AMERICAN UNIVERSITY OF BEIRUT

TARGETING DYSBIOSIS OF DIABETES AND COLORECTAL CANCER: GUT A FEELING, IT PLAYS A ROLE

by
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AN ABSTRACT OF THE THESIS OF

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Introduction:

In healthy individuals, the gut microbiota favors good butyrate-forming bacteria; this balance is shown to be revoked during diabetic-associated dysbiosis. Butyrate is a short chain fatty acid (SCFA) that acts as a histone deactylase inhibitor (HDAC) and is speculated to play a crucial role in mediating diabetic complications through inhibiting inflammation and ROS production. Previous work by our group demonstrated that ROS production is prevailing in the course of diabetes-associated complications. In this project we aim to study the role of diabetic and colorectal cancer dysbiosis and butyrate or probiotics on the development of diabetes-associated complications with a focus on diabetes and colon cancer onset and development.

Methods:

HT-29 were used in this project where proliferation via the viability assay was assessed. To confirm our in vitro observations, *MKR* and *APC* mice models were used. The effect of probiotics or butyrate supplement on the microbiota of the diabetic and the APC mice was assessed using biochemical and molecular techniques. After sacrifice, colons were harvested for biochemical, histological, and molecular analysis.

Results:

HT-29, colorectal cell line, were cultured either in 5mM media(Normal glucose; NG) or in diabetic mimicking media of 25mM (high glucose; HG). In parallel, experiment cells were treated with 0.75 mM butyrate in the presence or absence of HG. Butyrate treatment decreased HT-29 cellular proliferation starting at 12 hours and this was sustained throughout the whole length of the experiment. Furthermore, in animal models of diabetes and cancer, probiotics or butyrate treatment reversed the alteration seen in the autophagy pathway, where beclin-1 and LC-3β were comparable to that of control mice. By to by treatment with probiotics or butyrate reversed diabetes- and cancer-induced complications. Taken together, our results suggest that dysbiosis associated with both diabetes and colon cancer impair autophagy through oxidative stress and this can be reversed by butyrate or probiotic treatment.

Conclusion:

Dysbiosis characterized by reduction of butyrate-forming bacteria leading to a decreased butyrate is a key player in diabetes and colon cancer. Restoring gut microbiota homeostasis via probiotics or butyrate administration show a protective effect on reducing diabetes induced colon injury in *MKR* mice as well as a decrease in polyp formation in the *APC* cancer mice. This effect is paralleled by restoring autophagy homeostasis and decreasing oxidative stress. Pursuing this mechanism and the pathways it intercalates offers a promising insight into identifying novel treatment for diabetes, cancer, or diabetes-induced cancer.

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LIST OF ABBREVIATIONS

- DM: Diabetes Mellitus
- CRC: colorectal cancer
- NG: Normal Glucose
- HG: High Glucose
- ATG: Autophagy related protein
- HDAC: Histone deacelytase
- PAS: Periodic acid Schiff
- HPLC: High performance liquid chromatography
- ROS: reactive oxygen species
- PCR: polymerase chain reaction
- HbA1c: hemoglobin A1c
- T1DM: type 1 diabetes mellitus
- T2DM: type 2 diabetes mellitus
- GDM: gestational diabetes mellitus
- FBG: fasting blood glucose
- BG: blood glucose
- BW: body weight
- OGTT: oral glucose tolerance test
- ADA: American diabetes association
- WHO: world health organization
- IGF: insulin like growth factors
- NADPH: Nicotinamide adenine dinucleotide phosphate
- NOX: NADPH oxidase

CHAPTER I

INTRODUCTION

A. Diabetes Mellitus: General Overview

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. According to the International Diabetes Federation, it is estimated that DM affects 415 million of the world adult population (International Diabetes Federation, 2015). The incidence of the diabetes epidemic is on the rise and it is expected to rise beyond 592 million in 2035[1]. Furthermore, in the Middle East and North Africa region (MENA), the prevalence of diabetes has increased due to many factors mainly urbanization and lifestyle changes. In 2013, countries in the MENA region were among the areas with the highest rates of diabetes worldwide, whereby 9.2% of the adult population was diagnosed which is equivalent to 34.6 million people. This number is expected to increase to almost 67.9 million in 2035. In fact, Lebanon ranks the 7th in the top 10 countries for prevalence in the MENA region bearing 14.99% of diabetic cases[2]. In a population-based study predominantly type II DM was found to be a leading cause of mortality among the Lebanese population, with 8.5% issued a diagnosis[3].

Diabetes mellitus can be classified into 4 main categories, Type I DM, Type II DM, gestational diabetes and other specific types. Type 1 diabetes mellitus (T1DM), also called insulin-dependent diabetes, results from an autoimmune destruction of ß cells. Type 2 diabetes mellitus (T2DM), termed insulin-independent diabetes, encompasses patients with insulin resistance. The third type is gestational diabetes mellitus (GDM) which occurs during the second or third trimester of pregnancy. However, most cases of GDM resolve after pregnancy.

The commonly experienced and clinically relevant symptoms include frequent thirst, polyuria, tiredness, lack of interest and concentration, blurred vision, tingling sensation or numbness in the hands or feet, weight loss/gain, which is sometimes accompanied with polyphagia, and poor wound healing[4].

1. Classification of diabetes

a. Type 1 Diabetes Mellitus

Insulin Dependent Diabetes Mellitus (IDDM) or juvenile-onset diabetes is commonly referred to as Type I DM[4]. This form develops as a result of the autoimmune destruction of the insulin-producing β-cells of the pancreas, thus leading to insulin deficiency[5]. It is reported that Type I DM is associated with a genetic predisposition; however, there have been speculations that environmental factors may play a role in inducing autoimmune destruction of β-cells, but these are still poorly understood. Type I DM accounts for about 10% of patients with diabetes and is most commonly reported to occur in children and teenagers[6] although it may appear during adulthood as well. This disease is affiliated with a sudden onset whereby the absence of insulin production leaves patients dependent on exogenous insulin administration for their survival[7,8]. Despite active research, Type I diabetes has no cure, and carries the constant threat of devastating complications.

b. Type 2 Diabetes Mellitus

Previously referred to as Non-Insulin Dependent Diabetes Mellitus (NIDDM) or adult-onset diabetes mellitus[4], this form of diabetes is characterized by insulin resistance in peripheral tissues and a relative insulin deficiency[6]. It is the more prevalent form of diabetes mellitus and accounts for about 85% of the cases worldwide[6,9]. Type II diabetes is often associated with a strong genetic component, yet the risk of incidence increases with age, obesity, and lack of physical activity, which suggests that environmental factors are important contributors to the disease[8,10]. In fact, studies have shown that obesity itself or increased percentage of body fat in the

abdominal region is tightly correlated with insulin resistance[11,12]. Defective insulin secretion is central to the pathophysiology of type 2 diabetes. Patients with type 2 diabetes fail to adequately increase insulin secretion in order to overcome insulin resistance. The progression of the deterioration of pancreatic ß cell function results in a sustained increase of blood glucose. This deterioration might be a possible explanation of why patients with T2DM might develop into T1DM.

c. Gestational Diabetes

This type of diabetes is a major medical complication occurring during the second or third trimester of pregnancy. In some cases, GDM does not resolve after delivery thus increasing the risk of developing diabetes later in life[13]. The prevalence of gestational diabetes ranges from 1% to 14% and this is population-dependent with certain ethnicities being at a higher risk[4,8,14].

d. Other Specific Types

Diabetes may further manifest as other types which may be genetically defined or associated with other diseases or drugs such as: exocrine pancreas disease (ex. pancreatitis), viral infections, endocrinopathies (ex. Cushing's syndrome), and insulin receptor antibodies[15].

2. Diabetes Mellitus: Tests for Diagnosis

Diagnosis of diabetes mellitus is achieved through three main laboratory tests. Fasting blood glucose, also considered as the simplest diagnostic test, consists of measuring blood glucose after at least 8 hours of fasting. Another test is the glucose tolerance test where the patient is given a fixed amount of glucose (75g) to be ingested orally two hours before blood glucose measurement. This test allows the assessment how fast glucose is cleared from the bloodstream. Lastly, the most accurate test for the diagnosis of diabetes is Hemoglobin A_{1C} (HbA1c). Hemoglobin is a protein in red blood cells that carries oxygen throughout the body. When glucose builds up in the bloodstream, it tends to bind to hemoglobin in red blood cells. Red blood cells have a life span of around 3 months; hence, this test allows the screening for the

amount of glycated hemoglobin for the past 3 months. It is noteworthy to mention that, unfortunately, 1 in 4 people are unaware that they have diabetes. These diagnostic tests serve as screening tools for the early detection of diabetes mellitus and ultimately allow the early treatment before the development of its complications[16]. According to American Diabetes Association (ADA), diabetes may be diagnosed based on plasma glucose criteria, either the fasting plasma glucose (FPG) or the 2-h plasma glucose (2-h PG) value during a 75-g oral glucose tolerance test (OGTT), or A1C criteria. A patient is said to have diabetes if he or she presents with the following lab values: fasting plasma glucose > or = to 126 mg/dL, OGGT > or = to 11.1 mmol/L or A1C > or = to 6.5 %.

3. Diabetes Mellitus: Complications

It is no secret that diabetes mellitus is a ruthless disease not merely with the associated metabolic outcomes it presents and the strain it places on the body, but also with the vigorous complications it drags along. Chronic complications of diabetes are broadly divided into microvascular and macrovascular complications, with the former having a higher prevalence than the latter. Microvascular complications include neuropathy, nephropathy, and retinopathy, recently known as the triopathy, while macrovascular complications consist of stroke, cardiovascular disease, and peripheral artery disease[17]. Numerous epidemiological, observational and cohort studies have established the risk of cancer development in diabetic individuals with either Type I or Type II DM[18-20]. Furthermore, diabetes being a current epidemic predicted to rise in incidence in the future poses as a burden on diabetic individuals with the risk of cancer development.

4. Cancer in Diabetes

Among the leading causes of mortality worldwide, cancer prevalence is predicted to increase by 70% globally[21]. Several risk factors integrate with the risk of cancer incidence in patients, namely physical and chemical carcinogens such as UV, radiation, tobacco and alcohol,

and biological carcinogens such as infections. Other life-style based risks exist such as age, body-mass index, obesity, weight, poor diet, smoking and sedentary to low physical activity[21,22]. Some of these risk factors are shared with the 12th leading cause of death worldwide, diabetes. Thus, speculations to the extent to which diabetes may contribute to cancer development among patients were raised.

In a meta-analysis study, cancer incidence among individuals with preexisting diabetes was reported to be associated with a higher all-cause mortality risk relative to non-diabetics[23]. The data identified hyperinsulinemia (also known as secondary diabetes) as a diabetic factor rather than hyperglycemia as a contributor to an elevated risk of colon cancer among type II diabetic subjects in response to insulin treatment[24]. Finally, although most of the epidemiological evidence links the prevalence of cancer to the more prevalent form of diabetes, type II, a growing body of data, however limited, indicates an elevated risk among type I patients as well[25-27]. Nevertheless, the mechanisms by which cancer progression occurs in diabetes are still under investigation.

Diabetogenic cancer onset has been shown to develop by cause of numerous pathways. Metabolic disorders associated with diabetes such as hyperglycemia, hyperinsulinemia and inflammation have been described to be key modulators of neoplastic growth[28-32]. This multifactorial pathogenesis is dependent on mediators such as cytokines, hormones and growth factors that play a prominent role in metabolic signaling and function. The coupled diabetic duo, glucose and insulin, influence mitogenic and oncogenic pathways that are normally tightly regulated.

Insulin and the Insulin-like growth factor (IGF) receptors have been shown to be expressed on the surface of most cancerous cells. In a breast cancer cell line, it was shown that the downregulation of IGF receptor expression in cancerous cells elevated their sensitivity to insulin, and reduced the malignancy, proliferation and metastatic potential[33]. IGF receptors have

also been shown to play a role in hyperinsulinemia-dependent carcinogenesis. Through its indirect effects, exogenous insulin attenuates the levels of IGF binding protein production by the liver, which in turn leads to the increased availability of biologically active IGF levels in the blood, further enhancing the proliferative and mitogenic effect on neoplastic growth[29 34,35].

Additionally, diabetes and cancer reciprocate influence onto pathways that aggravate the complexity of the disease. With glucose being the central biological linkage that integrates with both etiologies, the Warburg hypothesis actually explains the energetics of tumorigenesis and suggests the significance of insulin-independent glucose uptake by cancerous cells and their reliance on glycolysis as a powerhouse for cellular survival[36-38].

Diabetes is a metabolic disorder that is associated with complex metabolic 'organs' such as adipose tissue and lipids. This imparts a risk in inflammatory cytokine signaling changes that manifest as inflammation. Such factors include adipokines which have been reported to be associated with insulin resistance and diabetes, that influence proinflammatory cytokine (interleukins and tumor necrosis factor) release[39,40]. In a recent study, the roles of the adipokines were examined for their insulin-modulating effect in diabetes. The results were indicative of fluctuating levels of certain adipokines in the serum of diabetic patients, and were reported to play a role in mesotheliomas, gastric cancer, as well as colon cancer[41]. These complex pathways have been linked to tumorigenesis and tumor invasive potential as well as an overall weakened immunity in patients[42]. Together, this suggests that diabetes-associated manifestations such as hyperglycemia and hyperinsulinemia may contribute to and facilitate carcinogenesis especially with chronic, poor glucose management in diabetic patients[43,44].

a. The Diabetic Colorectum

Colorectal physiology has been increasingly shown to be vulnerable to inflammation, infection, colitis, inflammatory bowel disease, constipation, diverticulitis and diverticulosis, polyp formation and cancer [45,46]. In fact, all of these are bridged to a number of risk factors such

as sedentary life styles, poor habits such as alcohol consumption and tobacco use, diet, obesity, genetic mutations, and most importantly diabetes[28,47]. With special emphasis on colonic complications and the overlapping risks with diabetes, colon pathophysiology has been shown to be especially influenced by diabetes and its complex pathologies[29,45,48].

Numerous studies in experimental models of diabetes have reported the implications of diabetes in colon remodeling and malfunction. The data revealed dimensional increases in area and dilation concurrent with significant reductions in muscular tunic, colonic wall thickness, muscular fiber tone and neuronal myeneteric innervation and population[49,50]. In a recent study, Siegman et al., (2016) described the effects of diabetes in Streptozotocin-treated Sprague-Dawley rats. Muscles cells from the colonic mucosa were reported to undergo hypertrophy characterized by elevated DNA levels and extracellular matrix protein (such as collagen) expression under hyperglycemia. These structural modifications were paralleled with muscular stiffness, which contributes to poor dilatory capacity and physiological dysfunction[51].

B. Colon Cancer

1. Staging and Oncogenesis

As mentioned earlier, epidemiological studies provide strong evidence that diabetic subjects are at a significantly higher risk of developing numerous forms of cancer and solid tumors, at a higher rate of occurrence and progression relative to the general population [52-54]. Colon cancers are actually among the frequently reported in diabetic patients with a risk ranging from 1.2 to 1.5[55]. Indeed, the impact of diabetes on colon cancer patients' disease-free and overall survival and recurrence of the malignancy was reported to be worse in comparison to non-diabetic patients[56].

Despite major advancements in cancer research, colon cancer remains the third most common type of cancer. A recent World Health Organization (WHO) report revealed that in

2015 alone, colorectal cancer estimated 774000 lives globally. For the year 2018, the American Cancer Society estimated 97,220 new cases of colon cancer and 43,030 new cases of rectal cancer in the United States.

Colon cancer affects the colon (Ascending, Descending, or transverse) and the rectum area of the large intestine. Three main phenotypes can be classified as follows; non-cancerous, pre-cancerous, and cancerous. Non-cancerous begins as a non-cancerous polyp that grows slowly on the inner lining of the colon or rectum. The precancerous phase is an adenomatous polyp or adenoma. Less than one tenth of these adenomas might progress into cancer. There are several different types of polyps of which the most prominent is adenomatous polyps. The second most common polyps are flat polyps (which are not easily detected with a colonoscopy), and the third is hyperplastic polyps. Furthermore, colon cancer may be divided into four main stages depicted in **Figure 1**.

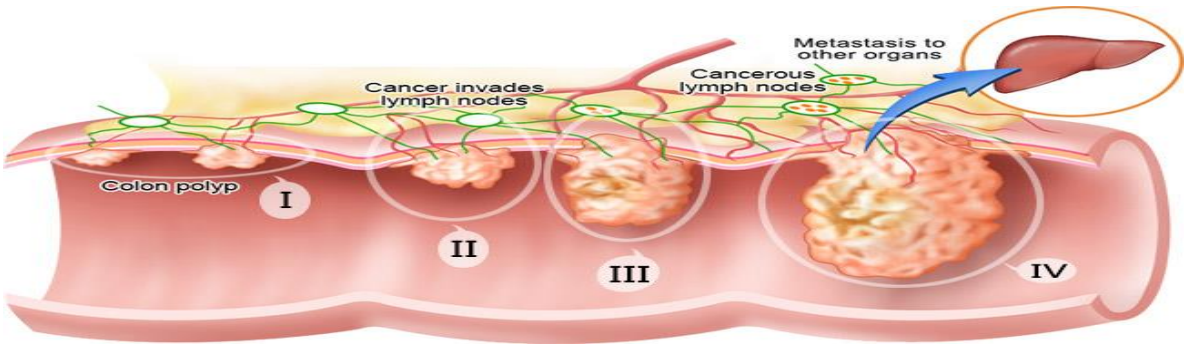


Figure 1: Colorectal cancer transformation happens at various stages 1 to 4. Adapted from: Modern Cancer Hospital Guangzhou, 2012 (http://www.asiancancer.com/cancer-topics/newintestinal/).

Stage I is when the tumor has grown through the inner bowel lining reaching the muscle wall of the bowel. Stage 2 is a tumor that has grown through the outer bowel lining but without the involvement of nearby tissue or lymph nodes. Stage 3 is when the tumor has grown into the muscle layers of the intestine and has spread to at least 1-3 lymph nodes. Stage 4 refers to cancer that has spread to a distant part of the body, such as the lungs or liver.

The oncologic pathogenesis of colon cancer is a multi-factorial etiology and much research goes to unravel the mechanisms through which benign growths turn into malignancies. Numerous studies show that colon cancer tumor development and malignant transformations possess genetic and epigenetic components that govern cellular pathways. One of the most important genes contributing to adenoma formation and responsible for the autosomal-dominant disease known as familial adenomatous polyposis is the adenoma polyposis coli (APC) gene[57]. The APC gene triggers colonic adenoma development throughout the colon of these patients at a young age[57]. Mutations in the APC gene have been linked to colon cancer progression in the early stages[58] and have been described to prevent the apoptotic death of colonic neoplastic growth in the early phases by conferring cyclin D1 over expression[59].

A second distinctive mechanism of colon cancer tumorigenesis involves defects in the DNA Mismatch Repair system which is pivotal for proofreading and nucleotide repair during replication[60]. These defects are further associated with microsatellite instability[61]. These represent molecular pathways whereby the aforementioned repair system is disabled exacerbating the rate of mutations typically described in another form of colon cancer, hereditary nonpolyposis colon cancer[60].

There are several phenotypic changes associated with colon cancer. Besides the malignant growth characterized as polyps, the length of the colon also varies depending on the degree of inflammation. Studies have shown that increased inflammation is associated with a shorter colon length due to decreased surface area, thus, leading to constipation and cramping [3]. Bleeding is also observed in the colon of colon cancer patients manifested as bloody stools.

2. The Role of Hyperglycemia/Hyperinsulinemia in Colon Cancer Progression

Several pathways by which colon cancer progresses are intimately affected by hyperglycemia/diabetes. Within this context, extensive research indicates that an association exists between elevated glucose levels or glycated hemoglobin levels and the predisposition to

colon cancer malignancies [28,42]. Indeed, clinical studies reported that patients with poorly controlled Type II DM have more right sided and advanced colon cancer, a younger age of presentation, greater use of exogenous insulin, and a poorer 5-year survival[62]. In another study, epidemiological data revealed the relationship between fasting serum glucose and DM and the risk of all cancers as well as specific cancers in men and women. The study found that elevated fasting serum glucose concentrations and DM are risk factors for the development of cancer in several tissues including colon cancer[63]. The age-adjusted incidence and mortality rate for colon cancer in this study were increased in both genders too[63]. Treatments with glucose-lowering agents in diabetic patients with developed colon cancer have showed an improved survival outcome[40]. Together these data imply that hyperglycemia plays a significant role in diabetes-induced cancer onset. However, further diabetes-associated abnormalities, such as hyperinsulinemia, are shown to also be involved in colon tumorigenesis as well[64,65]. This was observed in a retrospective cohort study whereby Type II DM patients going through insulin therapy were followed to investigate colon cancer occurrence. The data from this cohort indicated a positive correlation between duration of exogenous insulin administration and colon cancer risk[19]. Emphasis on insulin signaling is placed alongside hyperglycemia due to the complications that arise as a result of insulin treatments. In type 2 diabetes, insulin levels become exaggerated mainly due to peripheral resistance, which leads to the overproduction of insulin in a deleterious and cyclic manner. Insulin administered exogenously further exacerbates these outcomes, leading to colon cancer as epithelial cells of the colon gradually acquire characteristics typical of neoplasia[66]. Indeed, a significant and threefold increase in risk of colon cancer development has been reported among type 2 diabetic patients that are dependent on insulin[67]. Nevertheless, the metabolic and mitogenic mechanisms by which glucose or insulin signaling induce neoplastic growth and malignancies have not been clearly understood.

C. Microbiota

The gut microbiota is an essential part of the human body that is maintained through mutualistic association. It is highly diverse and varies between different individuals, yet when analyzed at the level of the phyla two main categories seem to be similar in everyone; Firmicutes and Bacteroidetes[68]. The gut microbiota plays several crucial physiological functions such as aiding in the digestion of indigestible dietary fibers, during which it produces short chain fatty acids mainly butyrate. Butyrate is the preferred energy source for colonic enterocytes, and it contributes to maintaining the cell homeostasis[68]. Some other functions it contributes to include the normal development of the gut and stimulating the host immune system. Techniques to study the microbiota have evolved over the years leading to whole genome sequencing. These advanced analysis methods would allow us to further understand the nature and characteristics of the families present and may aid in studying and preventing certain associated diseases. The gut microbiota population is usually strictly anaerobes including *Bacteroides, Eubacterium, Bifidobacterium, Fusobacterium, Peptostreptococcus,* and *Atopobium*[69], and some facultative anaerobes such as *Enterococci*, *Lactobacilli*, *Enterobacteriaceae*, and *Streptococci* that are a minority[69]. Assessing the gut microbiota in a state of disease versus healthy candidates can be a crucial insight into the progression of the diseases.

1. Microbiota in Diabetes

To recapitulate on what was previously mentioned, the gut microbiota are resident bacteria in our gut and are mainly comprised of four main phylla; *Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria*[70]. An alteration in the gut microbiota, known as dysbiosis, is implicated in several diseases of which is type 2 diabetes Mellitus[71]. One resident bacteria that plays a role in disease pathogenesis is *Bacteroides fragilis (B.fragilis*) that is represented by less than 1% in the gut[72]. *B.fragilis* can be of two types nontoxigenic and enterotoxigenic (ETBF)

with the enterotoxigenic being a key causative of diarrhea illness. Furthermore, *B.fragilis* underrepresentation is involved in the development of type 2 Diabetes Mellitus[73]. Two strains of bacteria play a role in promoting anti-oxidative stress, whereby *Bifidobacterium* was found to hoarder hydroxyl radicals and superoxide anion[46] and *Lactobacillus* promotes the production of anti-oxidants. Knowing that oxidative stress is one of the underlying pathogenic mechanism of diabetes, we can understand how the gut microbiota can function to alleviate this stress and enhance anti-oxidant properties.

2. Microbiota in Colon Cancer

The gut microbiota plays crucial physiological roles aiding in digestion and maintaining the protective shield of the colon[69]. As mentioned earlier, B. Fragilis is one of the resident bacteria that is present in our gut and shown to be implicated in the progression of colon cancer by increasing epithelial cell permeability.[72] These gut microbiota help in the digestion of dietary fibers leading to the production of short chain fatty acids including butyrate[74]. In turn, butyrate is then oxidized by colonocytes under normal physiological conditions to perform its functions such as increasing cell differentiation and inducing apoptosis in colon cancer cells.[75]

Previous reports have shown that the production of SCFA's decreases in colon cancer [76]. This suggests the mechanism behind the progression of colon cancer.

3. Diabetes, Colon Cancer, and the Microbiota: A love-hate relationship:

During normobiosis, a state where the gut microbiota is in normal physiological conditions, the biological state of an individual is often healthy in accordance. This positive synergism is disrupted in the case of diabetes leading to a state of dysbiosis. Dysbiosis, or altered gut microbiota, is thought to be the driving force that leads to diabetes-associated colon cancer. One of the main players that seem to monitor this progression is sodium butyrate by applying epigenetic modifications via histone deacetylation. During dysbiosis, the microbial signature is altered which leads to decreased production of butyrate resulting from a decrease in butyrate

forming bacteria. This, in turn, increases the activity of histone deacetylase (HDAC) that promotes inflammation via ROS production or immune responses. Therefore, these changes, altogether, favor the progression of diabetes-associated colon cancer.

4. Probiotics

Probiotics or "For Life" from its Greek origin has long been of interest to scientists to understand the role they play in normal physiology and pathophysiology[77]. Probiotics are live strains of bacteria administered in adequate doses for health stimulating purposes. Among those *Bifidobacteria* and *Lactobacilli* are the most studied strains. Several studies look at the effect the gut microbiota has on certain diseases; thus, probiotics were used to 'correct' the disturbed microenvironment to treat diseases such as irritable bowel syndrome (IBS), Crohn's diseases, type 2 diabetes, and colon cancer[77]. Probiotics exhibit various health beneficial properties including antioxidant properties. Several strains of probiotics displayed antioxidant behavior such as *Bifidobacterium* where it was found to scavenge hydroxyl radicals and superoxide anion[46]. *Lactobacillus* also play a key role in protecting against oxidative stress by increasing the levels of antioxidants in the body and decreasing ROS[46].

5. Short Chain Fatty Acids: Butyrate

As mentioned earlier, butyrate is a short chain fatty acid produced by the colon microbiota such as *Faecalibacterium* and is the preferred source of energy for colonocytes. Emerging research linking dysbiosis to disease states has highlighted the pivotal role butyrate plays including maintaining the gut barrier functions, inflammatory, and immunomodulatory functions[78]. Other studies underlined the interaction between other innate bacterium and butyrate, as well as with butyrate-producing colon bacteria. For instance, Bifidobacterium, which have a 5% prevalence in our colon, are crucial in our heathy gut microbiota for their function in aiding in digestion, producing antioxidants, and protecting against pathogens[78]. It has been shown that there is a cross-feeding relationship between butyrate-producing bacteria and bifidobacteria that enhances

the co-existence of these two crucial bacterium in our healthy gut microbiota[78]. Butyrate practices epigenetic modifications as a histone deacetylase inhibitor whereby it increases histone acetylation[75]. Butyrate is shown to be oxidized by colonocytes under normal physiological conditions to perform its functions like increasing cell differentiation and inducing apoptosis in colon cancer cells[75].

D. Autophagy: Overview and its implications in health and disease

Autophagy is described as a process that helps maintain cellular physiology by digesting and recycling unwanted organelles[79,80]. During autophagy, cytoplasmic material is delivered to lysosomes for degradation in a process termed macroautophagy. The other two types of autophagy are termed microautophagy and chaperone-mediated autophagy which involve direct invagination of the lysosomal membrane[81]. Materials that are engulfed during autophagy lead to the formation autophagosomes which in turn fuses with lysosomes to form an autolysosome that digests and recycles the materials inside[81]. Autophagy plays a double-ended sword role whereby it has tumor-suppressor and tumor-promoter functionalities. In cases where it promotes less inflammation and clearance of damaged organelles, it also ensures the maintenance of the cancer stem cells[81]. It is noteworthy that the autophagic pathway can be activated by stress such as nutrient deprivation, hypoxia, or oxidative stress[82]. During the formation of autophagosomes, a crucial step in autophagy, Beclin 1 (BECN1), also referred to as ATG-6, is at the center of the signaling complex that is activated and recruited[83]. When autophagosomes are formed, a cytosolic form of microtubule associated protein 1A/1B light chain (LC3) is fused into the membrane[84]. Another autophagy factor implicated in the process is Autophagy related 12 (ATG-12) which facilitates the lipidation of the LC3 family mentioned earlier[85].

1. Autophagy in Diabetes

Autophagy can play a dual role when it comes to maintaining the viability of a cell. When autophagy is stimulated under stress, it functions in clearing damaged organelles and thus sustain cell survival. However, when activated under over-stress, autophagy may drive the cells to their own death[80]. Increasing evidence have reported the role of autophagy in protecting pancreatic beta cells against oxidative stress[86]. Recent studies showed that mice with Autophagy related protein 7 (ATG7) knockout displayed degeneration of beta-cells, impaired glucose tolerance with reduced insulin secretion that further contributed to the progression of diabetes[87].

2. Autophagy in colon cancer

The role of autophagy in colon cancer remains to play a contradictory role. On one hand, autophagy is protecting benign cells from becoming malignant by clearing damaged organelles and reducing ROS[81]. On the other hand, it is aiding in tumor progression by allowing cancer cells to maintain their survival and gain access to nutrients[81]. Colon cancer cells are often under metabolic stress due to hypoxia and nutrient deprivation thus they demand more energy and resources which can be pertained through autophagy[81].

E. Oxidative Stress, Diabetes, and Colon Cancer

1. Reactive Oxygen Species and their Role in Homeostatic Maintenance

Reactive Oxygen species (ROS) are bioactive molecules that contain oxygen, and are produced as byproducts of ongoing biochemical cellular reactions. Several forms have been identified, and they include nitrogen based free radical species such as nitric oxide and peroxynitrite as well as superoxide free radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and singlet oxygen. ROS have evolved to play an important role in house-keeping physiologic and cellular processes and therefore, are crucial entities for cellular physiology that are involved in gene expression, cell signal transduction, cell and tissue growth and proliferation, host defense and

innate immunity and homeostatic maintenance. ROS and free radical formations also influence angiogenesis, salt and fluid homeostasis, biochemical reactions, apoptosis, etc.[88]

An imbalance between ROS generation and the ability of the antioxidant defense mechanism to neutralize the excess ROS and their intermediates, or repair the resulting damage, is considered deleterious and may lead to oxidative stress[89]. This is defined as the state whereby ROS overwhelms cellular defenses and leads to injury through lipid and protein oxidation, dysmetabolism, activation of intracellular signaling and transport pathways, and ultimately programmed cell death.

Hyperglycemia has been shown to disrupt the oxidant-antioxidant balance by triggering persistent ROS production[90], and lowering the antioxidant defenses. As a result, antioxidant therapy approaches have been studied and shown to act at different levels, inhibiting the formation of ROS, scavenging free radicals or increasing the antioxidants defense enzyme capabilities.

2. Oxidative Stress in Diabetic Complications

Diabetes and hyperglycemia are accompanied by increased generation of reactive oxygen species (ROS)[91]. Diabetes in all its forms is characterized by impaired insulin-stimulated glucose uptake in adipose and muscle tissues leading to the consequential elevation in circulating blood glucose levels and the subsequent uptake by insulin-independent tissues. As a result, the disregulated intracellular flux of glucose leads to ROS overproduction. In fact, long-standing hyperglycemia is reported to be associated with increased systemic and cellular oxidative stress, which is currently accepted to be the common pathway for cellular injury, leading to the onset and progression of diabetic complications[92].

3. Oxidative Injury and Carcinogenesis

ROS play a central role in intracellular signaling that may be a double-edged sword. ROS may play a role in cellular senescence or cellular survival depending on a variety of factors

and signaling pathways affected, which are actually susceptible to endogenous or exogenous sources of ROS, conferring support to the environmental contribution to carcinogenesis[93]. Consequently, cells may either maintain molecular interactions and remain anti-tumorigenic entities, or transform into oncogenic cancer cells[93]. Such alterations exacerbated by ROS production by cancerous cells alter several pathways that mediate oncogenesis and malignant transformations. Indeed, oxidative damage has been reported to be underlying the mechanism of carcinogenesis in several cancers[94] via facilitating genomic instability and by being a DNA mutagen.

4. NADPH oxidases in Colon and Carcinogenesis

NADPH oxidases are one of the many sources of ROS in biologic systems, and there are seven isoforms: NOX 1, NOX 2, NOX3, NOX4, NOX 5, DUOX 1, and DUOX 2 [95]. The NADPH oxidases have certain conserved structural characteristics that are common in all isoforms and include a NADPH-binding site at the COOH terminus, a Flavin adenine dinucleotide (FAD)-binding region in proximity of the COOH-terminal transmembrane domain, six conserved transmembrane domains, and four highly conserved heme-binding histidines. Under normal non-pathologic conditions, NADPH oxidases produce ROS for a positive impact such as regulating blood pressure via reducing nitric oxide (NO)[96]. However, under stressful conditions stimuli, such as cytokines or hyperglycemia, NADPH oxidases are suggested to be the driving force behind activating signaling pathways that lead to cellular death due to the overproduction of ROS[97]. NOX1 and NOX 4 are the predominant form in colon tissue and will be discussed in more details below[88]. For the purpose of this thesis, we assessed the expression of NOX 4 merely in colon tissue of the different mice groups.

Cancer cells, like other non-malignant cells, produce ROS. In tumors, these reactive oxygen metabolites can act as signaling molecules to promote cell survival over apoptosis[98]. Moreover, in malignant and non-malignant tissue, the expression of the NOX family of genes is

described to be highly organ-specific. The Nox1 and Nox4 isoforms are majorly expressed in colon cells. Nox4 has been shown to be prominent in tumorigenicity. Indeed, Nox4 plays a role in carcinogenesis in several cancers. For instance, Nox4-mediated ROS is reported to prevent apoptosis and promote tumor cell growth in pancreatic cancer[99], melanoma cancer cell proliferation and growth[100], breast cancer cells[101], glioblastoma cancer cells[102], and colon cancer cells[103]. Nox4 in these studies is shown to contribute to tumor cell resistance, metastasis and oncotic transformation. Indeed, in colon cancer patients overexpressing Nox4, findings have correlated Nox4 overexpression with a poorer prognosis in colon cancer patients[104] in concordance with a higher likelihood of metastatic growth[105], angiogenicity[106] and apoptotic death[107]. Interestingly, Nox4 overexpression has been also dubbed as a prognostic factor in metastatic colon cancer predictive of relapse in Stage II and III colon cancer and highly associated with metastatic profiles of tumors relative to primary stages[103]. In a study conducted by Bauer et al, results were indicative of the implication of Nox4-dependent cellular motility and thus, a Nox4 mediated contribution to aggressiveness of tumors in colon cancer by modulating cytoskeletal regulating proteins[103].

However, despite these studies, our understanding of the role(s) of the NOX family of genes in the development and growth of human cancer and especially in colon cancer is limited, nevertheless the role of hyperglycemia in colon cancer tumorigenesis.

F. TIGAR in Diabetes and Colon Cancer

P53 is a tumor suppressor transcription factor that serves in several cellular functions including apoptosis, senescence, and cell cycle. One of the targets of P53 is the protein TP53 induced glycolysis regulatory phosphatase (TIGAR) that acts as a fructose-2,6-bisphosphatase. Cancer cells typically switch from oxidative phosphorylation to glycolysis for generation of energy. This is a mechanism by which cancer cells evade cell death brought upon by stress and excessive

ROS production[108]. In this case, TIGAR lowers the glycolytic flux and promotes antioxidant properties. Consequently, TIGAR aids cancer cells 'unknowingly' by protecting them from oxidative stress and apoptosis[108]. We aim to assess the expression of TIGAR in cancer models to understand the pathways through which cancer cells sustain their growth.

G. Aim

The aim of this study is to assess the effect of probiotics and sodium butyrate supplements on slowing the progression of diabetes-induced colon dysfunction as well as their effect on colon cancer growth and aggressiveness. Understanding the role of microbiota in these 2 diseases, will set the stage for further studies aimed at understanding the role of diabetes in increasing the risk of colon cancer development. This study focuses on understanding the role of microbiota in regulating NADPH-induced reactive oxygen species generation and autophagy. In a nutshell, in this study, we assess dysbiosis in diabetes and cancer, in order to study the missing link tying these two diseases.

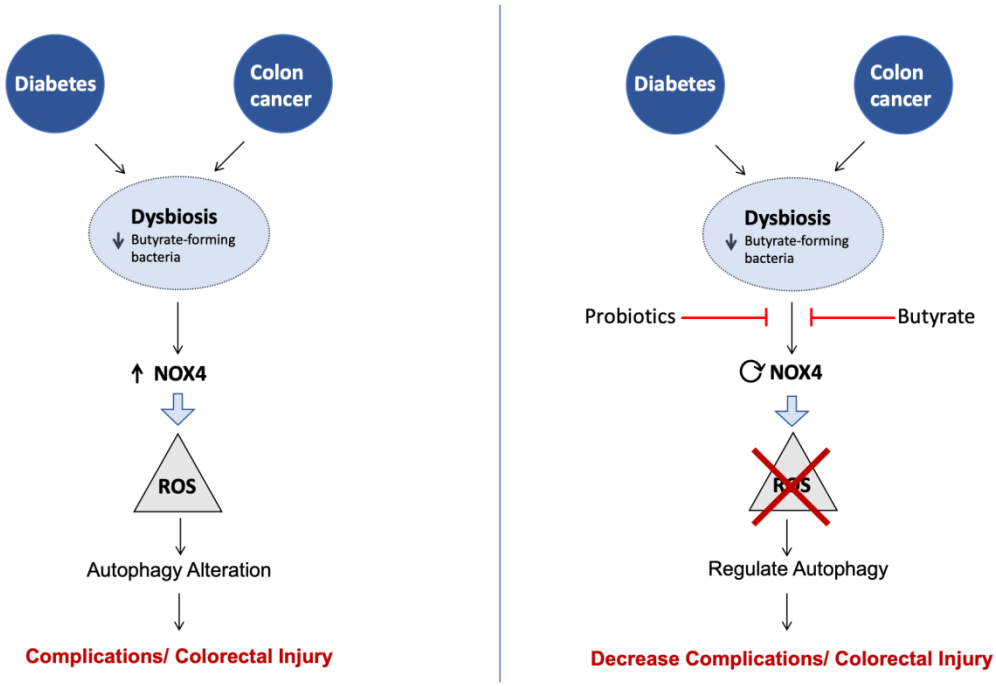


Figure 2: Schematic Representation of the hypothesis

CHAPTER II

MATERIALS AND METHODS

A. In vivo

1. Animal Models

Two different mice models were used for the purpose of these experiments. The first animal model used is the *MKR* mouse model which is genetically predisposed to develop T2DM. This is due to a mutation in its insulin receptor in the muscle. *MKR* mice are non-obese mice that develop mild hyperglycemia associated with insulin resistance[109]. Of interest these mice don't develop dyslipidemia as previously described[109]. The second type of mice were the *C57BL/6-Apc^{tm1Tyj}/J (APC)* mice that have mutations in the Adenomatous Polyposis Coli gene. This results in induction of sporadic colon cancer[110]. *FVB-NJ* and *C57BL/6J* mice served as the controls for *MKR* and *APC* mice respectively as they share the same genetic background. For the first set of experiments the groups were divided as follows: Control mice *FVB-NJ (n=3),* MKR mice (n=3), *MKR* mice treated with Probiotics (n=3), *MKR* mice treated with butyrate (n=3). The second set of experiments included control mice *C57* (n=3), *APC* mice (n=3), *APC* mice treated with Probiotics (n=3), and *APC* mice treated with Butyrate (n=3).

2. Drugs Administered

Upon reaching the age of 8 weeks and weighing around 30g, the mice underwent treatment. The probiotics used in this study is the Probiolife by Green Made which contains 25 Billion colony

forming units. The drug was administered by oral gavage daily to the mice with a dosage of 5mg/kg. The Probiolife contains several strains of bacteria belonging to the phylla Lactobacillus, Saccharomyces, and Bifidobacterium. Sodium butyrate supplement was prepared and administered by intra peritoneal (IP) injections initially with several titrated dosages to determine the LD_{50} which was set at 50 mg/kg. Hence, we opted to use this supplement with a dosage of 20 mg/kg. The control mice received injections of the vehicle (phosphate buffer saline "PBS"). Body weight and blood glucose were assessed every two weeks using a glucometer (Accu-Chek Performa, Roche, USA). Furthermore, fecal samples were collected every two weeks by placing each mouse in an autoclaved cage in order to collect them stress-free and in a short time to avoid any bacterial contamination.

3. Sacrifice and Organ Harvesting:

After 8 weeks of daily treatment, the mice were sacrificed and their organs were harvested. The colons were then cleaned with cold PBS and its length was measured. From the distal side of the colon in the APC mice groups the colon was opened up and polyps were counted if visible. A piece of the colon was stored in 4% formaldehyde and stained with Periodic Acid Schiff (PAS). The harvested organs were flash frozen in liquid nitrogen for proteins and RNA extraction for further molecular studies.

4. HbA1c Measurement

HbA1c done by HPLC using the variant II Hemoglobin Testing System (BIORAD) on blood samples collected from the mice models at sacrifice.

5. Colon Length and Polyps Counting

Colon length was measured using a ruler starting from the distal colon till the end (until it reaches the cecum). Polyps were observed by cutting open the colon gently, exposing the polyps, and counting with the naked eye.

B. In vitro

1. Cell Line

The cell line used in our study is the HT-29 which is a human colorectal adenocarcinoma cell line with epithelial morphology[111]. They grow in an un-polarized and undifferentiated multilayer unless their culturing conditions are altered or when treated. HT-29 cells were cultured in Dulbeccos's modified eagle medium DMEM (Sigma Aldrich, Steinheim, Germany) (5mM glucose) supplemented with 10% Fetal bovine serum and 1% Penicillin streptomycin. To mimick a diabetic environment when needed, we resorted to using high glucose DMEM media with 25 mM glucose after serum starvation for 12 hours. Cells were incubated at 37 ℃ in 5% CO_2 humidified air and 95% air.

2. Viability Assay: Trypan Blue Exclusion Assay

Trypan blue exclusion assay assessed the viability of HT-29 cells after treatment with butyrate (0.75 mM) for 24 hours. After seeding the cells in 6 well plates (triplicates) and serum starving them, we divided them into four conditions: Normal Glucose (NG), High Glucose (HG), Normal Glucose treated with butyrate, and High Glucose treated with butyrate. We then collected the supernatant. After trypsinization, cells were centrifuged at 1000 rpm for 5min. We took 50 uL of cells from the conical and 50 uL trypan blue Then 20uL from the mixture were used to count the cells using the hemocytometer. Viable cells were detected as translucent whereas dead cells were dark blue.

C. Molecular Work

1. Western Blot

Colon tissues that were pertained from the sacrifice were used to undertake western blots to evaluate the protein expression of our protein of interest. A small piece was cut, placed in Radio immunoprecipitation assay(RIPA) buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5%

sodium deoxylate, 150 mM sodium chloride, 100 mM EDTA, 50 mM Tris-hydrochloride, 1% Tergitol (NP40), 1% of the protease and phosphatase inhibitors and 1mM phenylmethylsulfonyl fluoride, and homogenized mechanically at first then in a tissue lyser. It was then left rotating overnight at 4 degrees. The following day, the eppendorf's were centrifuged at 4 degrees at 13.5 rpm for 30 minutes and the supernatant was collected. We performed the Lowry assay to quantify our protein and loaded 30ug/well. For immunoblotting, proteins were separated by 8% or 15% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (BioRad laboratory, USA). The membranes were blocked with 5% BSA and then incubated with the primary antibodies (Table 1). They were detected using a secondary antibody. Bands were visualized using the X-Omat developer. Quantification of the bands was performed on image J software and results were then assessed on GraphPad Prism. Primary antibodies that were used to detect our protein of interest are listed below.

Table 1: Primary Antibodies dilution and Source

Primary Antibody	Dilution	Company
NOX 4	1:500	Santa Cruz
Beclin-1	1:1000	Cell-Signalling
LC3	1:1000	Cell-Signalling
ATG-12	1:1000	Cell-Signalling
TIGAR	1:1000	abcam

2. Real- time PCR

RNeasy Micro Kit (Qiagen) was used to extract mRNA from colon tissues then transformed into cDNA via reverse transcriptase PCR. Samples then underwent real-time PCR using the Real time PCR biorad CFX384 machine. The following human and mice primers were assessed using SYBR green.

Table 2: Real time PCR Primers sequences

	Sequences
Buk-5F1	CCATGCATTAAATCAAAAAGC
Buk-5F2	CCATGCGTTAAACCAAAAAGC
Buk-6R1	AGTACCTCCACCCATGTG
Buk-6R2	AATACCTCCGCCCATATG
Buk-6R3	AATACCGCCRCCCATATG
Total Buk-F	GCAGGCCTAACACATGCAAGTC
Total Buk-R	CTGCTGCCTCCCGTAGGAGT

3. PAS staining

Sections of 4-μm thickness from paraffin-embedded tissues were stained with periodic acid-Schiff. (MetaMorph version 6.1; Universal Imaging). They were then observed under the microscope to detect crypts, nuclei, and polyposis.

4. Detection of SuperOxide via HPLC

The HPLC-based assay allows separation of superoxide-specific EOH from the nonspecific ethidium. Briefly, quantification of DHE, EOH, and ethidium concentrations was performed by comparison of integrated peak areas between the obtained and standard curves of each product under chromatographic conditions identical to those described above. EOH and ethidium were monitored by fluorescence detection with excitation at 510 nm and emission at 595 nm, whereas DHE was monitored by ultraviolet absorption at 370 nm. The results are expressed as the amount of EOH produced (nmol) normalized for the amount of DHE consumed (i.e., initial minus remaining DHE in the sample; μmol).

CHAPTER III

RESULTS

A. In Vivo

Several studies have revealed a variety of observational links between diabetes and cancer. Recent evidence suggests that the microbiome may affect the probability of both diseases. Several internal and external factors that are altered in diabetes, especially the increase in blood glucose levels, may affect this population and alter their composition, which in turn can constitute a major contributor to the onset and development of disease state. Furthermore, during dysbiosis, several bacteria related to inflammation may be altered, as well as the proliferation of butyrate-forming bacteria may be dysregulated. This provides a possible explanation for a correlation between these two diseases.

In this study, we assess dysbiosis in diabetes and cancer, in order to study the missing link tying these two diseases.

In our in vivo experiments, we first assessed the difference in gut microbiota of the diabetic and control mice. Then, we investigated the role of both probiotics and butyrate in slowing the progression of diabetic complications. In parallel experiments, the role of the identified microbial alteration observed in diabetes were studied in the APC mice to assess if they regulate colorectal cancer tumorigenesis.

1. Body weight and blood Glucose:

Body weight and random blood glucose were measured and recorded every two weeks. Our results show that body weight of the MKR mice was comparable to that of their control littermates. Same observations were made concerning the APC cancerous mice. Furthermore, our

results show that MKR mice have a significantly higher random blood glucose values when compared to the control mice, while the APC mice didn't show any changes in their glucose levels when compared to their control littermates. Moreover, HbA1c levels reflected blood glucose changes seen in the different groups of mice. These results were consistent throughout the whole study and sustained till the sacrifice date. Table 3 and table 4 highlight the body weight and random blood glucose levels at sacrifice. It is of utmost importance to highlight that treatment with neither butyrate nor probiotics altered HbA1c levels.

Table 3: Body Weight, Blood Glucose, and HbA1c of MKR mice Models

Conditions	FVB-NJ	MKR	MKR + Probiotic	MKR + Butyrate
Body Weight (g)	**30.6± 0.72**	**23.7± 1.38**	**29.4± 0.46**	**25.3± 1.32**
Glucose (mg/dL)	**150± 20.1**	**247± 18.6***	**191± 6.6#**	**149±7.9#**
HbA1c (%)	**5.5± 0.1**	**8.4± 0.08***	**7.6± 0.2***	**7.6± 0.08***

Table 3: Bodyweight, blood glucose, and HbA1c of MKR mice model(n=3).Data are expressed as mean± standard error of mean (SEM). * at *p*<0.05 vs FVB-NJ; # p<0.05 vs *MKR*

Table 4: Body Weight, Blood Glucose, and HbA1c of APC mice models

Conditions	C57	APC	APC + Probiotic	APC + Butyrate
Body Weight (g)	**28.5± 0.41**	**26.5± 1.2**	**18.4± 1.25**	**23.3± 0.188**
Glucose (mg/dL)	**165.5± 11.3**	**160.6± 6.8**	**139± 5.2**	**133.75± 6.7**
HbA1c(%)	**5.3± 0.15**	**5.1± 0.08**	**4.7± 0.15**	**4.8± 0.14**

Table 4: Bodyweight, Blood Glucose, and HbA1c of APC mice models (n=3).Data are expressed as mean± standard error of mean (SEM).

2. Microbiota of MKR diabetic mice contains less Bacteroid fragilis and butyrate-forming bacteria.

Butyrate-producing bacteria have recently gained attention, since they are important for a healthy colon, and when altered may contribute to emerging diseases such as CRC and type II diabetes. In that same spirit, and in the first set of experiments, we aimed to assess the difference in gut microbiota of the MKR mice compared to their control littermates. For that, real time –PCR (RT-PCR), using specific primers for different bacterial communities and primers for the total butyrate kinase genes (Table 2) was performed. Our results show that the most significant difference between microbiota of *MKR* mice and that of the controls lies in a strong reduction of *B. fragilis* **(Figure 3),** and the butyrate-forming bacteria (as reflected through reduced abundance of the total butyrate kinase gene **(Figure 4).** While both *MKR* mice and their control littermates had similar abundance of *Akkermansia muciniphila, Bacteroidaceae, Enterobacteriaceae, Faecalibacterium prausnitzii, Lachnospiraceae and Ruminococcaceae.*

Figure 3: Gut microbiota in control and diabetic mice

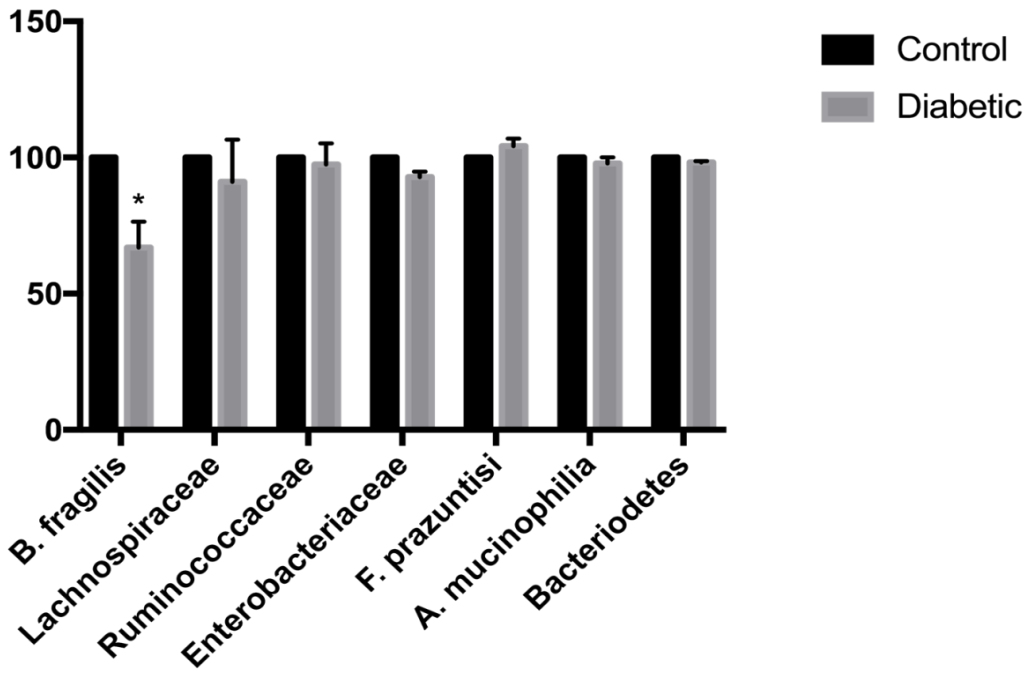


Figure 3: Microbial population of MKR and control mice (n=3). Data are expressed as mean± standard error of mean (SEM). * p<0.05 vs control.

Taken into account these results, we next assessed if the alteration in the butyrate-forming bacteria perceived in diabetes is replicated in colorectal cancer mice.

3. Fecal butyrate contents are significantly lower in diabetic MKR mice and APC mice when compared to control non-diabetic and control cancer free mice.

Since T2DM is correlated with less abundance of butyrate-forming bacterial population, we measured the fecal butyrate contents of *MKR, C57BL/6-Apc^{tm1Tyj}/J (APC)*, FVB-NJ control and C57BL/6J control mice. Our results show that butyrate kinase gene measured in the fecal samples of the different group of mice was decreased in the MKR and APC-/- mice when compared to their control littermates respectively **(Figure 4A and 4B).**

Figure 4: Butyrate producing bacterial fraction in MKR (a) and APC (b)

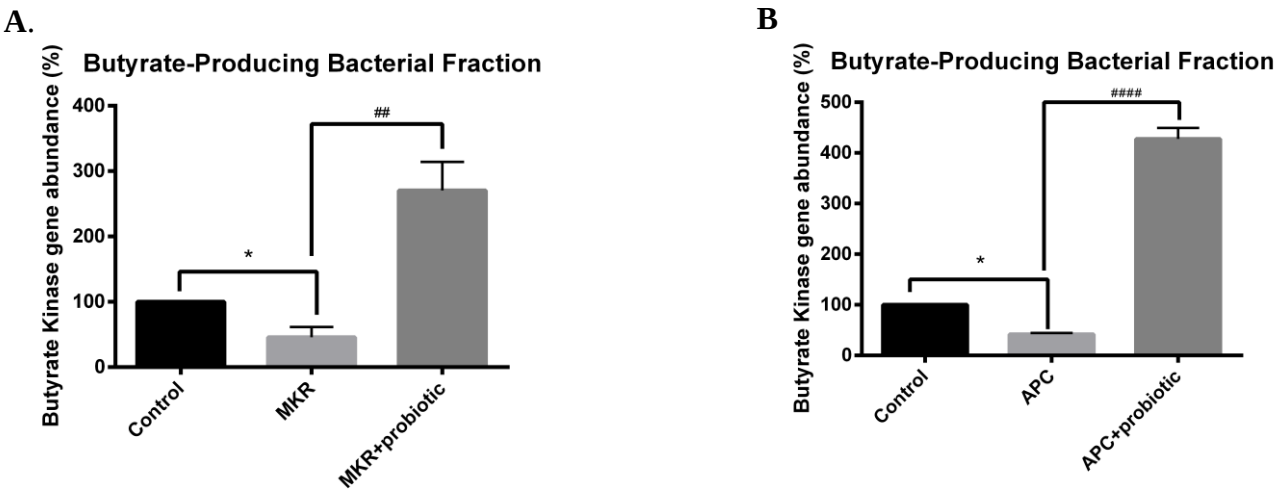


Figure 4: Butyrate producing bacterial fraction in MKR and APC mice models(n=3). Data are expressed as mean± standard error of mean (SEM). * p<0.05 vs control; # p<0.05 vs MKR(2A) APC (2B).

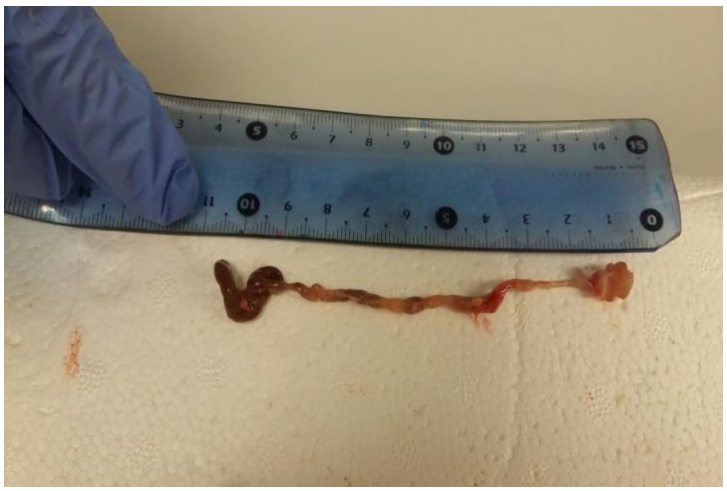
Taken together, our results highlight the alteration in the butyrate kinase gene in diabetes and in cancer mice. Therefore, in the next series of experiments we study the role of butyrate in diabetes-induced colon remodeling, as well as its role in colorectal cancer malignancy.

4. Gut Anatomy is altered in the diabetic animals.

Non-obese type 2 diabetic MKR mice and colorectal cancer mice model (APC) were treated with either probiotics (5mg/kg) or butyrate (20mg/kg). After 8 weeks of treatment, mice were sacrificed and colon tissues were isolated. Anatomical and histological analysis were performed. Our data show that diabetes induces intestinal remodeling, seen as a reduction in the colon length of MKR mice when compared to their control littermates (**Figure 5**). These anatomical changes were restored upon treatment with either butyrate or probiotics (**Figure 5b).** As for the APC mice, our results show that butyrate and probiotics reverse the aggressiveness of CRC, as assessed by the reduction in number of polyps observed in the APC treated mice when compared to the APC mice (**Figure 6b**). Furthermore, our results indicate that butyrate and probiotics reverse the histological changes observed in the APC mice as assessed by PAS staining. Results from the histological sections reveal an increase in the number of crypts per surface area, a decrease in goblet cells as well as a stratification of the nuclei. We can also observe the start of a microadenoma migrating towards the surface from the basement membrane. It is defined by an irregular prominent nuclei and the formation of microadenomatous polyps (**Figure 7**)

Figure 5: Colon length in MKR mice models

A



B

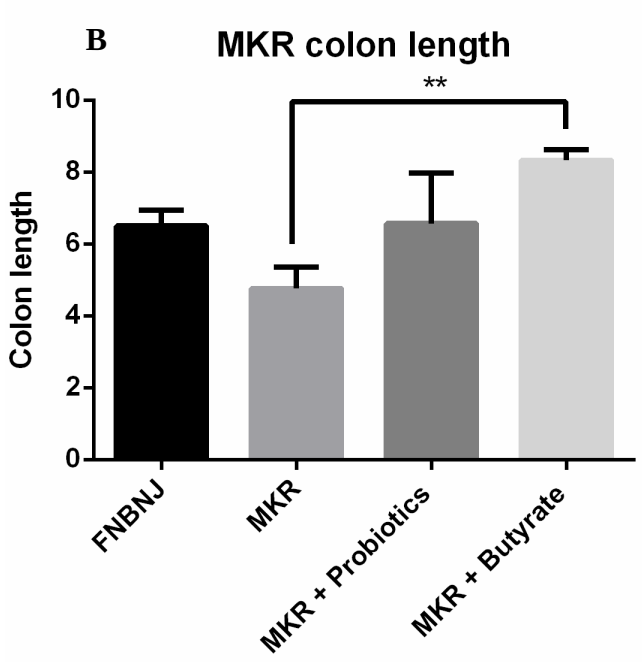
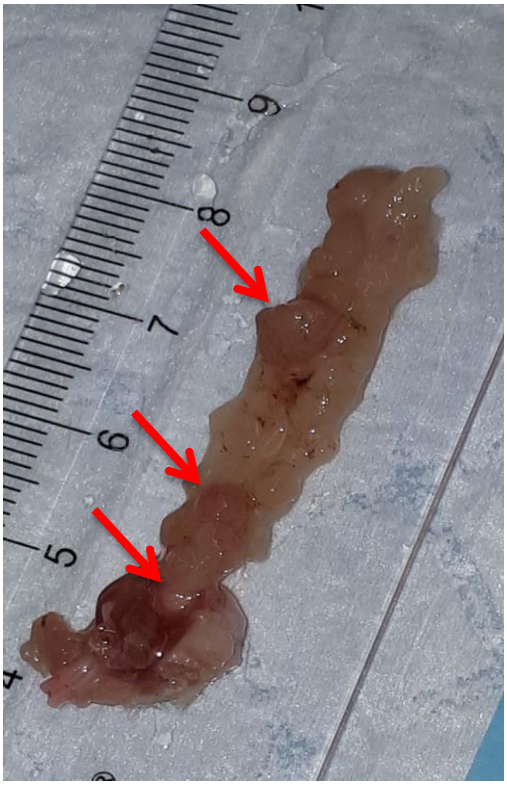


Figure 5: Colon Length in MKR mice models(n=3). Data are expressed as mean± standard error of mean (SEM). * p<0.05 vs MKR

Figure 6: Polyps number in APC mice models

A



B

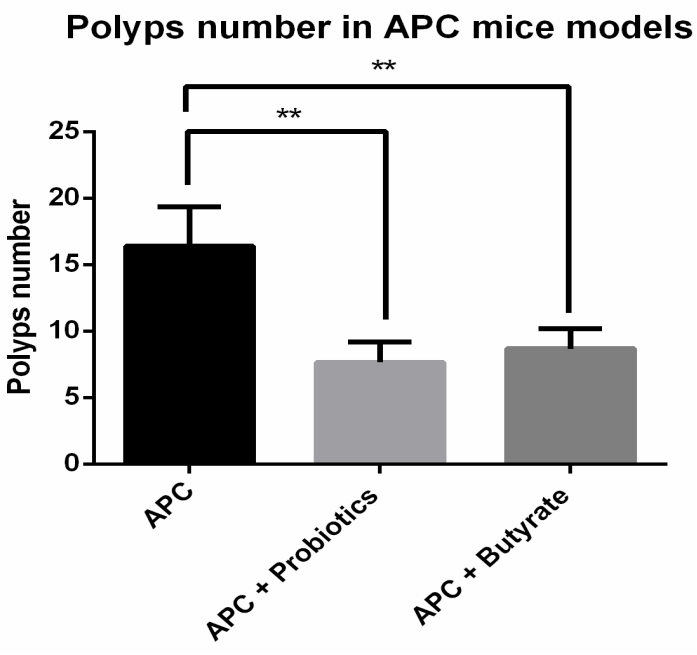


Figure 6: Polyps number in APC mice models(n=3). Data are expressed as mean± standard error of mean (SEM). * <0.05 vs APC.

Figure 7: Histological observations: PAS staining on colon tissue of APC mice models

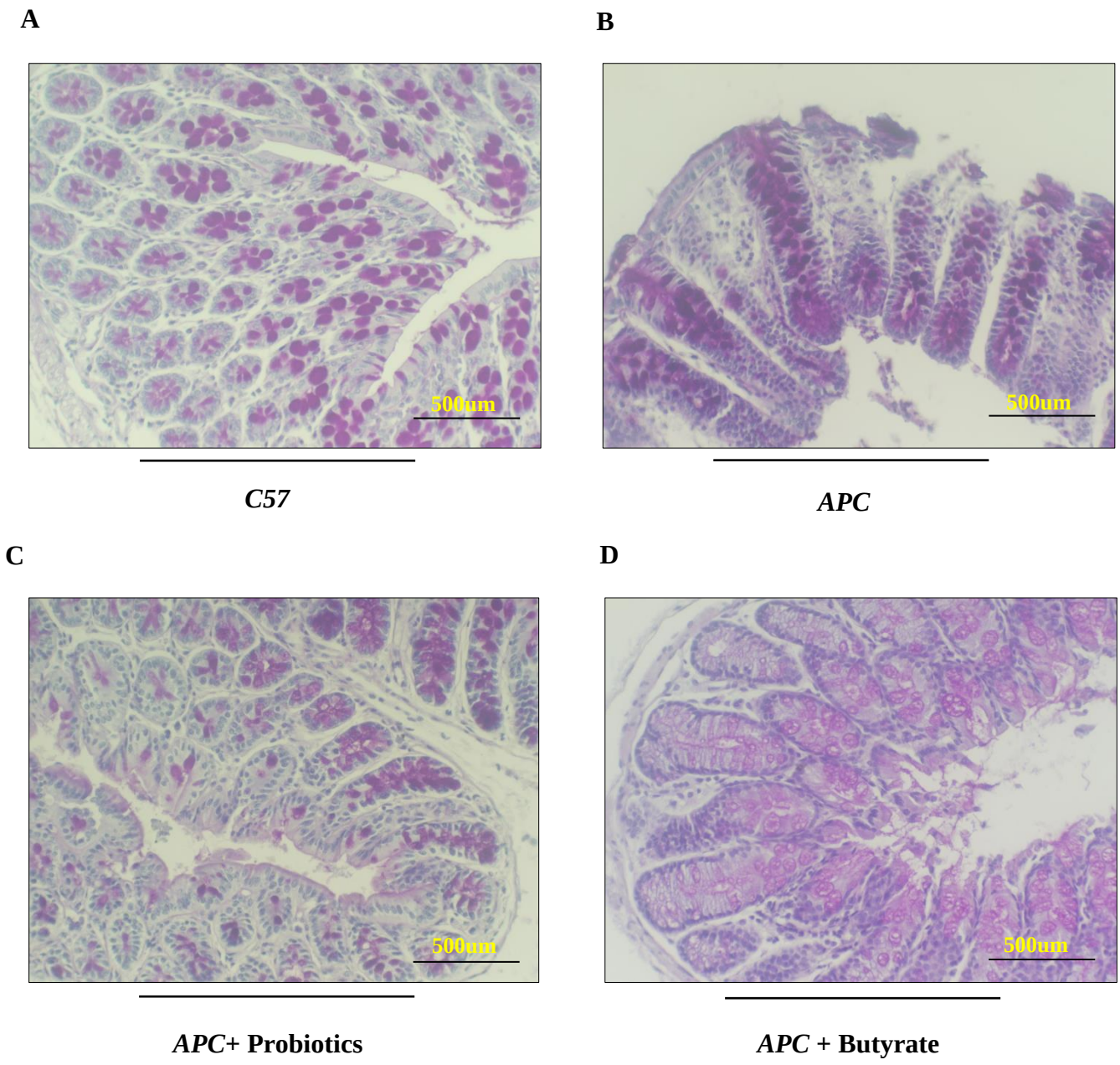


Figure 7: PAS staining Colon Histopathology: Colon sections were taken from the four different groups: C57, APC, APC+Probiotics, and APC+Butyrate cut into thin slices and stained with PAS. A stratification of nuclei, a decrease in goblet cells and an increase in number of crypts per surface area are histological changes observed in cancer. These changes are reversed upon treatment. (A) C57 (B) APC (C) APC treated with probiotics (D) APC treated with butyrate

B. Cell Culture:

We next wanted to validate our in vivo results using an in vitro model, especially regarding the phenotypic changes observed in cancer. For that, HT-29 colorectal cancer cells were grown in high glucose (HG) media in the presence or absence of butyrate.

High glucose increases the proliferation of colon cancer cells in culture

The trypan blue assay results indicate that cultured HT-29 cells in the diabetic mimicking milieu display an increase in cellular viability from 40% to around 70% that was reversed upon butyrate treatment **(Figure 8).**

Figure 8: Trypan Blue Exclusion Assay

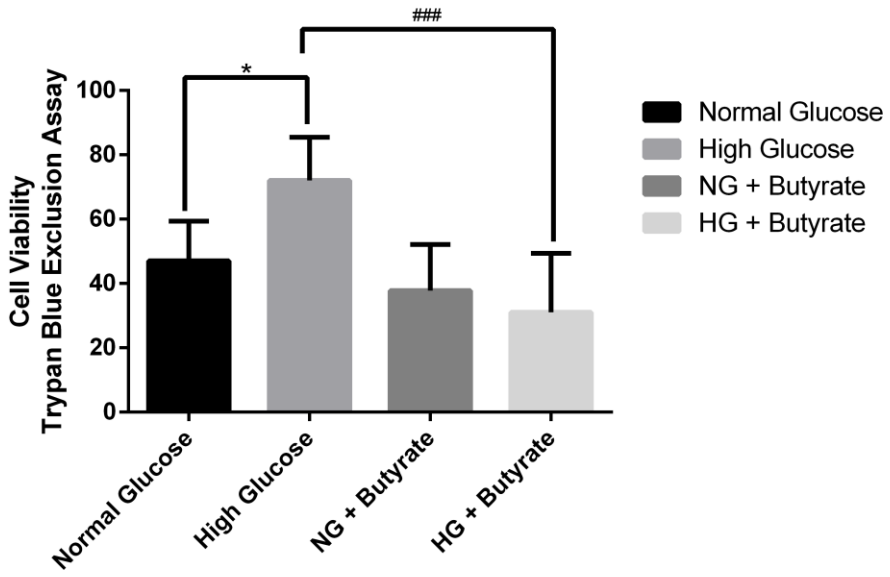


Figure 8: Trypan Blue Exclusion Assay(n=3). Data are expressed as mean± standard error of mean (SEM). * p<0.05 vs Normal Glucose. #p<0.05 vs High Glucose

Next we assessed the mechanisms by which anatomical and histological alterations were induced.

C. Autophagy alteration in diabetes and cancer was homeostatically restored upon butyrate and probiotic treatment.

Autophagy is a molecular mechanism for maintaining cellular physiology and promoting survival. Defects in autophagy lead to the etiology of many diseases, including diabetes mellitus and cancer. In the next series of experiments we assessed the protein expression of central autophagy proteins.

1. LC-3β

LC-3β is a protein well-involved in autophagy and a prominent marker of autophagosome formation. In the MKR mice model, a non-statically significant elevation in the protein expression of LC-3β was observed. Upon treatment with probiotics or butyrate LC-3β protein expression showed a tendency to decrease (**Figure 9**). In the APC mice model, the increase in LC-3β protein expression was significantly and remarkably higher with respect to the control mice. Butyrate or probiotic treatment show a tendency to reverse cancer-induced LC-3β overexpression (**Figure 10**).

Figure 9 : LC3-β expression in MKR mice models

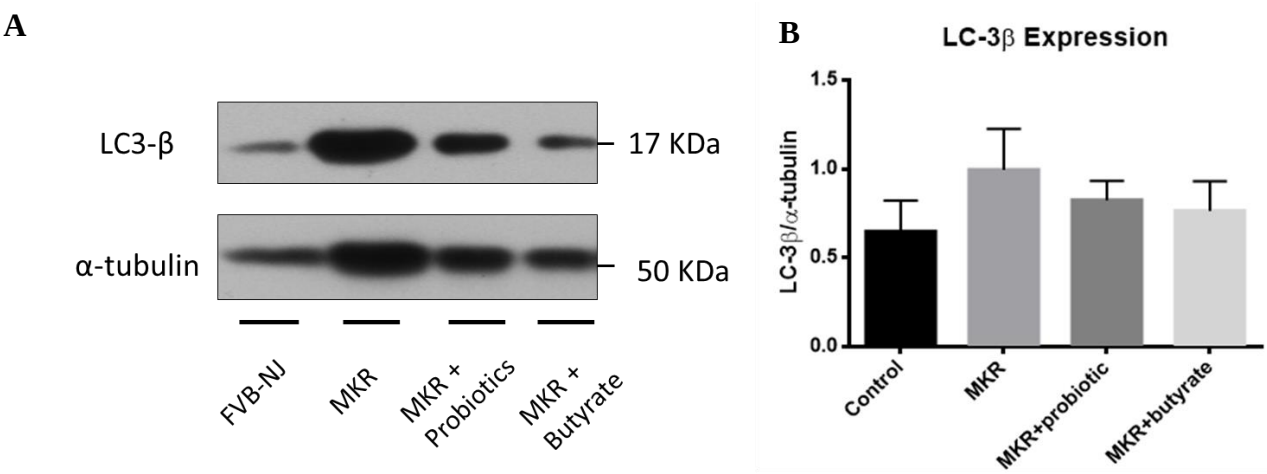


Figure 9: protein expression of LC3-β in MKR mice models(n=3).. (A)Representative western blots of LC3-β and α-tubulin performed on colon tissues of MKR mice models. (B) Histogram of the quantification results of protein LC3-β normalized against α-tubulin.

Figure 10 : LC3-β expression in APC mice models

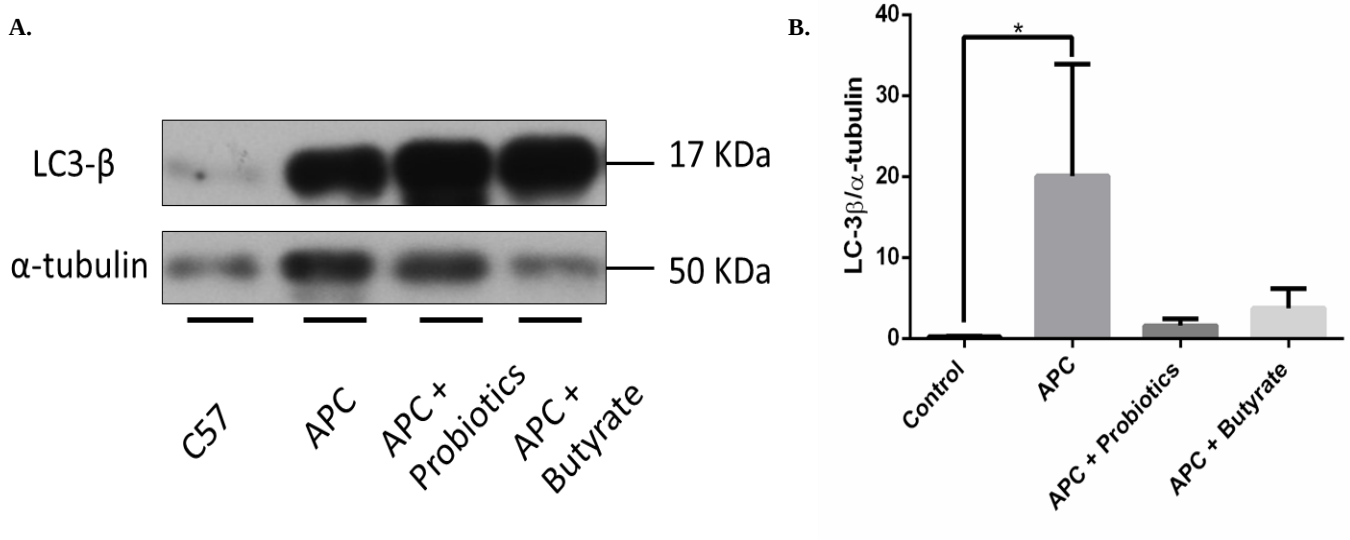


Figure 10 :LC3-β expression in APC mice models (n=3). Data are expressed as mean± standard error of mean (SEM). * p<0.05 vs control, # p<0.05 vs APC. (A)Representative western blots of LC3-β and α-tubulin and performed on colon tissues of APC mice models. (B) Histogram of the quantification results of protein LC3-β normalized against α-tubulin.

2. ATG-12

ATG-5, ATG-8, and ATG-12 belong to the same family of autophagy markers. In our results, ATG-12 was not affected by the diabetic milieu of the MKR mice (**Figure 11**). In contrast, it was significantly decreased in the APC mice with respect to its control littermates. This decrease was significantly reversed upon treatment with probiotics and butyrate (**Figure 12**).

Figure 11 : ATG-12 expression in MKR mice models

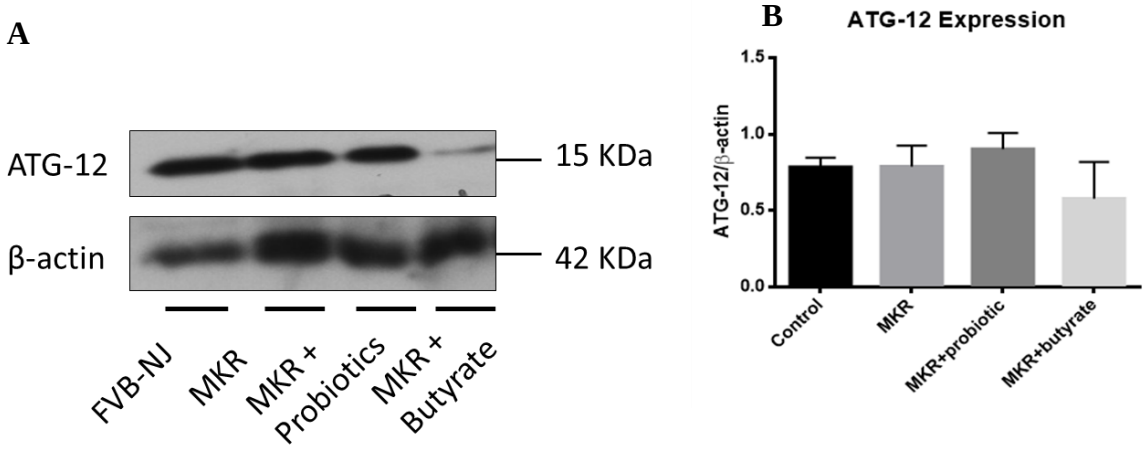


Figure 11: ATG-12 expression in MKR mice(n=3). (A)Representative western blots of ATG-12 and β-actin performed on colon tissues of APC mice models. (B) Histogram of the quantification results of protein ATG-12 normalized against β-actin.

Figure 12: ATG-12 expression in APC mice models

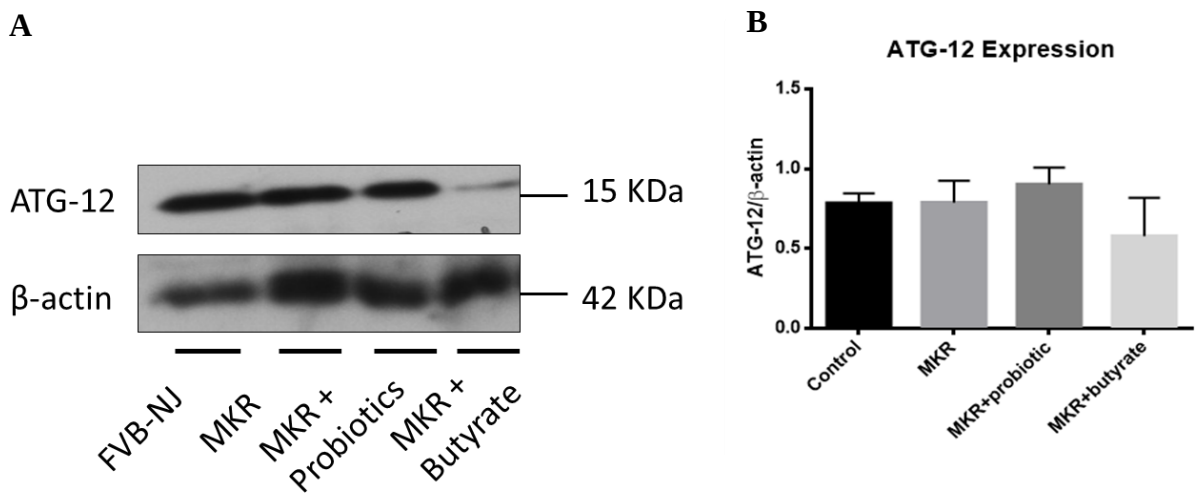


Figure 12: ATG-12 protein expression in MKR and APC mice models(n=3) .). Data are expressed as mean± standard error of mean (SEM). * p<0.05 vs control. . # p<0.05 vs APC (A)Representative figures of ATG-12 and β-actin performed on colon tissues of APC mice models. (B) Histogram of the quantification results of protein ATG-12 normalized against β-actin.

3. *Beclin-1*

Beclin-1, an autophagy marker that is implicated in inhibiting tumor growth in cancer cells, was decreased significantly in APC mice with colon cancer. Mice treated with probiotics or butyrate showed a homeostatic repair of Beclin-1levels. **(Figure 14**). This change was not observed in the MKR mice models (**Figure13**).

Figure 13: Beclin-1 protein expression in MKR mice models

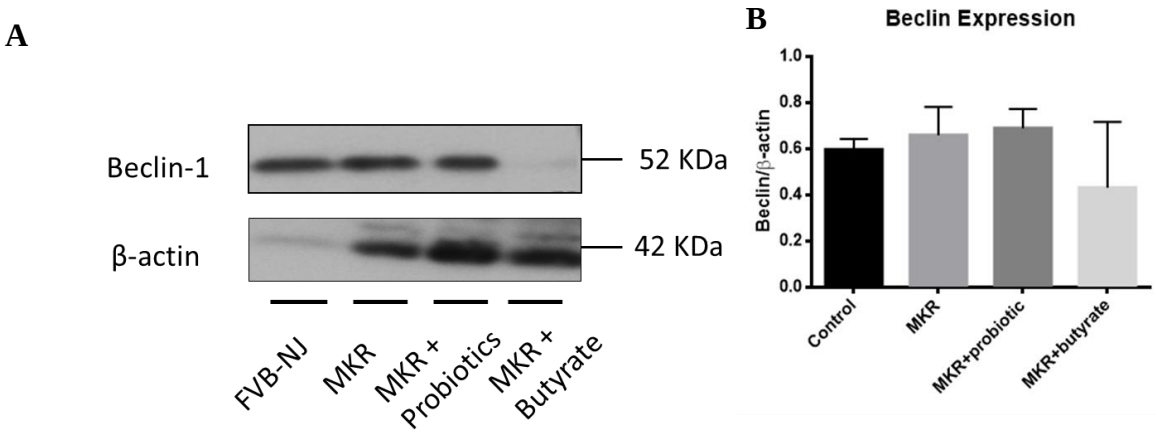


Figure13: Beclin-1 protein expression in MKR mice models(n=3). (A)Representative western blots of Beclin-1 and β-actin performed on colon tissues of APC mice models. (B) Histogram of the quantification results of protein Beclin-1 normalized against β-actin.

Figure 14: Beclin-1 protein expression in APC mice models

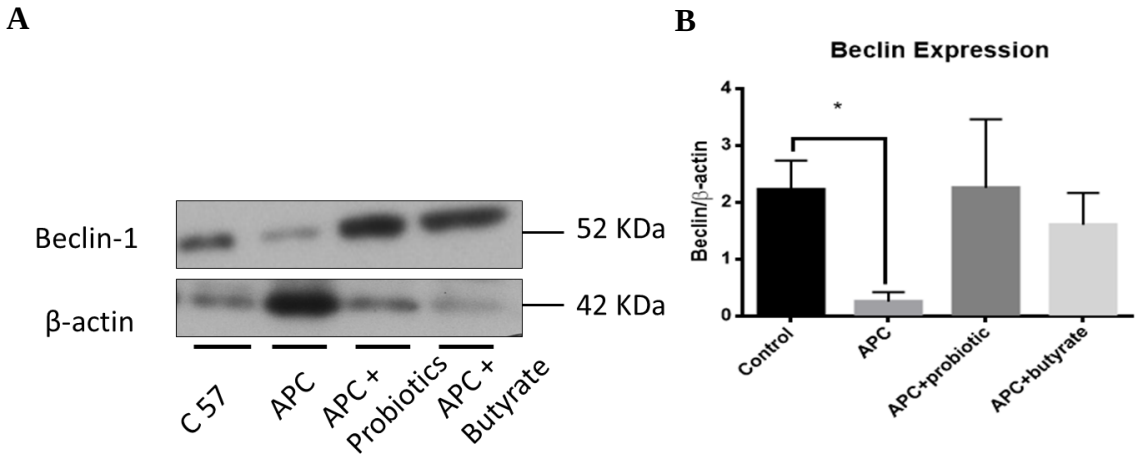


Figure 14: Beclin-1 protein expression in APC mice models (n=3). Data are expressed as mean± standard error of mean (SEM). * Statistically significant at p<0.05 vs control. (A)Representative figures of Beclin-1 and β-actin performed on colon tissues of APC mice models. (B) Histogram of the quantification results of protein Beclin-1 normalized against β-actin.

D. ROS production and NADPH Oxidase Activity

NADPH oxidases (NOXs) catalyze the transfer of electrons from NADPH to molecular oxygen to produce superoxide and/or hydrogen peroxide, two major reactive oxygen species (ROS). NOX-induced ROS production is now recognized to play a fundamental role in human health and disease especially in diabetes and cancer. Among the 7 different Nox isoforms, Nox4 has been shown to be majorly involved in both diabetes and cancer[112].

1. ROS Production

To assess ROS production, we measured DHE alteration on colon tissue extracts. The results show a significant increase in ROS production in *MKR* and *APC* mice models compared to their control littermates. Butyrate or probiotics treatment decreased ROS production significantly compared with untreated *MKR or APC* mice models (**Figure15**).

Figure 15: HPLC in MKR and APC mice models

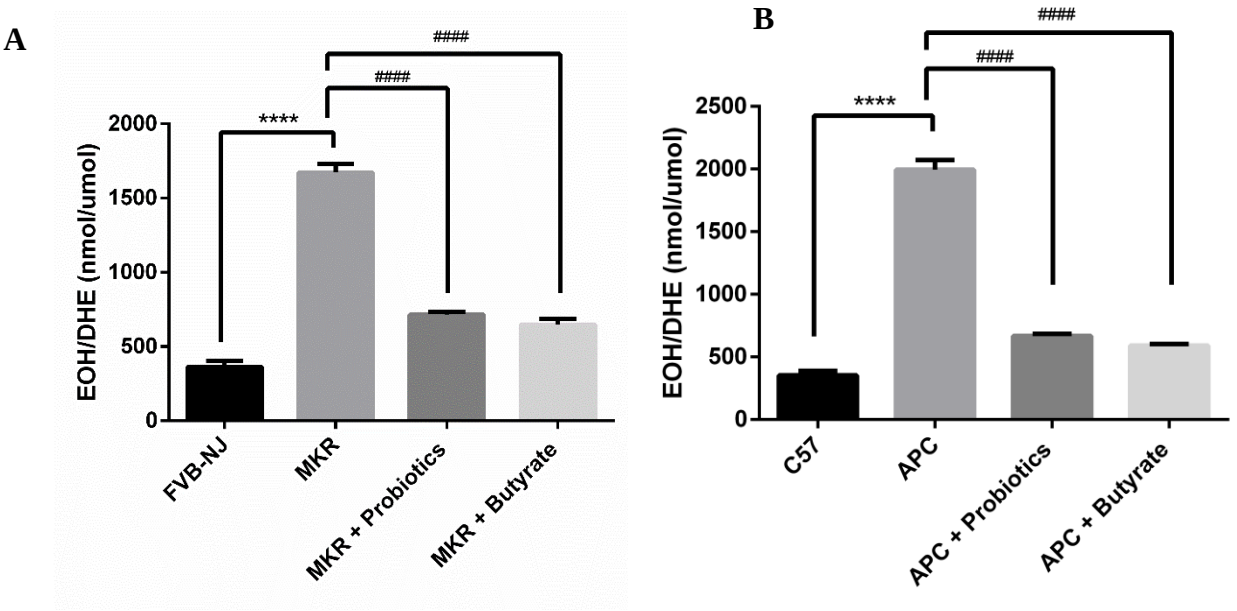


Figure 15: HPLC in MKR and APC mice models(n=3). Data are expressed as mean± standard error of mean (SEM). * p<0.05 vs control. . # p<0.05 vs MKR/APC. Superoxide generation evaluated using DHE and HPLC in MKR (A) and APC (B) mice models

1. NOX4 expression in MKR and APC mice

The level of expression of NOX4, a member of the NADPH oxidases family, was assessed in MKR and APC mice models. In both MKR mice and APC mice the protein expression levels of NOX4 were significantly increased. This increase was restored upon the administration of probiotics or butyrate (**Figure16-17**).

Figure 16: NOX4 expression in MKR mice models

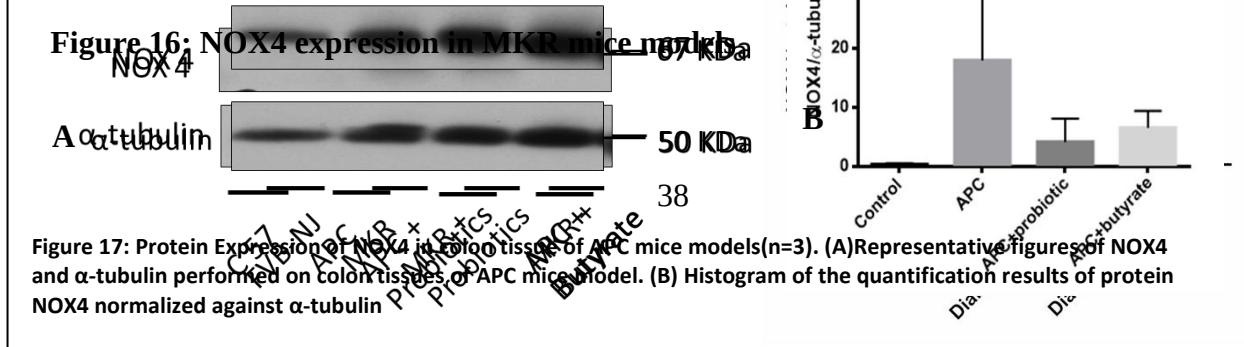


Figure 17: Protein Expression of NOX4 in Colon tissues of APC mice models(n=3). (A)Representative figures of NOX4 and α-tubulin performed on colon tissues of APC mice model. (B) Histogram of the quantification results of protein NOX4 normalized against α-tubulin

E. TIGAR Expression is altered in APC mice groups with no visible change in MKR mice groups:

The level of expression of TIGAR, a P53 target protein[113], is significantly increased in colorectal cancer as seen in APC mice models and restored to normal upon treatment with butyrate or probiotics (Figure **18**). No significant change was observed in MKR mice models **(Figure19**).

Figure 18 : TIGAR expression in MKR mice models

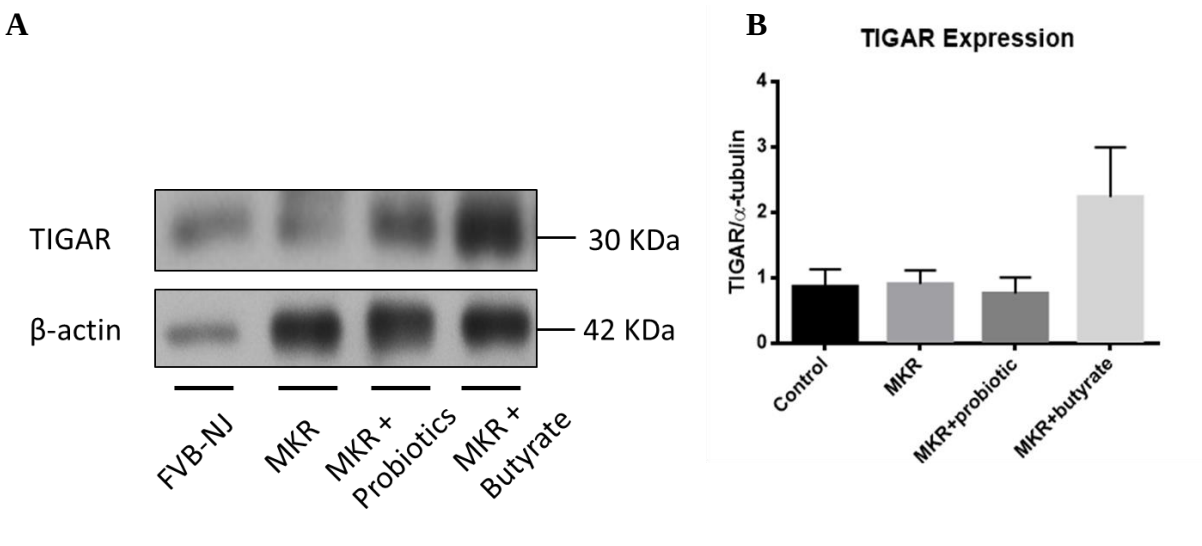


Figure 18: Protein Expression of TIGAR in colon tissue of MKR mice models(n=3). (A)Representative figures of TIGAR and β-actin performed on colon tissues of MKR mice models. (B) Histogram of the quantification results of protein TIGAR normalized against β-actin.

Figure 19 : TIGAR expression in APC mice models

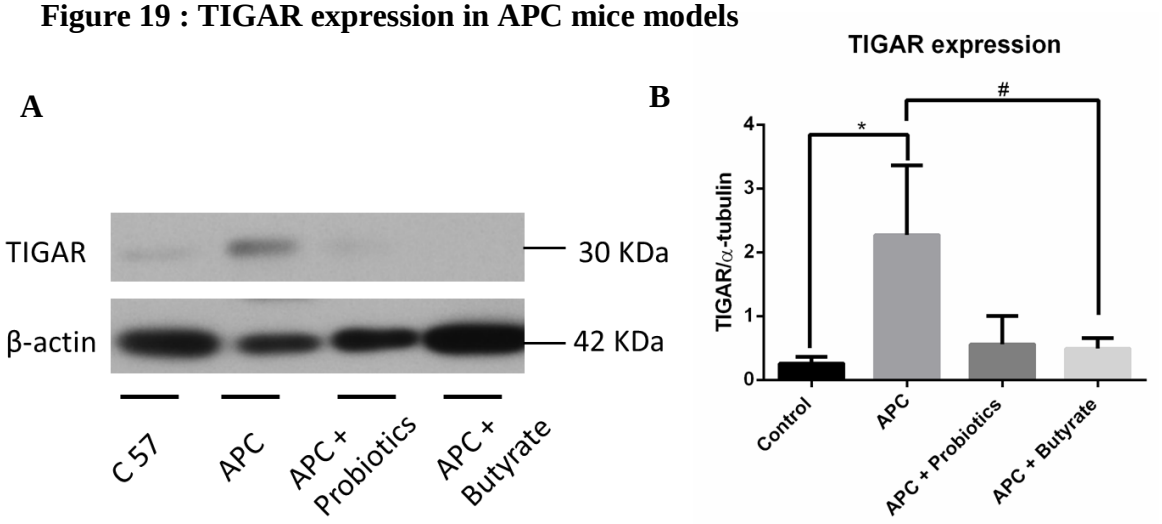


Figure 19: Protein Expression of TIGAR in colon tissue of APC mice models(n=3).). Data are expressed as mean± standard error of mean (SEM). * p<0.05 vs control. . # p<0.05 vs APC (A)Representative figures of TIGAR and β-actin performed on colon tissues of APC mice models. (B) Histogram of the quantification results of protein TIGAR normalized against β-actin

CHAPTER IV

DISCUSSION

The MKR mouse model, expresses a dominant-negative IGF1R in skeletal muscle under the muscle creatine kinase promoter. The resultant is a mouse that is insulin resistant, glucose intolerant but not obese[109]. Furthermore, the MKR mice display mild dyslipidemia, but normal levels of circulating inflammatory cytokines making them a good model to study the effects of T2DM on colon tumorigenesis independent of obesity. This allows us to attribute our observed results to hyperglycemia and insulin resistance merely. Measuring the HbA1c levels reflected that treatment with neither butyrate nor probiotics altered HbA1c. This suggests that the reversal of the high glucose/hyperglycemia-induced anatomical, histological and biochemical alterations are solely due to a distinct effect of the treatment rather than normalization of HbA1c levels.

We sought to identify the phylla of bacteria resident in the gut of control and diabetic mice. The results showed that *B.fragilis* had a significant decrease in the gut of the diabetic mice. This is concomitant with previous findings that suggest the association of decreased *B.fragilis* with the progression in T2DM[73]. Furthermore, PCR analysis of DNA from fecal samples showed a significant increase in butyrate-forming bacteria in both diabetic and colon cancer mice models after probiotics administration. This was interpreted by assessing the percentage of butyrate kinase gene abundance. In both *MKR* and *APC* mice models treated with probiotics we saw a significant elevation of butyrate kinase gene.

Phenotypic changes marked by colon length and polyps formation were assessed in *MKR* and *APC* mice. In *MKR* mice models, measurements of the length of the colon revealed a shortening in the colon of diabetic mice which was restored upon treatment. Although the literature shows that colon length is often increased in diabetic mice for higher absorption of

glucose[114], however, we postulate that the shortening of colon length was due to inflammation. In fact, recent studies show that during colon cancer and when the colon is inflamed, the colon length is shortened[115]. It is probable that the elongation of the colon initially in diabetes is present for increased absorption, but later as it becomes inflamed it shortens. In *APC* mice models, having adenomatous polyposis coli mutation, our results indicate an increased number of polyps. This number was decreased in mice treated with either probiotics or butyrate. Alteration in the microbial diversity offers a safe ground for adenomatous polyps to develop into colon cancer[116] and this effect is somewhat reversed upon the administration of probiotics. Increasing evidence highlight the role of probiotics in altering the composition and diversity of the gut microbiota and explains how probiotics modulate the levels of the so called 'good' and 'bad' bacteria[117]. Histological observations of the colon tissue of treated and untreated *APC* mice were performed using PAS staining. Our results revealed tissues with colon cancer to have an increase in the number of crypts per surface area, a decrease in goblet cells, and a stratification of nuclei[118] that is reversed upon treatment.

To confirm our results, we performed a trypan blue exclusion assay to assess the viability of colon cancer cell line HT-29 in normal or high glucose conditions in the presence or absence of butyrate. Our results displayed an increase in the viability of colon cancer cells in high glucose simulating diabetic conditions. This was reversed upon treatment with butyrate. Although butyrate is considered the energy supply of colon cells it did not promote their growth out of control.

When analyzing the levels of autophagic marker LC3β, our results showed a tendency of an increase in mice with colon cancer which was reversed upon treatment with probiotics or butyrate. Previous studies suggest that increased autophagy enhances the aggressiveness of human colon cancer by evading apoptosis and utilizing autophagy as a mean for cell survival[119,120]. Furthermore, several reports demonstrate the effect of butyrate on the AMPK

pathway by studying macrophages. Their results show that butyrate has increased AMP which is an inducer of AMPK that in turn inhibits mTOR; a well-known regulator of autophagy.[121] AMPK and mTOR involvement will be assessed in future experiments. Other studies display the role of probiotics on regulating the initiation and development of autophagy by suppressing its maturation via decreasing P62; an ubiquitin-binder of LC-3β[122]. Probiotics were shown to decrease autophagic vacuoles after LPS treatment[122] This stimulated us to hypothesize that since LC-3β is required for the formation of autophagosomes and since probiotics decrease autophagic vacuoles, then LC-3β is decreased upon the administration of probiotics. One more noteworthy point found in literature was that LC3β overexpression may be a compensatory mechanism due to a decrease in expression of other autophagic molecules[123].

Another key autophagic maker is autophagy related protein 12 (ATG-12) that was downregulated in APC mice and restored close to control after probiotics or butyrate administration. ATG-12 belongs to a family of autophagy related proteins and is covalently attached to ATG-5. Studies show that ATG-5 is speculated to act as a tumor suppressor and is highly downregulated in colon cancer due to deletions in 6q21 loci[124]. Furthermore, ATG-5 and ATG-12 are required to form a complex in early stages of autophagosome formation, yet this process is downregulated altogether[124]. In parallel, another study attributes this decrease in ATG-12 to frameshift mutations leading to disruption of amino acids synthesis of the target protein hence losing its function[123]. Probiotics were shown to increase the expression of ATG-12 by interfering with the ATG-5/ATG12 complex[122].

Beclin-1, also known as ATG-6, belongs to the autophagy related proteins family responsible for autophagosome formation. In our data, Beclin-1 observed a similar pattern as ATG-12 expression. This is concomitant with the literature whereby beclin-1 and ATG-12 work together in promoting autophagy[124]. Beclin-1 expression is significantly downregulated in colon cancer. This is speculated to be the result of allelic loss or microRNA regulatory activity[125].

Upon treatment with probiotics, studies show that Beclin-1 was upregulated along with ATG5-ATG12-ATG16 leading to induction of autophagy[126]. This could suggest that probiotics induce autophagy in colon cancer cells as a defense mechanism to protect them from cell death. Similarly, treatment with butyrate showed an increase in beclin-1 expression with respect to control and this was shown to be due to butyrate stimulating the production of reactive oxygen species (ROS) thus leading to the activation of autophagy[127].

ROS production was measured using HPLC to assess the level of oxidative stress in diabetes and colon cancer states. Our results indicate that superoxide levels increase in diabetic mice and mice with colon cancer. This was reversed upon treatment with either butyrate or probiotics. NADPH oxidases are one of the many sources of ROS in biologic systems. Within the family of NADPH oxidases, NOX4, being found in the colon epithelium, was assessed. In both diabetic and mice with colon cancer, the pattern of NOX4 protein expression observed was similar with an increase in diabetic and APC mice and a decrease upon administration of the treatment. In MKR mice, NOX4 is a known accomplice of diabetes and a contributor to oxidative stress[112]. Thus observing an increase in NOX4 in diabetic mice came as no surprise as elevated levels of glucose induce increased production of ROS through augmented NADPH oxidases activity[97]. Butyrate, a histone deacetylase was shown to reduce the basal level of NOX4 expression and H_2O_2 in endothelial cells[128]. Similarly, probiotic supplements were shown to counteract the increased ROS production via anti-oxidative mechanisms like increased *Bifidobacterium*[129]. In colon cancer, NOX4 increased expression is linked to promoting tumor progression via cell–cycle, apoptosis, and migration[104].

Cancer cells find several means to elongate their lives and escape cell death, one of which is shifting their metabolism from oxidative phosphorylation to glycolysis in order to avoid oxidative damage[108]. P53 is a tumor suppressor gene that was reported to help in controlling metabolism, making it rather an oncogene at certain times. TIGAR is a target of P53 protein that

protects from oxidative stress and lowers the glycolytic flux. As a result, by protecting the cells from oxidative stress, TIGAR unintentionally aids cancer cells in escaping apoptosis[108]. In our APC mice models, we saw an increase in TIGAR expression indicating that it was shielding colon cancer cells from apoptosis[113]. This expression was decreased upon the administration of butyrate or probiotics.

CONCLUSION

In conclusion, diabetes and colon cancer- induced dysbiosis is leading to the decrease in butyrate forming bacteria. This, in turn, is increasing NADPH oxidases specifically NOX4 protein expression that is responsible for the production of ROS. The oxidative stress is the resulting in autophagic alterations that are further ameliorating colon injury. Upon the administration of probiotics or butyrate, we are restoring normo-biosis and adjusting the action of NOX4. Therefore, ROS production becomes under control and autophagy is regulated. Altogether, this improved colon injury and slows down the progression of cancer.

LIMITATIONS

In this work, the first limitation is related to the small number of animals used (n=3). This was a pilot study to assess the role of microbiota in diabetes and cancer. Also, this pilot study was aimed to identify the dose of butyrate needed for treatment. In the ongoing and future studies, we aim at increasing the number of animals to pertain more statistically significant and accurate results. Our in vitro work was merely used as a confirmation for our in vivo work, yet we seek to expand it more. We will use a battery of cell lines including ones that represent different stages of colon cancer such as SW480, SW620, Caco-2, LoVo, and HCT-116. We will also attempt to use several drugs to repurpose them for colorectal injuries. It is important to pursue this road down further and to seek plausible treatments for these debilitating diseases. I am grateful to be able to contribute even in this minute amount one step further towards aiding those in need. As someone once said ‘’we didn’t come this far to only come this far’’.

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