were carnitine-independent. Carnitine decreased the action of butyrate as an HDAC inhibitor and suppressed induction of H3 acetylation by butyrate in colorectal cancer cells. Thus, diminished oxidation of butyrate is associated with decreased HDAC inhibition and histone acetylation. In relation to the mechanism, we find that dichloroacetate, which decrease phosphorylation of pyruvate dehydrogenase, increased butyrate oxidation and that this effect was carnitine-dependent. In conclusion, these data suggest that
colorectal cancer cells decrease butyrate oxidation through inhibition of pyruvate dehydrogenase, which is carnitine-dependent, and provide insight into why butyrate shows selective effects toward colorectal cancer cells.

2.2 Introduction

Diet is one of the risk factors associated with colorectal cancer susceptibility and dietary modifications have been proposed to lower colorectal cancer incidence and mortality (Bruce, Wolever, & Giacca, 2000; Ryan-Harschman & Aldoori, 2007; Safari, Shariff, Kandiah, Rashidkhani, & Fereidooni, 2013; Vargas & Thompson, 2012; Vargas et al., 2012; Willett, 2001). Several studies have shown that consumption of a high-fiber diet is associated with a reduction in colorectal cancer incidence and development (Bingham et al., 2003; Giovannucci, Stampfer, Colditz, Rimm, & Willett, 1992; Reddy, 1999; Schatzkin et al., 2007). In contrast, several other human epidemiological studies have failed to demonstrate a beneficial effect toward preventing colorectal cancer (Fuchs et al., 1999; Lanza et al., 2007; Mai et al., 2003). Thus, the role of dietary fiber in colorectal cancer prevention remains unclear and unresolved. The major metabolites derived from fiber include acetate, propionate, and butyrate. Of these metabolites, butyrate has been proposed to be a primary candidate in fiber’s suppressive effects toward colorectal cancer (Louis, Hold, & Flint, 2014; Wollowski, Rechkemmer, & Pool-Zobel, 2001). Butyrate is a short-chain fatty acid, produced in the colon by bacteria through fermentation of fiber, and is the preferred energetic substrate of the colonocytes (Donohoe et al., 2011; Flint,
Scott, Duncan, Louis, & Forano, 2012; WE Roediger, 1980). In addition, to butyrate’s role as an energetic substrate in colonocytes, butyrate inhibits cell growth, promotes cellular differentiation, and induces apoptosis in cancer cells in vitro at physiologically relevant doses (Archer, Meng, Shei, & Hodin, 1998; Chopin, Toillon, Jouy, & Bourhis, 2002; Velcich et al., 1995).

Several previous studies have demonstrated that, the ability of butyrate to inhibit cell proliferation and induce apoptosis is directly associated with its metabolic fate (Andriamihaja, Chaumontet, Tome, & Blachier, 2009; Donohoe et al., 2012; Leschelle, Delpal, Goubert, Blottiere, & Blachier, 2000). Specifically, in colorectal cancer cells, increased glycolysis or the Warburg effect regulated the intracellular butyrate level, which was associated with butyrate inducing pro-apoptotic genes through inhibition of histone deacetylases (HDACs; Donohoe et al., 2012). As an alternative to inhibiting HDACs, butyrate was oxidized completely through the Krebs cycle (to CO$_2$) or butyrate directly gave rise to cytosolic acetyl-CoA (through citrate originating from the mitochondria). Cytosolic acetyl-CoA then donated acetyl groups to histone acetyltransferase (HATs) to regulate gene expression or it was used for de novo lipogenesis (Andriamihaja et al., 2009; Donohoe et al., 2012; Leschelle et al., 2000; Wellen et al., 2009). Thus, to understand butyrate’s inhibitory and selective effects toward colorectal cancer, it is essential to characterize the mechanisms that regulated butyrate oxidation.

Carnitine and the carnitine palmitoyltransferase (CPT) system regulate fatty acid transport into the mitochondria. The CPT system, which utilizes carnitine palmitoyltransferase 1 (CPT1), carnitine acyltransferase, and carnitine
palmitoyltransferase 2 (CPT2), transports fatty acids across the outer and inner mitochondrial membranes into the matrix, and has been long known to have selectivity toward transporting long-chain fatty acid (Fritz, 1961). In the first reaction, carnitine palmitoyltransferase 1 (CPT1), uses carnitine for its transport mechanism as it converts the fatty acyl-CoA into the fatty acyl-carnitine ester. The fatty acyl-carnitine, is then shuttled through the CPT system/transporter into the mitochondrial matrix. In the final step, CPT2 converts the fatty acyl-carnitine back into the original fatty acyl-CoA, which is released in conjunction with carnitine into the matrix. This transport process is considered the major rate-controlling step in fatty acid oxidation (Ceccarelli, Chomienne, Gubler, & Arduini, 2011). The uptake of butyrate and other short-chain fatty acids (SCFAs) into the mitochondria has been shown not to require carnitine or the CPT system (Ceccarelli et al., 2011; Fritz, 1959, 1961). However, it is important to recognize that many of these earlier studies that characterized fatty acid oxidation utilized lower SCFA concentrations (in the micromolar range), and cell-types, such as heart and liver, where SCFAs are not the primary energy source under physiological conditions. Thus, in the case of butyrate oxidation in the colonocytes, where colonic butyrate level reach millimolar concentrations, and butyrate serves as the preferential energetic substrate by the colonocytes, it is unknown as to whether carnitine and the CPT system, impact the oxidation of butyrate.

In the study, experiments were performed to distinguish butyrate oxidation in the cancerous and non-cancerous colonocytes, while also further
characterizing the mechanisms that impact oxidation of butyrate in the cancerous colonocytes, and thus influence butyrate's action as an HDAC inhibitor. Cancerous colonocytes showed diminished butyrate oxidation compared to non-cancerous colonocytes. This result coincided with lower intracellular carnitine levels and decreased levels of organic cation/carnitine transporter 2 (OCTN2), a major carnitine transporter, in the cancerous colonocytes compared to non-cancerous colonocytes. As a consequence, the role of carnitine in regulating butyrate oxidation in the cancerous colonocytes was interrogated further. Toward this end, butyrate oxidation was found to be both carnitine-dependent and carnitine-independent in the cancerous colonocytes. Furthermore, the carnitine-dependent regulation of butyrate oxidation was mediated through the phosphorylation of pyruvate dehydrogenase, which is a major contributor to the Warburg effect (Fan et al., 2014; Hitosugi et al., 2011). This study provides an initial framework into understanding how shifts in cellular mechanism alter the fate of microbial-derived butyrate and determine its selective effects toward the colorectal cancer cell.

2.3 Materials and Methods

2.3.1. Cell culture and transfections

HCT116 cells (ATCC, CCL-247) were grown in DMEM formulated with 25mM glucose and 10% FBS. FHC cells (ATCC, CRL-1831) were grown in DMEM:F12 medium with 20% FBS. RNAi transfection in HCT116 cells was
performed as described (Donohoe et al., 2012), and siRNA pools for human Cpt1a (Dharmacon, L-009749-00), and siMock non-targeting control (Dharmacon, D-001810-01-05) were used at a 20nM final concentration. The optimized time for each siRNA transfection was confirmed with Western blotting.

**2.3.2. Flux experiment**

XF²⁴ Analyzer (Seahorse Bioscience) was used to measure % change in the oxygen consumption rates (% OCR) in HCT 116 and FHC cells. Experiments were conducted following manufacturer guidelines. Cells (FHC and HCT116 cells) split and seeded (at an identical cell number per well) into XF²⁴ cell culture microplates (Seahorse Bioscience, 100777-004). Before running seahorse, cell plates are incubated with 1X KHB (2.5mM glucose, with or without 50μM carnitine) in non-CO2 incubator at 37°C for 1h. All Seahorse experiments were run with identical condition (unless otherwise noted). Briefly, KHB media or short chain fatty acids (SCFAs) including sodium acetate, propionate or butyrate (Sigma P1880, S8750 and B5887) at 1 and/or 5mM final concentration were injected and the change in OCR was measured from baseline (%OCR). Next, 2-deoxyglucose (Sigma, D8375) was injected and %OCR was measured again. Finally, 10% sodium azide was injected to block Complex IV and mitochondrial respiration. Azide was used as a positive control to show that mitochondrial respiration is responding as expected and our compounds have an effect on mitochondrial function. In all cases, azide decreased OCR to at or below baseline respiration. As we were initially developing the methodology to measure butyrate
oxidation with the Seahorse we did not use azide. When testing other cell lines (non-colonocyte) we found that they showed no response to butyrate. However, they did respond to azide (OCR dropped), thus suggesting that the lack of response to butyrate was real, and not an artifact of the Seahorse technology. In some situations, additional chemical compounds were injected: etomoxir (Tocris Bioscience, Minneapolis, MN, 4539), palmitate/or BSA from XF Palmitate-BSA FAO substrate kit (Seahorse Bioscience, #102720-100), and 5mM DCA (Sigma, 347795).

2.3.3. Western blotting

Proteins from FHC and HCT116 cells were extracted with RIPA buffer (Cell Signaling, #9806), 1mM PMSF (Cell Signaling, #8553) and phosphatase inhibitor cocktail (Cell Signaling, #5872). Protein concentrations were measured by Bradford assay. Gel electrophoresis and transfer were conducted using standard protocol for Western blot. Antibodies that were used included pan-acetylated-histone H3 (Active Motif, Cat#39139), total Histone H3 (Active Motif, Cat#39736), MCT1-c terminal (Abcam, Cat# ab179832), OCNT2 (Abcam, Cat# ab79964), CPT1A (Cell Signaling, Cat# 122525), phospho-PDH E1- α (S293) (Abcam, Cat# ab92696), PDH E1- α (Abcam, Cat# ab110330), and β-actin (Sigma, Cat# A1978). Chemiluminescence or fluorescent detection was performed with the Odyssey Fc and bands were quantified with Image Studio Software (LI-COR Biosciences, Lincoln, NE).
2.3.4. Carnitine assay

Carnitine levels in FHC and HCT116 cells were measured with a carnitine assay kit (Biovision, K642-100). Conditions were kept identical to the Seahorse experiments. The assay was performed as described the protocol from the manufacturer. Cells were deproteinized immediately before the assay with a PCA deproteinization kit (Biovision, K808-200). The carnitine contents in cells were normalized to protein amounts.

2.3.5. Statistical analysis

For biochemical assays, Seahorse Experiments, and Western blots, the differences between experimental groups were determined by ANOVA followed by a Turkey post-hoc test. All data are expressed as mean ± SE.

2.4 Results

2.4.1. Diminished butyrate oxidation in colorectal cancer cells

To test whether butyrate oxidation is altered in colorectal cancer cells compared to non-cancerous colonocytes, we first sought to develop methodology that would allow us to analyze butyrate oxidation over time, and to probe the mechanisms that regulated the process in more detail. Therefore, we utilized the Seahorse XF Analyzer to measure changes in the oxygen consumption rate (OCR) in cultured cells. In all of our Seahorse experiments, the assay is run in KHB media, were the only exogenous energetic substrate is glucose (2.5mM).
Thus, initial OCR measurements represent glucose oxidation. Injection of butyrate results in both butyrate and glucose contributing to OCR. To block glucose oxidation, and thereby leave butyrate as the sole exogenous substrate (butyrate oxidation), we decided to use 2-deoxyglucose (2DG), which is a competitive inhibitor of glucose and abolishes glucose oxidation (Figure 12A). We first tested this strategy utilizing HCT116 colorectal cancer cells. The % change in the OCR was measured as cells were treated with increasing concentrations of butyrate (0.5, 1.0, 2.0, and 5.0mM). Although the top three doses were elevated over 0.5mM, there was no distinct dose-response relationship. However, upon injection of 2DG (5mM) a dose-response could clearly be identified, presumably as butyrate as the only exogenous substrate available (Figure 12B). We then tested whether butyrate oxidation was different between non-cancerous Fetal Human Colonocytes (FHC) and cancerous HCT116 cells utilizing the same strategy. The % change in the oxygen consumption rate was much greater in FHC compared to HCT116 cells, suggesting that in general FHC have greater response to butyrate (Figure 12C). Moreover, after addition of 2DG, the butyrate oxidation was much greater in FHC compared to cancerous HCT116 cells (Figure 12D). This provided evidence that butyrate oxidation is suppressed in cancerous colonocytes compared to non-cancerous colonocytes.

Butyrate, uptake into the cell is accomplished through the monocarboxylate transporter I (MCT I). We next tested whether the cancerous cell line (HCT116) had diminished transport of butyrate compared to the non-
cancerous cell line (FHC). However, when analyzing MCT I levels in the two cell lines, we actually find that MCT I expression was higher in HCT116 cells compared FHC. This suggests that butyrate uptake is not suppressed in HCT116 cells (Figure 12E).

2.4.2. Butyrate oxidation is regulated by carnitine-dependent and -independent mechanism

Transport of SCFAs into the mitochondria is recognized to be independent of carnitine palmitoyltransferase I (CPT I) (Ceccarelli et al., 2011; Fritz, 1961). However, butyrate oxidation has been shown to be elevated through carnitine supplementation (Fritz, Kaplan, & Yue, 1962; Hird & Weidemann, 1966). These conflicting results are likely due to differences in cell-type and SCFA concentrations used in the studies. Nevertheless, we sought to determine whether carnitine and CPT impacts butyrate oxidation in our system. First, we measured intracellular carnitine levels in non-cancerous FHC and cancerous HCT116 cells. With assay conditions kept identical to when we measured butyrate oxidation, we found that the intracellular carnitine level was over 12 times higher in FHC compared to HCT116 cells. (303 vs. 24μmols/g of protein) (Figure 13A). Next, we analyzed whether OCTN2, which is the major carnitine transporter (Ohashi et al., 2001; Seth, Wu, Huang, Leibach, & Ganapathy, 1999; Wu et al., 1999), is expressed differently in the FHC and HCT116 cells. The expression of OCTN2 was found to be elevated in FHC compared to HCT116 cells (Figure 13B). To probe the role of carnitine in the oxidation of butyrate, we
performed the assay with KHB media containing carnitine and compared it to KHB media void of carnitine. If butyrate oxidation was carnitine-independent then we postulated that oxidation should not be affected by absence of carnitine. However, this was not the case, as the addition of 50μM carnitine to KHB media, the % change in oxygen consumption rate following HCT116 cells treated with 5mM butyrate was higher than KHB media without carnitine (Figure 13C). This was especially evident after 2DG injection as the butyrate oxidation showed both a carnitine-independent (even without carnitine, butyrate significantly increased the %OCR) and carnitine-dependent responses (Figure 13D). To test whether butyrate oxidation was carnitine-dependent at lower doses, we treated HCT116 cells with 1mM butyrate in the presence or absence of 50μM carnitine. At this lower doses, 1mM HCT116 cells did not exhibit a carnitine-dependent response (Figure 13E). Thus, butyrate oxidation was not significantly impacted by carnitine (Figure 13F). These data reveal a carnitine-dependent mechanism that functions at higher butyrate doses to regulate oxidation in the cell.

2.4.3. Carnitine-dependent mechanism is selective for butyrate oxidation

To gain further insight into the impact of carnitine on butyrate oxidation in cancerous colonocytes, we tested increasing concentrations of carnitine (6, 12.5, 25, and 50μM) with a fixed butyrate dose (5mM). As carnitine concentrations increased, so did the % change in the oxygen consumption rate after butyrate treatment (Figure 14A). This dose-response was especially apparent after 2DG treatment to block glucose oxidation, as butyrate oxidation was highest at 50μM
(Figure 14B). We next sought to determine whether this carnitine-dependent mechanism was prevalent for oxidation of other SCFAs. Thus, the major objective of this experiment was to test how carnitine impacted the oxidation of SCFAs, not how the oxidation of the SCFA differed from each other. Using a set SCFA concentration of 5mM, we found that treatment of colorectal cancer cells (HCT116) with acetate or propionate failed to show the same carnitine-dependent response as butyrate (Figure 14C). Moreover, only butyrate oxidation was significantly affected by carnitine (Figure 14D). This suggests that the carnitine-dependent oxidation is selective for butyrate in these colorectal cancer cells.

2.4.4. CPT1A-dependent oxidation of butyrate

Carnitine regulates fatty acid oxidation through carnitine palmitoyltransferase (CPT), which transfers carnitine onto the fatty acid in exchange for coenzyme A (CoA). We reasoned that since butyrate oxidation was, in part, carnitine-dependent, at higher doses, then inhibiting or knocking down CPT1A (the major isoform in colonocytes) would have a similar outcome as taking away carnitine, where the oxidation of butyrate would be diminished. To test this idea, we decided to inhibit CPT1A with the pharmacological agent etomoxir, and transiently knockdown CPT1A with RNA interference. In the first set of experiments, we treated HCT116 cells with etomoxir (ETO) after the 2DG injection (so butyrate is the only available exogenous substrate), and found that the % change in the oxygen consumption rate decreased (Figure 15A). Thus,
butyrate oxidation was significantly diminished by the CPT1 inhibitor etomoxir (Figure 15B). To further confirm CPT1 involvement and selectively target CPT1A, we performed a time course knockdown of CPT1A in HCT116 cells. The time course was meant to determine the maximal knockdown of CPT1A, and set the conditions for the Seahorse assay. At 72hr, we observed maximal knockdown of CPT1A protein (Figure 15C). To confirm fatty acid oxidation was decreased, we conducted a series of experiments to test if palmitate oxidation was lower in cells where CPT1A was knockdown. This was indeed the cases, as knockdown CPT1A, completely abolished palmitate oxidation (Figure 15D). This result showed us that knockdown of CPT1A was having a functional consequence on fatty acid oxidation. We next tested whether butyrate oxidation was altered by CPT1A knockdown in HCT116 cells. CPT1A knockdown diminished the change in % oxygen consumption rate after butyrate injection (Figure 15E). CPT1A knockdown completely blocked butyrate oxidation in HCT116 cells (Figure 15F). These data point at role of CPT1A in regulating butyrate oxidation in the colonocytes. This is consistent with CPT1A having diminished expression in colorectal cancerous cells (HCT116) compared to non-cancerous colonocytes (FHC) (Figure 19).

2.4.5. Lower butyrate oxidation results in elevated HDAC inhibition and H3 acetylation

As a result of diminished butyrate oxidation from lack of carnitine, we hypothesized that suppressed butyrate oxidation, would increase butyrate
concentration, and that would increase HDAC inhibition and histone acetylation. We discovered that HDAC activity was diminished in cells treated with 5mM butyrate whole media lacked carnitine (Figure 16A), thereby suggesting enhanced inhibition of HDACs at this concentration of butyrate. Absence of carnitine had no effect on HDAC activity for HCT116 cells treated with 1mM butyrate. If inhibition of HDACs was indeed enhanced then histone acetylation should be elevated. This was the case, as we demonstrated that HCT116 cells treated with butyrate without carnitine had greater H3 acetylation (H3ac) than cells treated with equal concentration of butyrate with carnitine (Figure 16B and C).

2.4.6. Phospho-pyruvate dehydrogenase lowers oxidative metabolism and butyrate oxidation

As cancer cells begin to increase glucose uptake and utilization through the Warburg effect, the pyruvate dehydrogenase complex (PDH) becomes inactive through phosphorylation of Ser 239 on the E1 subunit. We constructed a model based on targeting phospho-pyruvate dehydrogenase as a central player in mediating the diminished oxidation of butyrate in the colorectal cancer cells (Figure 17A). In this simplified model, pyruvate is shunted toward lactate as PDH becomes inactive through phosphorylation. The net result is lowered glucose oxidation (per molecule of glucose taken up), and an overall decrease in mitochondrial oxidation, including butyrate oxidation. In addition, elevated glucose utilization, and glycolysis, suppress OCTN2 expression (this results in
decreased carnitine uptake and concentration in cell), and lower butyrate oxidation through the carnitine-dependent mechanism. To test this model, we used the compound dichloroacetate (DCA) to inhibit pyruvate dehydrogenase kinase and decrease phosphorylation of PDH (increase in activity). Therefore, treatment of colorectal cancer cells with DCA should increase butyrate oxidation through this mechanism. DCA behaved as expected in our cells, as DCA inhibited phosphorylation of PDH (Ser 293) (Figure 17B). We next tested the effects of DCA on glucose and butyrate oxidation. Consistent with the model, initial injection of DCA (before butyrate oxidation), increased the % change in the oxygen consumption rate, demonstrating that DCA elevated oxidative metabolism of glucose (Figure 17C), and butyrate oxidation (Figure 17D).

2.4.7. Link between phospho-pyruvate dehydrogenase and carnitine

To test whether there is a relationship between suppressed butyrate oxidation from phospho-PDH and carnitine, we sought to find out if the elevated butyrate oxidation observed after DCA treatment was dependent in any way on carnitine. We first tested whether DCA had a significant impact on OCNT2 and/or CPT1A expression in cancerous HCT116 cells undergoing the Warburg effect. DCA elevated OCTN2 and CPT1A expression (Figure 18A) in HCT116 cells. We then repeated the DCA experiments with and without carnitine, and analyzed butyrate oxidation with the Seahorse XF24 analyzer. Removing carnitine fully attenuated the butyrate oxidation response due to DCA treatment (Figure 18B). DCA and carnitine were required for maximal response (Figure 18C). Taken
together, these data point toward the phosphorylation (and thus, inhibition) of PDH as a key event in suppressing butyrate oxidation in the colorectal cancer cell. This also demonstrates that phosphorylation of PDH targets the carnitine-dependent oxidation of butyrate through regulation of carnitine uptake and expression of CPT1A.

2.5 Discussion

The colonocytes is unique because butyrate serves the role of being the preferred energetic substrate (WE Roediger, 1980). It is also recognized that, cancerous colonocytes shift their metabolism toward increased glucose uptake and utilization as suggested by FDG uptake in Positron Emission Tomography – Computed Tomography (PET-CT) scans (de Geus-Oei et al., 2006; Delbeke & Martin, 2011; Kawada et al., 2015; van Kouwen et al., 2006). The change in cellular metabolism toward glucose utilization and the Warburg effect alters butyrate metabolism in the cell (Andriamihaja et al., 2009; Donohoe et al., 2012; Leschelle et al., 2000). As a result, butyrate levels in the cells are affected, as it inhibition of histone deacetylase (HDACs) by butyrate (Donohoe et al., 2012; Donohoe et al., 2014; Leschelle et al., 2000). In this regard, suppression of cell proliferation and induction of apoptosis in colorectal cancer cells by butyrate has been associated with inhibition of HDACs (Archer et al., 1998; Chopin et al., 2002; Velcich et al., 1995). Therefore, it is essential to characterize the oxidation of butyrate in the cancer cell to understand the impact of butyrate as an HDAC inhibitor. If cancer cells are predisposed to accumulate butyrate compared to
normal colonocytes under the proper conditions, this could help explain why cancer cells are sensitive to fiber diets.

In the work presented here, we define a diminished oxidation of butyrate in cancerous colonocytes and interrogate mechanism(s) that include carnitine and pyruvate dehydrogenase. We also demonstrate that with lower butyrate oxidation, HDAC inhibition and histone H3 acetylation increase in colorectal cancer cells.

Previous studies have defined a role for carnitine in the oxidation of long-chain fatty acids, and short-chain fatty acid oxidation is mainly accepted to be carnitine-independent (Ceccarelli et al., 2011; Fritz, 1959, 1961). In contrast, we find butyrate oxidation to be partially carnitine-dependent in cancerous colonocytes. This difference can be attributed to doses or concentrations of butyrate use, where butyrate concentrations were in the micromolllar range. In our case, we used 5mM butyrate, which is a physiologically relevant concentration in the colon. Toward this end, we demonstrate at 1mM there is no carnitine-dependent mechanism involved in butyrate oxidation. This is consistent with the carnitine-dependent mechanism only functioning at higher butyrate doses, and this mechanism only being physiologically relevant to the colon. De Preter et al. (2011) found that carnitine did not enhance the oxidation of 1mM $^{14}$C-butyrate to $^{14}$CO$_2$ in colonic biopsies from normal and ulcerative colitis patients (De Preter et al., 2011). A previous publication found that carnitine elevated butyrate oxidation in an ADP-dependent fashion (Hird & Weidemann, 1966).
The fact that carnitine levels were lower in HCT116 cells compared to FHC cells suggest that carnitine may be an important determinant in lowered butyrate oxidation observed in HCT116 cells. Thus, in addition to diminished butyrate-producing bacteria and butyrate levels that has been observed in colorectal cancer patient (Balamurugan, Rajendiran, George, Samuel, & Ramakrishna, 2008; Wang et al., 2012), butyrate oxidation may be decreased due to a reduction in carnitine in the colorectal cancer cell. We find that OCTN2, the major carnitine transporter, is higher in FHC compared to HCT116 colorectal cancer cells. This is consistent with a previous report that highlights a downregulation in OCTN2 in cancer cells, in general (Scalise et al., 2012). The mechanism causes the diminished carnitine level and decreased OCTN2 in cancer cells is unknown; however, it is interesting to consider increased glycolysis and diminished oxidative metabolism as a plausible starting point. Dichloroacetate (DCA) is a compound that has been used to decrease the phosphorylation of pyruvate dehydrogenase, and therefore, increase oxidative metabolism (Izquierdo-Garcia et al., 2015; Whitehouse, Cooper, & Randle, 1974). We found that DCA could exert this effect on HCT116 cells, where phosphorylation of PDH was diminished by 10-fold in DCA treated cells. Moreover, HCT116 colorectal cells treated with DCA displayed elevated butyrate oxidation, which is consistent with phospho-PDH playing a role in the diminished butyrate oxidation observed in the colorectal cell. In a previous paper, Roediger and Nance (1990) reported that addition of DCA had no effect on conversion of $^{14}$C-butyrate to $^{14}$CO$_2$ (WEW Roediger & Nance, 1990). This suggests that the
increased butyrate oxidation caused by DCA, was specific for the cancer cell, where PDH was inactivated through phosphorylation. The selective effects of butyrate on colorectal cancer cells as opposed to non-cancerous cells may be the result of altered cellular metabolism with inactivation of PDH at the center of the cause through diminishment of butyrate oxidation.

To date, there have been no human studies testing the effect of carnitine supplementation on colorectal cancer. However, dietary sources high in carnitine, such as red meat, have been associated with an increased risk for the development of colorectal cancer. Our data might suggest that consumption of red meat (high in carnitine) would increase the oxidation of butyrate and subsequently diminish HDAC inhibition. As a results of increase butyrate oxidation, butyrate's protective actions toward colorectal cancer would be diminished. In addition, carnitine supplementation in humans has been shown to increase trimethylamine-N-oxide(TMAO) in a microbiota-dependent manner (Koeth et al., 2013). Elevated blood/plasma TMAO has been suggested to be a causal factor in colorectal cancer (Bae et al., 2014; Xu, Wang, & Li, 2015). Thus, carnitine supplementation may have tumor-promoting effects in humans that are fiber-or butyrate-dependent (carnitine’s role in regulating butyrate oxidation) and independent (formation of TMAO from carnitine).
2.6 References


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2.7 Appendix
Figure 12 Butyrate oxidation in non-cancerous and cancerous colonocytes.

(A) Diagram showing experimental strategy that will be used to measure butyrate oxidation in cells over time with XF24 Analyzer. (B) Percent change in oxygen consumption rate (OCR) relative to baseline for 0.5, 1.0, 2.0, and 5.0mM butyrate in HCT116 cells. Total contribution of butyrate toward OCR (%) is observed after injection of 2-deoxyglucose (2DG). (C) Percentage change in OCR relative to baseline was determined in HCT116 and FHC cells with or without butyrate injected into the wells (final concentration of butyrate is 5mM). 2DG was then injected into wells and butyrate oxidation was analyzed. (D) Area under the curve analysis from OCR measurements taken after 2DG injection, but before azide injection (80-128min). These measurements represent the butyrate oxidation (arbitrary units), as butyrate is the only exogenous oxidative substrate available. (E) Representative western blot of MCT1 from FHC and HCT116 cells with β- as loading control. The glucose concentration in the in the fatty acid oxidation media for panels B and C was kept constant at 2.5mM. Data points represent the average OCR (%) over 3-5 replicates per experimental condition. Error bars are +/- the SEM.
Figure 12 continued
Figure 13 Butyrate oxidation is carnitine-dependent and -independent.

(A) Intracellular carnitine level in FHC cells and HCT116 cells. (B) Representative western blot showing OCTN2 expression in FHC and HCT116 cells with β-actin serves as loading control. (C) Percent change in oxygen consumption rate (OCR) relative to baseline in HCT116 cells treated with and without butyrate (5mM) and carnitine (50μM). Total contribution of butyrate toward OCR (%) is observed after injection of 2-deoxyglucose (2DG). (D) Area under the curve analysis from OCR measurements taken after 2DG injection, but before azide injection (56-84 min). These measurements represent butyrate oxidation (arbitrary units) at 5mM. (E) Percent change in OCR relative to baseline in HCT116 cells treated with and without butyrate (1mM) and carnitine (50μM). As before, total contribution of butyrate toward OCR (%) is observed after injection of 2DG. (F) Area under the curve analysis from OCR measurements taken after 2DG injection, but before azide injection (56-84 min). These measurements represent butyrate oxidation (arbitrary units) at 1mM. For butyrate oxidation measurements data points represent the average OCR (%) over 3-5 replicates per condition. Error bars are +/- SEM.
Figure 13 continued
Figure 14 Carnitine selectively affects butyrate oxidation in a dose-dependent manner.

(A) Percent change in oxygen consumption rate (OCR) relative to baseline in HCT116 cells treated with and without butyrate (5mM) and increasing carnitine concentrations (12.5, 25, and 50μM). Total contribution of butyrate toward OCR (%) is observed after injection of 2-deoxyglucose (2DG). (B) Area under the curve analysis from OCR measurements taken after 2DG injection (All measurements after 50min). These measurements represent butyrate oxidation (arbitrary units) at 5mM with increasing carnitine concentrations. (C) Percent change in oxygen consumption rate (OCR) relative to baseline in HCT116 cells treated with and without SCFAs (ACE, acetate; PRO, propionate; BUT, butyrate) and carnitine (50μM). Final concentration for all SCFAs was 5mM. (D) Area under the curve analysis from OCR measurements taken after 2DG injection, but before azide injection (80-108 min). These measurements represent butyrate oxidation (arbitrary units). For butyrate oxidation measurements data points represent the average OCR (%) over 3-5 replicates per condition. Error bars are +/- SEM.
Figure 14 continued
Figure 15 Butyrate oxidation is CPT1A dependent.

(A) Percent change in oxygen consumption rate (OCR) relative to baseline in HCT116 cells treated with and without butyrate (5mM) and etomoxir (after 2DG injection). Total contribution of butyrate toward OCR (%) is observed after injection of 2-deoxyglucose (2DG). Effect toward blocking CPT1 is shown after etomoxir injection. (B) Area under the curve analysis from OCR measurements taken after 2DG injection, but before azide injection (82-112 min). These measurements represent butyrate oxidation (arbitrary units) with or without etomoxir injection. (C) Representative western blot of RNAi knockdown (siMock and siCpt1A) showing CPT1A expression over time course of 72h. CPT1A expression was maximally knocked down after 72h treatment. β-actin serves as loading control. (D) Palmitate oxidation (arbitrary units) calculated from subtraction of OCR measurements before and after etomoxir injection. (E) Percent change in OCR relative to baseline in HCT116 cells that received siMock or siCpt1A treated with and without 5mM butyrate. (F) Area under the curve analysis from OCR measurements taken after 2DG injection, but before azide injection (80-100 min). Measurements represent butyrate oxidation (arbitrary units). For butyrate oxidation measurements data points represent the average OCR (%) over 3-5 replicates per condition. Error bars are +/- SEM.
Figure 15 continued
Figure 16 Carnitine suppressed HDAC inhibition and H3 acetylation.

(A) HDAC activity as measured in HCT116 cells treated with or without butyrate and carnitine (50μM) for 1h. Average values represent three replicates per condition. (B) Representative western blot showing relative H3 acetylation in HCT116 cells treated ± butyrate (1 or 5mM final concentration) and carnitine for 24h. (C) Quantification of H3 acetylation levels relative to total H3 levels. β-actin was used as loading control. Error bars are +/- SEM.
Figure 17 Impact of PDH inactivation on butyrate oxidation.

(A) Working model of pathways that regulate butyrate oxidation. In this model, butyrate is oxidized by a CPT1-independent and -dependent mechanism. Carnitine levels and CPT1 expression are both mediators of butyrate oxidation. Decreased butyrate oxidation occurs as PDH becomes inactivated (through phosphorylation of E1) and the Warburg effect decrease OCTN2 and the carnitine-dependent mechanism. (B) Representative western blot showing phospho-PDH (Ser293) and total PDH in HCT116 cells treated ± DCA (5mM) for 6h. β-actin was used as loading control. (C) Percent change in oxygen consumption rate (OCR) relative to baseline in HCT116 cells treated with and without butyrate (5mM) and dichloroacetate (50μM). Total contribution of butyrate toward OCR (%) is observed after injection of 2-deoxyglucose (2DG). (D) Area under the curve analysis from OCR measurements taken after 2DG injection, but before azide injection (74-100 min). These measurements represent butyrate oxidation (arbitrary units). For butyrate oxidation measurements data points represent the average OCR (%) over 3-5 replicates per conditions. Error bars are +/- SEM.
Figure 17 continued
Figure 18 Carnitine-dependent mechanism is regulated through inactivation of PDH.

(A) Representative western blot showing CPT1A, OCTN2, phospho-PDH (Ser293), and total PDH in HCT116 cells treated ± DCA (5mM) for 6. β-actin was used as loading control. (B) Percent change in oxygen consumption rate (OCR) relative to baseline in HCT116 cells treated with and without butyrate (5mM), carnitine (50μM), and DCA. Total contribution of butyrate toward OCR (%) is observed after injection of 2-deoxyglucose (2DG). (C) Area under the curve analysis from OCR measurements taken after 2DG injection, but before azide injection (88-116 min). These measurements represent butyrate oxidation (arbitrary units). For butyrate oxidation measurements data points represent the average OCR (%) over 3-5 replicates per condition. Error bars are +/- SEM.
Figure 19 CPT1a expression in FHC and HCT116 cells

(A) Western blot showing CPT1A expression in FHC and HCT116 cells. (B) Quantification of CPT1A levels relative to β-actin levels.
CHAPTER III

BUTYRATE REGulates ITS OWN METABOLIC FATE AS AN

HDAC INHIBITOR IN COLORECTAL CANCER CELLS
A version of this chapter is submitted by Anna Han, Bettaieb Ahmed, Jay Whelan, and Dallas R. Donohoe entitled “Butyrate regulates its own metabolic fate as an HDAC inhibitor in colorectal cancer cells” in Journal of Cell Biology (April 2017).
3.1 Abstract

Colorectal cancer is characterized by a metabolic shift toward increased glucose utilization. Butyrate, the major energy source for non-cancerous colonocytes that is produced from the fermentation of fiber, is underutilized and its role as an HDAC inhibitor is altered in cancerous colonocytes. Understanding this metabolic shift is important in deciphering the protective effects derived from consuming a fiber diet in colorectal cancer. We discover that butyrate itself diminishes its own oxidation in cancerous colonocytes. This reduction in butyrate oxidation is associated with decreased expression of acyl-CoA dehydrogenase-short chain (SCAD) an important component that mediates the oxidation of short-chain fatty acids such as butyrate. Butyrate does not alter SCAD levels in non-cancerous colonocytes. Trichostatin A, a structurally unrelated HDAC inhibitor, and propionate also diminish SCAD alluding to HDAC inhibition as part of the mechanism. Moreover, butyrate specifically inhibits HDAC1 to suppress SCAD expression. Knockdown of HDAC1, but not HDAC 2 or 3 abrogated the effects of butyrate on SCAD expression. This work identifies a mechanism by which butyrate selective targets colorectal cancer cells through changes in metabolism.
3.2 Introduction

Colorectal cancer (CRC) is the third most common and lethal cancer in the United States (Siegel, Miller, & Jemal, 2016). Considering diet is one of the strongest influential risk factors of CRC development, intervention of diet has been suggested as an effective way to decrease CRC development and mortality (Haggar & Boushey, 2009; Johnson et al., 2013; Vargas & Thompson, 2012). Many studies have observed the beneficial effect of dietary fiber against CRC, although some studies have reported contradicting findings (Bingham et al., 2003; Dahm et al., 2010; Michels et al., 2000; Romaneiro & Parekh, 2012). The fermentation of dietary fiber in the proximal colon produces bacterial derived-short chain fatty acids (SCFAs) including acetate, propionate and butyrate. Among these SCFAs, butyrate has been considered a critical metabolite that drives the tumor repressive effects of dietary fiber against CRC (Blackwood, Salter, Dettmar, & Chaplin, 2000; Macfarlane & Macfarlane, 2012; Scharlau et al., 2009).

Unlike other SCFAs, butyrate is primarily metabolized by colonocytes as an energy source and also plays a role in epigenetic modification as a histone deacetylase (HDAC) inhibitor (Donohoe et al., 2011; Hamer et al., 2008; Steliou, Boosalis, Perrine, Sangerman, & Faller, 2012). At physiologically relevant doses, butyrate regulates cell proliferation, differentiation and apoptosis in colorectal cancer cells (Archer et al., 2005; Davie, 2003; Hinnebusch, Meng, Wu, Archer, & Hodin, 2002). Interestingly, the role butyrate plays in cell proliferation and apoptosis is related to its metabolic fates in colonocytes (Andriamihaja,
Chaumontet, Tome, & Blachier, 2009; Donohoe, Collins, et al., 2012). In normal colonocytes, butyrate is oxidized through mitochondrial β-oxidation and then utilized to produce energy through the tricarboxylic acid (TCA) cycle or cytosolic acetyl-CoA. This acetyl-CoA can be used as a cofactor for histone acetyltransferases (HATs) or substrates for lipogenesis (Donohoe, Collins, et al., 2012; Rémésy, Demigne, & Morand, 1992). However, cancerous colonocytes favor glucose over butyrate utilization as a result of a metabolic transformation called the Warburg effect. This glucose-preferred environment results in suppressed oxidation and elevated cellular butyrate levels, which help promote its action as an HDAC inhibitor (Andriamihaja et al., 2009; Donohoe, Collins, et al., 2012). Therefore, to understand butyrate’s inhibitory and selective effect against CRC, the investigation regarding butyrate metabolism in cancerous colonocytes is crucial.

Short chain acyl-CoA dehydrogenase (SCAD) is an enzyme that catalyzes the first step of butyrate mitochondrial β-oxidation in the cells (M Astbury & M Corfe, 2012). SCAD deletion reduces butyrate catabolism whiles simultaneously increasing butyryl-CoA accumulation and excretion, illustrating its role in butyrate metabolism (Augenlicht et al., 1999; Bhala et al., 1995; Wood et al., 1989). Additionally, the removal of SCAD in colonocytes leads to actual decreasing of butyrate oxidation (Kaiko et al., 2016). Previous studies have observed reduced protein expression related to mitochondrial metabolism in CRC including SCAD at both mRNA and protein levels (Birkenkamp-Demtroder et al., 2002; Jankova et al., 2011; Kim et al., 2006; Kitahara et al., 2001). However, there little is known
about the mechanism that controls SCAD expression. In this study, we report that butyrate regulates its own metabolism in colorectal cancer cells through suppressing SCAD expression. The mechanism as to how butyrate impacts SCAD levels in colorectal cancer cells is explored and appears to be mediated through HDAC inhibition. Importantly, non-cancerous colonocytes do not show this effect, thus it is selective to colorectal cancer cells.

3.3 Materials and Methods

3.3.1. Cell culture and siRNA transfection

HCT116 cell (ATCC, CCL-247) were grown in DMEM supplemented with 25mM glucose and 10% FBS. FHC cells (ATCC, CRL-1831) were grown in complete growth DMEM:F12 medium following the recommended recipe from ATCC with 20% FBS. RNAi transfection in HCT116 cells was performed as previously described (Donohoe, Collins, et al., 2012), and siRNA pools for human ACL (Dharmacon, #L-004915-00), human HDCA1 (Dharmacon, #L-003493-00-0005), human HDAC2 (Dharmacon, #L-003495-02-0005), human HDCA3 (Dharmacon, #L-003496-00-0005) and non-targeting control (Dharmacon, D001810-01-05) were used at a 20mM final concentration. The optimized time for each siRNA transfection was confirmed with Western blotting.

3.3.2. Colonocytes isolation

C57B1/6J were purchased from Jackson Laboratories (Bar Harbor, ME) and were maintained on a 12-hour light-dark cycle with free access to water and
standard laboratory chow (Purina lab chow, Cat # 5001). Mouse studies were conducted according to federal regulations and were approved by the Institutional Animal Care and Use Committee at the University of Tennessee-Knoxville. Isolation of colonic epithelial cells from mice were performed from 8-12 weeks old male mice as previously described (Donohoe, Wali, Brylawski, & Bultman, 2012). Colons were washed several times with sterilized phosphate-buffered saline (PBS). Then, the colon was incubated in PBS containing 5 mM EDTA (Fisher Scientific, Cat# S311-500) and 1% FBS, with or without butyrate (5 and 10 mM), for 45 mins at 37 °C. After 45 mins, the tissues were removed and isolated colonocytes were collected through centrifugation.

3.3.3. Biochemical Assays

HDAC activity assay was performed according to manufacturer specifications (BioVision, Cat# K339-100). Briefly, HCT116 cells seeded into 96well plates and treated with butyrate (Sigma, B5887) and trichostatin A (Promega, G6560). Following treatment times, assay was performed. All values were normalized to total protein in each well.

3.3.4. Flux experiment

To measure percentage change of oxygen consumption rates (% OCR) in HCT116 cells, Seahorse XF²⁴ Analyzer (Seahorse Bioscience) was used. All Seahorse assays were conducted according to the company guidelines, and the experimental design to measure butyrate oxidation in HCT116 cells were followed as stated (Han et al., 2015). HCT116 cells were seeded into XF24 cell
culture microplates (Seahorse Bioscience, 100777-004) with an identical cell number per well. Cell plates are incubated with 1X KHB (2.5 mM glucose and 50 μM carnitine) in non-CO₂ incubator at 37°C for one hour before Seahorse assay. All Seahorse experiments were performed with identical conditions (unless otherwise stated). In brief, KHB media or sodium butyrate (Sigma, B5887) at 5 mM final concentration were injected and the change in OCR was measured from baseline (% OCR). Then, 2-deoxyglucose (Sigma, D8375) was injected and % OCR was measured again. At last, 10% sodium azide was injected to block mitochondrial respiration by inhibiting complex IV, hence after azide injection OCR decreased at or below baseline respiration.

3.3.5 Western blot

From FHC, HCT116 cells and isolated colonocytes, the proteins are extracted with RIPA buffer (Cell Signaling, #9806), 1mM PMSF (Cell Signaling, #8553) and phosphatase inhibitor cocktail (Cell Signaling, #5872). Quantifications of protein were measured by Bradford assay. Gel electrophoresis and transfer were performed using standard protocol for Western blotting. Antibodies that were used included pan-acetylated-histone H3 (Active motif, Cat# 39139), total Histone H3 (Active motif, Cat# 39763), total PDH (Abcam, Cat# ab110330), ACL (Cell Signaling, Cat # 4332), SCAD (Abcam, Cat# 154823), HDAC1 (Cell signaling, Cat# 34589), HDAC2 (Cell signaling, Cat# 57156), HDAC3 (Cell signaling, Cat# 85057) and β-actin (Sigma, Cat# A1978).
Chemiluminescence detection was conducted with the Odyssey Fc and bands were quantified with Image Studio Software (LI-COR Biosciences, Lincoln, NE).

3.3.6. mRNA expression

Total RNA from (un) treated HCT116 cells were extracted using Trizol reagent (ambion, Cat# 15596-026). The concentration and integrity of RNA were measured by Nano-drop 1000. Reverse transcription was performed with RevertAid RT kit (Thermo Scientific, Cat# K1691) by following the company’s protocol. The amounts of product from RT-qPCR was measured by SYBR Green fluorescence (applied Biosystems, Cat# 4309155). SCAD primers for isoform 1 (Forward: GCGACTCATGGGTTCTGAAT and Reverse: TGCGACAGTCCTCAAAGATG), isoform 2 (Forward: GCCCGACTGGACCTATTTTT and Reverse: TGCGACAGTCCTCAAAGATG) and total (Forward: CAGGGATGGGCTTCAAGATA and Reverse: TGTCTGCCAACTTGAAGCTTGAAGATA and Reverse: TGTCTGCCAACTTGAAGATA and Reverse: TGTCTGCCAACTTGAAGATA and Reverse: TGTCTGCCAACTTGAAGATA and Reverse: TGTCTGCCAACTTGAAGATA) were designed and their efficiency was confirmed by gel PCR. Relative gene expression levels were calculated through the ΔΔCt method and normalized to human 18S rRNA.

3.3.7. Statistical analysis

For biochemical assays, Seahorse experiments, and Western blotting, the differences between experimental groups were determined by ANOVA followed by a Tukey post-hoc test. All data are expressed as mean ± SEM. Groups are considered significantly different at p < 0.05.
3.4 Results

3.4.1. Butyrate diminishes its own oxidation by inhibiting SCAD expression in colorectal cancer cells

In recent years our lab has sought to identify factors or conditions that impact the oxidation of butyrate in colorectal cancer cells. Since butyrate modulates gene expression through inhibiting HDACs, we postulated that butyrate could directly affect its own metabolic fates. To begin to test this possibility, HCT116 colorectal cancer cells were pretreated with or without butyrate (5 mM). Then, the percentage change in oxygen consumption rate (% OCR) was measured after 24 hours. In pretreated cells, butyrate repressed its own oxidation (Figure 20A). In order to inhibit glucose oxidation resulting in OCR values only contributed by butyrate, 2-deoxyglucose (2DG), which blocks glucose utilization was injected. After 2DG injection, butyrate oxidation was significantly lower in pretreated cells as compared to non-pretreated controls (Figure 20B).

Butyrate is transported into the colonocyte via a monocarboxylate transporter-1 (MCT1), where it taken up into the mitochondria and oxidized through carnitine-dependent and independent mechanisms (Han et al., 2015). After butyrate moves into the mitochondria, short chain acyl-CoA dehydrogenase (SCAD) catalyzes the first dehydrogenation step of butyryl-CoA to produce acetyl-CoA (M Astbury & M Corfe, 2012) (Figure 20C). Western blot analysis of colorectal cancer cells showed that butyrate suppressed the expression of SCAD (Figure 20D).
3.4.2. Butyrate reduces SCAD expression only in colorectal cancer cells

Butyrate decreased the expression of SCAD in colorectal cancer cells; however, it was not clear whether butyrate would have this same effect in non-cancerous colonocytes. Cancerous colonocytes have a reduced ability to oxidize butyrate compared to non-cancerous colonocytes which result in part from lower cellular carnitine levels and reduced protein expression related to carnitine-dependent mechanisms, such as organic cation/carnitine transporter, OCTN2 and carnitine palmitoyltransferase, CPT1A (Han et al., 2015). Thus, the reduced ability of cancerous colonocytes to oxidize butyrate may be due to the reduced SCAD levels compared to the non-cancerous colonocytes. To begin to address this, we compared SCAD levels in cancerous (HCT116 cells) and non-cancerous (FHC cells) colonocyte lines. To our surprise, SCAD expression was actually higher in HCT116 cells than FHC cells (Figure 21A). This may be due to the fact that the expression and activity of enzymes that related to fatty acid metabolism (i.e. SCAD) are dramatically elevated after birth in tissues that strongly metabolize fatty acids (Nagao, Parimoo, & Tanaka, 1993). Since FHC cells originated from a 13-week fetus, we concluded that the FHC cells are not a representative cell line for non-cancerous colonocytes. However, using a primary colonocytes, Kaiko et al. (2011) confirmed that normal colonocytes have high SCAD expression compared to the stem cells in the colonic crypts (Kaiko et al., 2016).

Cancerous colonocytes increase aerobic glycolysis (utilize the Warburg effect), which may alter the expression of proteins such as SCAD and impact the
oxidation of butyrate. To directly test the importance of the Warburg effect in butyrate-mediated SCAD reduction, we cultured cells in media with varying glucose concentrations; high (25 mM) or low (0.5 mM). Lower SCAD expression was observed in the HCT116 cells under both high (the Warburg effect) and low glucose condition (suppressed the Warburg effect) (Figure 21B and Figure 25). However, only high glucose condition led to a significant decrease in SCAD expression (Figure 21B and 21C). Throughout this paper (unless otherwise mentioned), pyruvate dehydrogenase (PDH) is used as a loading control instead of β-Actin due to the fact that PDH is also a mitochondrial protein like SCAD, and its expression is not affected by butyrate in cancerous colonocytes. Based on the importance of the Warburg effect, non-cancerous colonocytes were isolated from C57BL/6J wild type mice to test whether butyrate’s influence SCAD expression in normal colonocytes that are not undergoing the Warburg effect. While butyrate decreases SCAD expression in the colorectal cancer cells, normal isolated colonocytes showed a trend toward increasing SCAD expression, but this was not statistically significant (Figure 21D and 21E). Since butyrate also increases PDH expression in the normal isolated colonocytes, we normalized SCAD expression with β-Actin (Figure 26).

To confirm whether butyrate suppressed SCAD at the mRNA level, we conducted qRT-PCR with all isoforms of Scad (Scad1 and Scad2) and total Scad. At a high dose (5 mM), butyrate significantly reduced mRNA levels for Scad1, Scad2, and total Scad in HCT116 cells (Figure 22). Additionally, at a low butyrate concentration (1 mM), Scad2 and total Scad mRNA levels were
significantly decreased. These data suggest that butyrate suppresses SCAD expression at the transcriptional levels in the colorectal cancer cells, which may involve HDAC inhibition.

3.4.3. Butyrate mediates SCAD expression as an HDAC inhibitor, not as a metabolite

The fermentation of dietary fiber in the colon produces additional SCFAs, which include acetate and propionate. Therefore, we sought to investigate whether these other SCFAs reduced SCAD expression in colorectal cancer cells like butyrate. Both propionate and butyrate significantly suppress SCAD expression, while acetate does not influence SCAD expression in HCT116 cells (Figure 23A and 23B). Along with butyrate, propionate has been shown to be an HDAC inhibitor (Aoyama, Kotani, & Usami, 2010). This alludes to HDAC inhibition as a key component in regulating SCAD expression in colorectal cancer cells.

Butyrate is involved in epigenetic modifications through two mechanisms (Figure 23C) (Donohoe, Collins, et al., 2012). Mitochondrial butyrate oxidation results in the biogenesis of Acetyl-CoA, which can be utilized as a cofactor for HATs through ATP-citrate lyase (ACL). In addition, butyrate directly goes into the nucleus where it functions as an HDAC inhibitor. First, to test the importance of HDAC inhibition, a structurally distinct HDAC inhibitor, trichostatin A (TSA), was used as a positive control. Both butyrate and TSA significantly reduced SCAD expression (Figure 23D and 23E). Next, a siRNA knockdown of ACL was
performed and SCAD expression was evaluated with and without butyrate. Since ACL catalyzes the reaction that converts citrate into acetyl-CoA in the cytosol, an ACL knockdown would block butyrate’s involvement as a HAT cofactor. However, knockdown of ACL did not impact SCAD suppression caused by butyrate, indicating that this mechanism is unrelated to SCAD regulation. There was no difference in the percentage change in OCR between siMock and siACL transfected cells (Figure 27A and 27B). In addition, ACL knockdown did not alter SCAD expression compared to siMock HCT116 cells, while butyrate still significantly reduced SCAD expression in the both conditions (Figure 27C). Taken together, this data point to HDAC inhibition as the major mechanism as to how butyrate regulates SCAD expression in the colorectal cancer cells.

3.4.4. Butyrate decreases SCAD levels through selective inhibition of HDAC1 in colorectal cancer cells

In CRC, HDAC 1, HDAC 2, and HDAC 3 are highly expressed in order to accelerate cell proliferation, growth and survival (Weichert et al., 2008; Wilson et al., 2006). In general, butyrate effectively inhibits most HDACs resulting in decreased cell proliferation and induction of cell apoptosis in the cancer cells (Davie, 2003; Donohoe, Collins, et al., 2012). Although we found that butyrate suppresses SCAD levels through HDAC inhibitor action, it was still unclear whether a specific HDAC was involved in regulating SCAD level. Therefore, HDAC1, HDAC2, or HDAC3 were knocked down by RNAi and these cells were treated with butyrate to test whether butyrate-induced SCAD reduction was
augmented with each selective knockdown condition. While butyrate and TSA significantly decreased SCAD levels in siMock cells, siHDAC1 transfected cells did not show decreased SCAD levels after butyrate treatment (Figure 24A and 24B). RNAi knockdown of HDAC2 and HDAC3 in HCT116 cells did not impact SCAD levels like as HDAC1 (Figure 28). These findings demonstrate that butyrate targets HDAC1 to suppress SCAD levels in the colorectal cancer cells.

3.5 Discussion

Normal colonocytes prefer to oxidize butyrate as a primary energy source whereas cancerous colonocytes increases glucose utilization, which is demonstrated through increased flurodeoxyglucose (FDG) uptake as measured by Positron Emission Tomography – Computed Tomography (PET-CT) (de Geus-Oei et al., 2006; Delbeke & Martin, 2011; Miles, 2015; Roediger, 1980). In addition, colorectal cancer cells display a reduced capacity to oxidize butyrate compared to normal colonocytes, which could result from diminished intracellular carnitine, and CPT1A levels (Han et al., 2015). These metabolic alterations significantly impact butyrate’s role as an HDAC inhibitor in colorectal cancer (Donohoe, Collins, et al., 2012). As an HDAC inhibitor, butyrate represses cell proliferation and induces cell death in colorectal cancer cells (Archer et al., 2005; Archer, Meng, Shei, & Hodin, 1998; Medina et al., 1997). Thus, gaining knowledge toward the mechanisms that regulate butyrate oxidation in cancer cells is an important step in understanding butyrate’s role as an HDAC inhibitor, which is associated with protective action of butyrate against CRC. Here, we
demonstrated that butyrate suppresses its own oxidation in cancerous colonocytes through the regulation of SCAD levels, a critical enzyme in the oxidation of butyrate. Butyrate decreases SCAD levels as an HDAC inhibitor. Specifically, SCAD is regulated via HDAC1.

Many studies have reported the role of SCAD in regulating butyrate oxidation (Bhala et al., 1995; M Astbury & M Corfe, 2012; Wood et al., 1989). SCAD contributes to energy maintenance in the colon via participating in SCFA oxidation (Augenlicht et al., 1999). Recently, it was reported that colonocytes isolated from SCAD$^{-/-}$ mice showed diminished butyrate oxidation compared to those from control WT mice (SCAD$^{+/+}$), which confirms the importance of SCAD in mediating butyrate oxidation in colonocytes (Kaiko et al., 2016). In contrast to this study, a cancerous colonocyte cell line was used due to previous reports demonstrating diminished butyrate oxidation in this cell line (Donohoe, Collins, et al., 2012; Han et al., 2015). The fact that butyrate reduces its own oxidation in these cells reveals a potential mechanism as to why colorectal cancer cells are sensitive to butyrate’s HDAC inhibitory effects.

This mechanistic relationship between butyrate and SCAD in the colorectal cancer cells was significantly influenced by the Warburg effect. Previously, it was found that butyrate (1 mM) increased SCAD expression in a colorectal cancer cell line (HT15), however similar to a non-cancerous cell line, these cells still preferentially utilized butyrate over glucose thereby negating the impact of the Warburg effect (Serpa et al., 2010). The colonic administration of butyrate in healthy subjects increases gene transcription relating to energy
metabolism and fatty acid metabolism, while SCAD was not altered (Vanhoutvin et al., 2009). Germfree mice, which lack a microbiome and the capability to produce butyrate from fiber, show reduced SCAD expression compared to normal mice (Donohoe et al., 2011). Their findings are consistent with butyrate modulating SCAD levels differently in non-cancerous colonocytes.

In general, butyrate inhibits most HDACs, except class 2 (HDAC 6 and 10) and class 3, and specifically inhibits HDAC 1 and 3 in colorectal cancer cells (Davie, 2003; Thangaraju, Carswell, Prasad, & Ganapathy, 2009). As an HDAC inhibitor, butyrate effectively impedes cancer cell survival and growth (Bultman, 2014; Davie, 2003). In addition, butyrate suppresses intestinal inflammation and oxidative stress, while also protecting the intestinal epithelial barrier via its HDAC inhibitor roles; there are likely to help reduce CRC susceptibility (Canani, Di Costanzo, & Leone, 2012; Leonel & Alvarez-Leite, 2012; Plöger et al., 2012).

Separate from the non-cell autonomous effects, butyrate availability can influence cancerous colonocyte metabolism and HDAC inhibition. The suppressive action of butyrate on SCAD expression and its own oxidation in the colorectal cancer cells is mediated by its function as an HDAC inhibitor. Butyrate specifically inhibits HDAC1 to have these regulatory actions as knockdown of this protein negates any changes in SCAD caused by butyrate. These data also allude to butyrate promoting its own action as an HDAC inhibitor in colorectal cancer cells through altering suppressing its own metabolism. It will be interesting to conduct further research regarding why butyrate behaves in this way and whether this mechanism helps mediate its specificity toward cancer.
cells. CRC (all stages) has altered gene expression characterized by lower mitochondrial metabolism and a down-regulation in SCAD expression (Birkenkamp-Demtroder et al., 2002; Kitahara et al., 2001). Future studies are needed to investigate the outcome of reduced SCAD expression in cancerous colonocytes especially as it relates to the Warburg effect and diminished butyrate oxidation.
3.6 References


Han, Anna, Bennett, Natalie, MacDonald, Amber, Johnstone, Megan, Whelan, Jay, & Donohoe, Dallas R. (2015). Cellular Metabolism and Dose Reveal Carnitine-Dependent and-Independent Mechanisms of Butyrate Oxidation in Colorectal Cancer Cells. *Journal of cellular physiology*.


3.7 Appendix
Figure 20 Butyrate suppresses its own oxidation by inhibiting SCAD expression in colorectal cancer cells.

(A) Percentage change in oxygen consumption rate (OCR) relative to baseline in pre-treated HCT116 cells with and without butyrate (5 mM, 24 hrs). (B) Area under the curve measurement from OCR analysis taken after 2DG injection but before azide injection (56-104 min). (C) Schematic diagram of butyrate oxidation in the cancerous colonocytes. Cancerous colonocytes oxidize butyrate through carnitine-dependent and/or independent pathway. In the mitochondria, SCAD plays a role in first step of butyrate β-oxidation. (D) Western blot confirming the reduced SCAD level by butyrate. Data for butyrate oxidation measurement represent the average OCR (%) over 3-5 replicates per condition. Error bars are ± SEM. MCT1; monocarboxylate transport protein 1, OCTN2; organic cation/carnitine transporter, ACSS; Acyl Co-A synthetases, CPT1/2; carnitine palmitoyltransferase 1/2, SCAD; short chain acyl-CoA dehydrogenase
Figure 20 continued
Figure 21 Butyrate reduces SCAD expression in colorectal cancer cells.

(A) Western blot showing SCAD expression in FHC cells (non-cancerous colonocytes) and HCT116 cells (colorectal cancer cells). (B) Western blot describing SCAD expression in FHC cells and HCT116 cells that were treated with (0 mM, CON) or without butyrate (5 mM, B5). HCT116 cells were grown under the absence (2.5 mM glucose) and presence (25 mM glucose) for the Warburg effect. (C) Quantification of SCAD levels relative to PDH levels. (D) Western blot showing SCAD levels in isolated normal colonocytes with butyrate (0 mM, CON; 5 mM, B5 and 10 mM. B10). (E) Quantification of SCAD expression relative to β-actin levels. For statistical analysis, western blot was conducted three times per condition. Error bars are Mean ± SEM. *p<0.05 indicates significant difference between cells treated with butyrate Vs controls.
Figure 21 continued
Figure 22 Butyrate reduces the mRNA levels of SCAD in colorectal cancer cell.

(A) mRNA expression of isoform 1, isoform 2 and total SCAD was evaluated by semi-quantitative RT-PCR (0 mM, CON; 5 mM, B5 and 10 mM, B10). The relative mRNA level was normalized to 18S rRNA and shown as fold of the control value. For statistical analysis, qRT-PCT was conducted three times per condition. Error bars are Mean ± SEM. *p<0.05 and **p<0.01 indicates significant difference between cells treated with butyrate Vs controls.
Figure 23 Butyrate mediates SCAD expression as an HDAC inhibitor in colorectal cancer cells

(A) Western blot showing SCAD expression in HCT116 cells that were treated with a set of SCFAs (ACT; acetate, PRO; propionate, BUT; butyrate and APB; all of SCFAs) at 5 mM. (B) Quantification of SCAD levels relative to PDH levels. (C) A figure of mechanisms that butyrate plays in epigenetic role in colonocytes. In this figure, butyrate acts as a co-factor for HATs through the involvement of ATP-citrate lyase (ACL). Also, butyrate directly inhibits HDACs. (D) Western blot describing SCAD expression in HCT116 cells that were treated with butyrate (5 mM) or TSA (1 μM). (E) Quantification of SCAD expression relative to PDH levels. For statistical analysis, western blot was conducted three times per condition. Error bars are Mean ± SEM. *p<0.05 and **p<0.01 indicates significant difference between cells treated with butyrate Vs controls.
Figure 23 continued
Figure 24 Butyrate selectively inhibits HDAC 1 to reduce SCAD expression in colorectal cancer cells.

(A) Western blot showing SCAD expression in siMock and siHDAC1 transfected HCT116 cells after treatment without butyrate and with butyrate (5 mM) or TSA (1 μM) for 6hrs. (B) Quantification of SCAD levels relative to PDH levels. For statistical analysis, western blot was conducted three times per condition. Error bars are Mean ± SEM. *p<0.05 indicates significant difference between cells treated with butyrate Vs controls.
Figure 25 The Warburg effect involves in SCAD levels in colorectal cancer cells.

(A) Western blot showing SCAD expression in FHC cells (non-cancerous colonocytes) and HCT116 cells (colorectal cancer cells) with or without Warburg effect. (B) Quantification of SCAD levels relative to PDH levels. For statistical analysis, western blot was conducted three times per condition. Error bars are ± SEM.
Figure 26 Butyrate increases PDH levels in normal isolated colonocytes.
Quantification of PDH expression relative to β-actin levels. For statistical analysis, western blot was conducted three times per condition. Error bars are ± SEM.
Figure 27 Butyrate does not regulate SCAD expression as a metabolite in colorectal cancer cell.

(A) Percent change of oxygen consumption rate (OCR) relative to baseline in siACL and siMock transfected HCT116 cells. OCR (%) after 2DG injection is total contribution of butyrate. (B) Area under the curve analysis from percentage change in OCR of siMock and siACL transfected HCT116 cells with and without butyrate (5 mM). OCR measurement taken after 2DG injection, but before etomoxir (ETO) injection (48-72 mins) (C) Western blot presenting SCAD levels siMock and siACL transfected HCT116 cells after treatment of butyrate or not. Data for butyrate oxidation measurement represent the average OCR (%) over 3-5 replicates per condition. Error bars are ± SEM. For statistical analysis, western blot was conducted three times per condition. Error bars are Mean ± SEM.
Figure 28 HDAC 2 and HDAC 3 are not involved in SCAD suppression by butyrate in colorectal cancer cells.

(A) Western blot presenting SCAD levels in siMock and siHDAC2 transfected HCT116 cells after treatment without butyrate and with butyrate (5 mM) or TSA (1 μM) for 6 hrs. (B) Quantification of SCAD levels relative to PDH levels. (C) Western blot showing SCAD levels in siMock and siHDAC3 transfected HCT116 cells after pre-treatment without butyrate and with butyrate (5 mM) or TSA (1 μM). (D) Quantification of SCAD levels relative to PDH levels. For statistical analysis, western blot was conducted three times per condition. Error bars are Mean ± SEM. *p<0.05 indicates significant difference between cells treated with butyrate Vs controls.
CONCLUSION AND FUTURE DIRECTION

In this dissertation, it has been presented (1) the capacity of butyrate oxidation is reduced in cancerous colonocytes compared to non-cancerous colonocytes; (2) butyrate is oxidized in the colorectal cancer cells by both a carnitine-dependent and -independent mechanisms at physiologically relevant levels, which are selective for butyrate only among other SCFAs oxidation; (3) carnitine-dependent butyrate oxidation mechanism is suppressed in colorectal cancer cells through PDH inhibition; (4) butyrate decreases its own oxidation by suppressing SCAD expression in cancerous colonocytes; (5) butyrate functions as an HDAC inhibitor and selectively inhibits HDAC 1 in order to reduce SCAD levels and its own oxidation in colorectal cancer cells.

Our findings bring a better understanding of butyrate metabolism in cancerous colonocytes that allows insight into why butyrate has selective and inhibitory effects against colorectal cancer cells. Moreover, we report here for the first time that butyrate is involved in its own oxidation by specifically inhibiting HDAC1.

In the future, the studies of the effect of carnitine supplementation on colorectal cancer by using animal model and/or human study would be needed to test the results using in vitro models suggested in chapter II. Dietary sources with high carnitine (i.e. red meat) have shown an increased susceptibility of CRC development. Based on our findings in chapter II, the consumption of high carnitine dietary sources (i.e. red meat) might increase butyrate oxidation resulting in reduced HDAC inhibition and subsequently reduce butyrate’s
protective effects against CRC. Successful animal and/or human studies will offer a justification of butyrate’s selective and protective effects toward CRC.

Moreover, based on the conclusions of chapter III, we need to answer why butyrate regulates its own oxidation in cancerous colonocytes. This future studies will suggest another mechanism whereby butyrate shows anti-cancer effects toward CRC. Also, investigating role of SCAD in butyrate oxidation in cancerous colonocytes would be helpful to explain why cancerous colonocytes have reduced butyrate oxidation capability compared to non-cancerous colonocytes and its subsequent results in colorectal cancer development.
VITA

Anna Han was born in Stuttgart, Germany on December 15, 1986. She obtained a Bachelor of Science in Food Science and Human Nutrition from Chonbuk National University in 2009. After that she acquired a Master of Science in Food and Nutrition from Seoul National University in 2011. Anna Han began her doctoral program under Dr. Dallas Donohoe at University of Tennessee, Knoxville in 2013 and completed in 2017.