AMERICAN UNIVERSITY OF BEIRUT

A NOVEL ROLE OF mTORC2 IN DIABETES-ASSOCIATED COLORECTAL CANCER

by
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ABSTRACT

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Title: A Novel Role of mTORC2 in Diabetes-Associated Colorectal Cancer

Background: Type 2 Diabetes mellitus (T2DM) is a chronic systemic dysfunction characterized by persistent metabolic disturbances which result in a high rate of micro and macrovascular events to which cancer was recently annexed. In fact, diabetes increases the risk of colorectal cancer (CRC) by 1.2 to 1.5 folds, leaving patients with increased aggressiveness and poorer 5-year survival. However, the cellular and molecular pathways involved in diabetes-induced CRC progression are not well elucidated. mTORC signaling pathway has been described to play a role in several disease including diabetes or cancer. Yet, the role of the rapamycin insensitive unit, mTORC2, in diabetes-associated cancer is yet to be described. This study aims to identify a potential common mechanistic pathway between diabetes and CRC, especially the one orchestrated by mTORC2. Furthermore, in this study we will evaluate the effect of probiotics treatment on the control and prevention of diabetes-associated CRC.
Aims: We hypothesize that the onset of diabetes, cancer or their association induce dysbiosis that stimulates NADPH oxidases-activated ROS production by inducing the mTORC2 signaling pathway alteration.
Results: Our data suggest that diabetes and or cancer induce dysbiosis that in turn leads to gastrointestinal complications and induces colorectal cancer aggressiveness. These results are paralleled by an increase in ROS production by activating the NADPH oxidase 4. Of interest, diabetes-associated CRC also exhibits an increase in the mTORC2 signaling pathway. These changes are reversed when treated with probiotics, which corrects the dysbiosis associated with diabetes, cancer, or their association, reduces ROS production, and correct the mTORC2 expression suggesting a beneficial effect on the progression of these chronic comorbidities.
Conclusion: Our results clearly support the role of mTORC2 in diabetes associated cancer and advances the protective effect of probiotics treatment in diabetes, cancer, or their association.

TABLE OF CONTENTS

ILLUSTRATIONS

Figure

TABLES

Table

ABBREVIATIONS

DM	Diabetes Mellitus
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
OGTT	Oral glucose tolerance test
ADA	American Diabetes Association
MENA	Middle East North Africa
GDM	Gestational diabetes
CRC	Colorectal Cancer
CAC	Colitis-associated colorectal cancer
SCC	Sporadic colorectal cancer
IBD	Inflammatory Bowel Disease
mCRC	Metastatic CRC
VEGF	Vascular Endothelial Growth Factor
FDA	Food and Drug Administration
mTOR	Mechanistic Target of Rapamycin
mTORC1	Mechanistic Target of Rapamycin Complex 1
mTORC2	Mechanistic Target of Rapamycin Complex 2
PIKK	PI3K-related Kinase Family
RTKs	Receptor Tyrosine Kinases
ROS	Reactive Oxygen Species
NOX	NADPH Oxidase
NOX4	NADPH oxidase 4

DUOX	Dual Oxidase
TLR	Toll-like Receptor
LPS	Lipopolysaccharides
IL	Interleukins
SCFA	Short-Chain Fatty Acid
GPCRs	G Protein–Coupled Receptors
GLP	Glucagon-like peptide
GIP	Gastric Inhibitory Peptide
GABA	Gama-Amino Butyric Acid

CHAPTER I

INTRODUCTION

A. Diabetes Mellitus (DM)

1. Overview

Diabetes Mellitus is a group of metabolic diseases essentially portrayed by hyperglycaemia that results from defects in insulin secretion, insulin action, or both. Long-term damage, dysfunction, and failure to different organs can be associated with the chronic hyperglycaemia induced by diabetes. The development of diabetes is attributed to several pathogenic processes, ranging from the autoimmune destruction β-cells of the pancreas with its consequent insulin deficiency to the physiological abnormalities that yield resistance to insulin action. In diabetes, abnormalities in carbohydrate, fat, and protein metabolism are due to the deficient action of insulin on focus tissues. Deficient insulin action is caused by the inadequate secretion and/or the reduced tissue response to insulin along the complex pathways of hormone action. The impaired secretion of insulin and defects in insulin action and tissue response often coexist in a single patient and in this case it's uncertain which abnormality, if either alone, is the primary cause of the hyperglycaemia [1]**.** Diabetes Mellitus induced metabolic disturbances belong to two major etiopathogenetic groups. These are Type 1 Diabetes Mellitus (T1DM) and Type 2 Diabetes Mellitus (T2DM). T1DM is characterized by autoimmune destruction of pancreatic β cells and an absolute deficiency of insulin secretion [2]**.** T1DM prone patients can often be identified by serological evidence of an autoimmune pathologic process occurring in the pancreatic islets and by genetic markers [1]. However, T2DM, the more prevalent type constituting

up to 90% of all cases, is caused by both, a resistance to insulin action and an inadequate compensatory insulin secretory response. T2DM may be present for a long period of time before detection as the degree of hyperglycaemia sufficient to cause pathologic and functional changes in various target tissues, but without clinical symptoms [1, 3]**.** Both types of DM share classic symptoms, and these include polyuria, polydipsia, weight loss/gain, blurred vision, fatigue, and body pain. The acute and life-threatening consequences of uncontrolled or undetected diabetes are hyperglycaemia with ketoacidosis or non-ketone hyperosmolar syndrome [3].

Screening for diabetes mellitus can be either as a 2-hour oral glucose tolerance test (OGTT) or via an HbA1c test, as recently recommended by the American Diabetes Association (ADA) [3]**.** Over the past two to three decades, there has been growing awareness of the magnitude of the problem posed by the complications of diabetes, which represent a large part of the social and financial burden of diabetes in low- and middle-income countries.

2. Epidemiology

Globally, the burden of diabetes has increased dramatically in recent decades and is expected to keep the trend in the years to come. DM represents a huge health and economic burden in the region of Middle East North Africa (MENA) [4]. In 2019, approximately 54.8 million adults (20-79 years) were living with diabetes in the MENA region; by 2045 this number is estimated to double **(Figure 1)**. Among 54.8 million patients, 24.5 million are not diagnosed and not treated until they start experiencing the diabetes-related complications [4]. According to the International Diabetes Federation, it is estimated that in 2019, there were 418,900 deaths attributed to diabetes and

diabetes-related complication in the MENA region, which represents 16.2% of all-cause mortality. This makes diabetes the ninth leading cause of death worldwide [4].

In fact, Lebanon with 14.99% of diabetes cases ranks 7th among countries in the MENA region [5]. In fact, among the Lebanese population the prevalence of T2DM was 8.5%, which is the leading cause of death [6]**.** Diabetes spreads faster in low- and middle-income countries than in high-income countries.

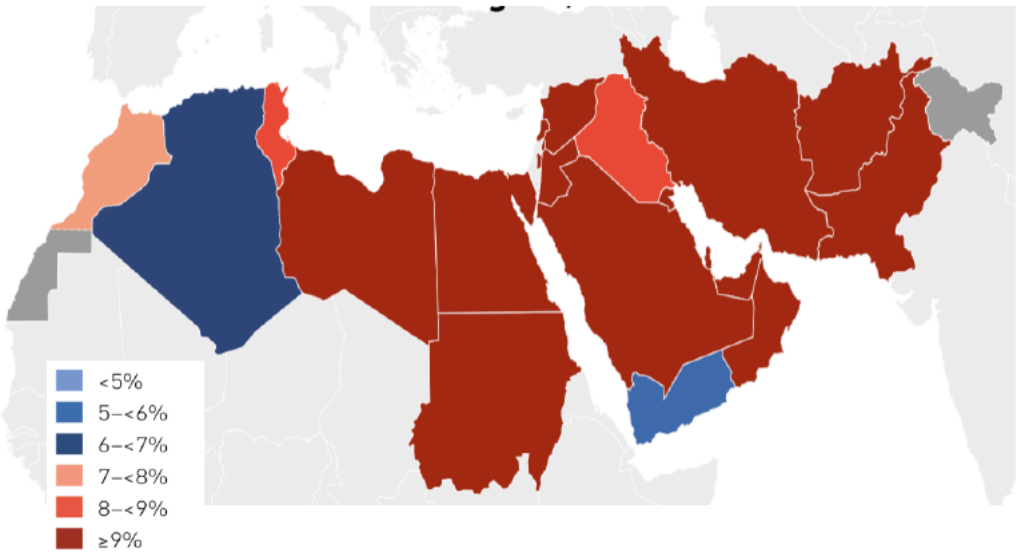

Figure 1: Epidemiology of Diabetes Mellitus in the MENA Region [4]

3. Diabetes Mellitus Type 2 (T2DM)

T2DM affects 90–95% of people with diabetes and was previously known as non-insulin-dependent diabetes, or adult-onset diabetes, characterized by insulin resistance and relative (rather than absolute) resistance. These people do not require insulin medication to survive, at least at first and often throughout their lives. This type of diabetes is likely caused by a variety of factors. Although the exact causes are undetermined, there is no autoimmune destruction of cells. Most people with T2DM are

obese, and obesity itself induces insulin resistance. Ketoacidosis is uncommon with this kind of diabetes; when it does develop, it is usually associated with the stress of another illness, such as infection [7]. As mentioned earlier, because hyperglycaemia develops gradually and is often not severe enough for the patient to detect any of the usual signs of diabetes in its early stages, T2DM frequently goes untreated for many years. Despite this, such patients are more likely to suffer microvascular and macrovascular problems [3, 7] Patients with T2DM may have insulin levels that appear normal or increased, but if their β cell function was normal, the higher blood glucose levels in these diabetic patients would result in even higher insulin values. As a result, insulin secretion in these people is impaired, and it is insufficient to compensate for insulin resistance [7]. A drop in weight and/or the use of pharmaceuticals to control high blood sugar is usually improve insulin resistance, but this seldom happens. T2DM is far more likely to occur in older people, obese people, and those who are inactive. It appears to be more common in women with pre-existing gestational diabetes (GDM) and in individuals with hypertension or dyslipidaemia, and it varies by racial/ethnic group. Also known as Type 1 Diabetes Insipidus, it is more typically associated with a significant genetic predisposition, rather than an autoimmune disease. The genetics of this type of diabetes are complicated, and it is not fully understood yet [7].

Several studies have connected dysbiosis of the gut microbiome to the rapid evolution of insulin resistance in T2DM, which accounts for over 90% of all diabetes cases globally. Changes in intestinal permeability, endotoxemia, interaction with bile acids, changes in the proportion of brown adipose tissue, and the impact of medications like metformin are all factors that link the microbiota to the formation of insulin resistance and diabetes [8].

B. Colorectal Cancer (CRC)

1. Overview

Colorectal cancer usually starts as a noncancerous tumor on the inner lining of the colon and/or rectum that grows slowly over a period of 10 to 20 years. Adenomatous polyps, also known as adenomas, are the most frequent type of colorectal cancer, accounting for approximately 95% of all cases [9]. Adenomas arise from glandular cells that produce mucus to keep the colorectum lubricated. Even though all adenomas have the potential to become malignant, only about 10% of them will advance to invasive carcinoma when they acquire a series of genetic or epigenetic modifications that provide them a selective advantage [10]. Colorectal cancers are a broad category of diseases that are caused by a variety of mutations and mutagens. Mutations can be inherited or acquired, and they are more likely to develop in the intestinal crypt stem cell [9]. According to genomic mutation diversity, CRC is classified into two typical types: colitis-associated colorectal cancer (CAC) and sporadic colorectal cancer (SCC) [11]. These two types have relatively independent phenotypes with totally different inner involved signal pathways; however, they still share a few sequential genetic mutations [12]. CAC develops from long-standing colitis in inflammatory bowel disease (IBD) patients. Evidence has shown that autophagy of the colon cells plays a vital role during the tumorigenesis and CAC development [13]. However, SCC refers to the sporadic, non-inherited colorectal cancer. Contributors to the development of SCC include exposure to carcinogens, diet, smoking and obesity [14].

Investigations using large cohorts and animal models have shown that the cause of CRC include genetic background and environmental risk factors [12]. Only less than

10% of colorectal cancer patients have genetic predisposition or an evident causative genetic event for the initiation of the cancer [15]. Age is a significant risk factor thus, approximately 90% of CRC patients are above the age of 50 [16]. However, modifiable environmental factors such as tobacco use, poor nutrition, excessive alcohol consumption, physical inactivity, and obesity are responsible for more than half of all cases and deaths, making them potentially preventable [17]. This heterogeneous disease has been found to have three major molecular groups. The first, and most common group, is the chromosomal instable group, which is characterized by mutations in specific oncogenes and tumor suppressor genes. The second group is the microsatellite instable group that is characterized by genetic hypermutability caused by dysfunction of DNA mismatch repair genes. The third group, the CpG Island Methylation phenotype characterized by hypermethylation [15]. In addition to that, gut microbiota in some studies has shown to participate in the initiation and progression of CRC [12]. The gut microbiota is a new but essential field of study for understanding the impact of the environment on the CRC. The enrichment of several bacterial species in the intestine has been identified as contributing to colorectal carcinogenesis by inducing tumor proliferation, promoting inflammation, degrading ADN, and protecting the tumor from immune attacks [18]. However, several bacteria that have been linked to a lower risk of CRC, including probiotics, have been found to be ineffective in CRC patients.

CRC symptoms are like those of a variety of other disorders, making it easy to misdiagnose without a colonoscopy. Unusual anaemia, weight loss, bloating, changes in bowel movements, bloody stools, vomiting, and pelvic pain are all symptoms of CRC [19]. Despite rapid advancements in diagnostic and treatment techniques, the 5-year survival rate for colorectal cancer is generally less than 50 percent. This is because most

patients with colorectal cancer are diagnosed at an advanced stage, which accounts for the poor prognosis [17]. As a result, early detection of colorectal cancer is extremely critical. In a population-based study done by the American Gastroenterology Association, it was found that a colonoscopy can drastically reduce the absolute risk of CRC and that the genetically predetermined risk of CRC can be further reduced by adherence to a healthy lifestyle **(Figure 2)**. The results show the magnitude of CRC prevention possible through colonoscopy and lifestyle at a predefined genetic risk [20].

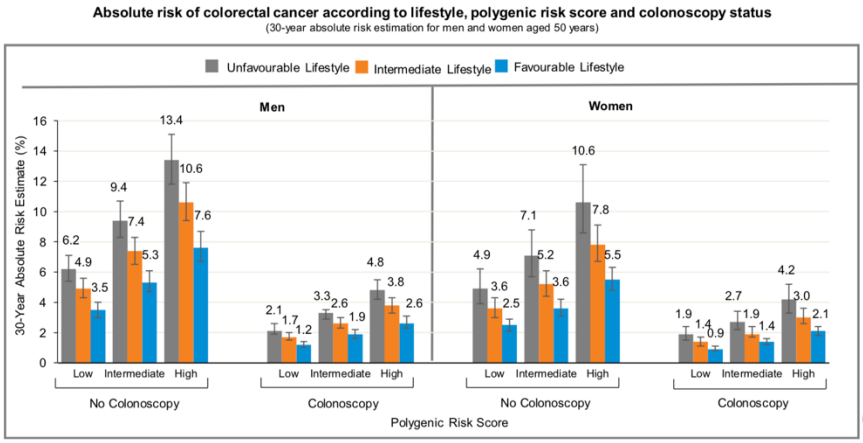

Figure 2: Absolute risk of colorectal cancer according to lifestyle, polygenic risk score and colonoscopy status [20]

2. Epidemiology

CRC is one of the most common malignancies in the world [21]. CRC is the third leading cause of cancer death, and its incidence is steadily rising in developing nations with 1.8 million new cases and almost 861,000 deaths in 2018 according to the World Health Organization [10]. CRC is the third most prevalent cancer in both men and women globally, with males being at higher risk at any age, even though it is a preventable disease. In the United States in 2020, there were 147,950 new cases of CRC

and 53,200 fatalities, including 17,930 cases and 3,640 deaths in adults under the age of 50 [22]. Although population-based colonoscopy screening and therapy can lower the incidence and death of CRC in some highly developed nations, several poorer nations continue to see an increase in incidence and mortality [18]. By 2030, the global burden of CRC will have increased by 60%, with more than 2.2 million new cases and 1.1 million annual deaths [10].

CRC incidence rises fast with age, roughly doubling every 5 years until the age of 50, then increasing by about 30% after the age of 55 [10]. Because the incidence of CRC is dropping in older age groups while increasing in younger people, the overall population of CRC patients is increasingly getting younger.

3. CRC Stages

CRC is a malignant disease that progresses through three distinct stages: **(I) Initiation**, which is a process that alters the normal cell's molecular message, **(II) Promotion**, which is a set of aberrant signal transduction cascades, and **(III) Progression**, during which the cells are transformed and phenotypically modified [16]. The severity of the condition and the treatment choices available are determined by the stages. **Stage 0** is characterized by the formation of a tumor in the mucosa or inner lining of the colon. **Stage 1** develops when cancer cells appear in the lining, but their invasion capability is limited to the muscular area and absent from the surrounding colon tissue [23]. **Stage 2** is classified into three types according on the degree of invasiveness in the colon's walls, the muscular layer of the abdominal lining, and the surrounding tissues [24]. **Stage 3** is divided into three categories based on the cancer's progression. Cancer grows in the inner lining of the colon's muscle layer and produces

lymph nodes in the surrounding tissues. This stage is referred to as 3A, 3B, or 3C depending on the number of nodules formed [16]. **Stage 4** is the most advanced stage of cancer, in which the disease has progressed to other organs such as the liver and lungs [25]. **Figure 3** below illustrates the different stages during the progression of colorectal carcinogenesis.

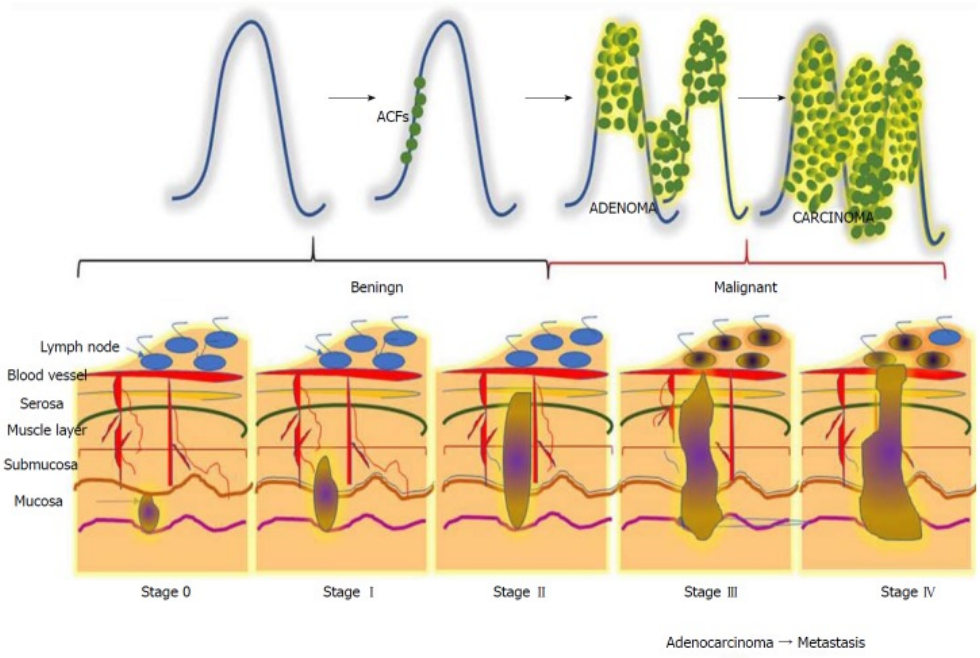

Figure 3: Different stages during the progression of colorectal carcinogenesis [16]

Stage 0: The cancerous cells grow within the inner lining of the mucosa.

Stage I: The cancerous cells grow throughout the mucosa and submucosa. The cancerous growth invades into the muscular layer of the colon.

Stage II: The cancerous growth penetrates through the wall of the colon without spreading to neighbouring tissues or lymph nodes.

Stage III: The cancer penetrates through layers of muscle into the serosa, the layer of visceral peritoneum. The cancer begins to spread to the lymph nodes.

Stage IV: A tumour nodule forms in the tissue surrounding the colon, cancer cells appear within the lymph nodes, and the cancer begins to metastasize.

4. Current Treatment of CRC

Because CRCs are a heterogeneous set of diseases produced by a wide range of mutations and mutagens, developing a molecular "catchall" therapy has been problematic because not all CRCs have the same motor mutations [10]. Targeted medicines are becoming commonplace, and the intriguing potential of therapy regimens tailored to individual tumor mutation profiles is beginning to emerge [26]. In general, the best treatment for CRC is total removal of the tumor and metastases, which requires surgery. However, over a quarter of CRCs are detected at an advanced stage with metastases, making surgical control difficult [27]. For patients with unresectable tumours or who are surgically intolerant, the most common therapies are radiation therapy and chemotherapy, with the goal of reducing the tumor and preventing its spread and development. To maximize tumor shrinkage and stabilization, chemotherapy or radiotherapy might be administered as a neoadjuvant or adjuvant treatment before or after surgery [27]. As a result of a greater understanding of cancer's molecular biology, a range of selective medicines have been discovered, and when paired with chemotherapy. They have proved to improve outcomes in patients with metastatic CRC (mCRC) [28]. For instance, Bevacizumab, a monoclonal antibody that targets vascular endothelial growth factor (VEGF), as well as cetuximab and panitumumqb, which are anti-EGFR monoclonal antibodies, were among the first agents to be developed and approved for use in mCRC by the Food and Drug Administration (FDA).

C. Diabetes and Colorectal Cancer

People with T2DM, unlike those with T1DM, rarely require insulin treatments to live. While there is no cure for T2DM, food intake, physical activity, and pharmacological therapy are regarded to be essential in the treatment of patients with diabetes [29]. However, strict control is difficult to maintain, and despite the advances, the risk of complications is still linked to a ten-year reduction in life expectancy [30]. Although little is known about the role and processes by which hyperglycaemia raises the risk of oncogenesis in diabetes, recent data shows the significance of insulin as a risk factor for cancer progression in diabetes. Patients with T2DM, on the other hand, have a 20-40% higher risk of colorectal cancer than the general population [31]. Furthermore, recent study suggests that cancer may raise the risk of diabetes. As a result, diabetes and cancer share several risk factors, but the cellular and molecular mechanisms that link diabetes and colorectal cancer remain unknown.

The association between T2DM and CRC has piqued experts' interest, encouraging them to explore for the association between the two disorders. There is a 1.2-1.5 relative risk of CRC when a patient also has diabetes mellitus [32]. Surprisingly, diabetic drugs have been linked to a higher or reduced risk of cancer, whilst antineoplastic drugs such as immune checkpoint inhibitors have been linked to a higher risk of diabetes mellitus [33]. Diabetes is associated with a greater death rate in patients with colorectal cancers as compared to individuals with normal blood glucose levels [34].

This link is thought to be mediated by the gut microbiota and its microbial residents. Changes in gut microbiota homeostasis have been shown to have far-reaching effects on local and systemic immunity, and to play a role in the pathogenesis of

gastrointestinal diseases like inflammatory bowel disease (IBD), irritable bowel syndrome, and colorectal cancer, as well as extra-intestinal systemic diseases like obesity, diabetes, and atherosclerosis [35]. Dysbiosis, the alteration in the normal microbiota profile, according to some authors, may be the link between chronic inflammation, IBD, CRC, and T2DM via interaction across numerous molecular pathways, including TGF, NFB, TNF, ROS, and others [35]. Many variables affect the amount and variety of the gut microbiota, including nutrition, physical and psychological stress, age, various illnesses, surgery, drugs, radiation, and toxins [36]. Food appears to be the most influential of these factors, as the interaction between the microbiota and diet, as well as their participation in inflammatory or metabolic pathways, defines the state of the host's gastrointestinal tract [37].

The disturbance of the healthy gut microbiome is closely linked to T2DM. Despite the diverse variety of bacteria that live in the digestive tract, researchers have been able to discover distinct flora signatures for T1DM, T2DM, and obesity [38]. This research was based on incorrect mechanistic assumptions and statistical correlations. As a result, more study is needed to determine the molecular mechanisms by which the gut microbiota contributes to T2DM development and consequences. The interaction between diabetes, obesity, and the microbiota in the development of diabetes complications is illustrated in **(Figure 4).**

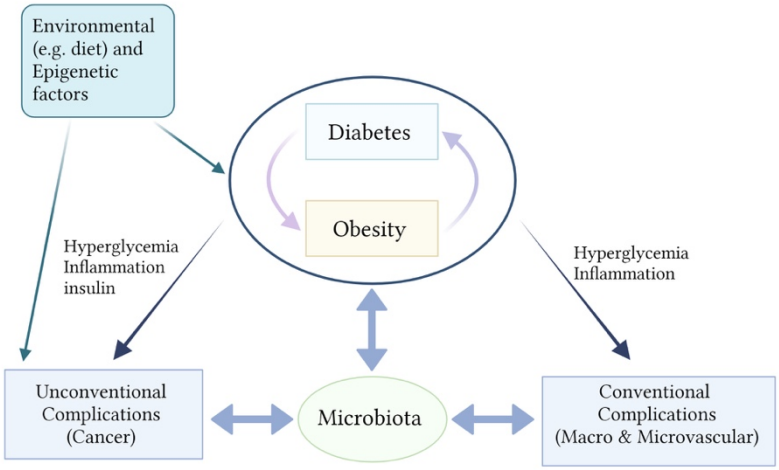

Figure 4: Interaction between diabetes, obesity, and the microbiota in the development of diabetes complications [39]

D. Gut Microbiota in Diabetes and Cancer

The gut microbiota is a concept originating from the collection of eukaryotic, bacterial, and archaeal organisms that are found to colonize the gastrointestinal tract. This collection, which consists of over 10^{14} organisms, has been found to co-exist with the host evolutionarily where both entities benefit from each other. The microbiota provides several physiological functions, such as contributing to gut shaping and integrity, immune responses, and energy provision [40]. Data assembled from The MetaHit and the Human Microbiome Project studies identified 2172 species, which are classified into 12 different phyla1. 386 of the identified species in humans were found to be anaerobic and located in mucosal regions such as the oral cavity and the GI tract [40].

A study that catalogued the functional capacity of gut microbiota suggests that its composition is influenced by environmental factors and genetics due to identifying

1 Phylum: a principal taxonomic category that ranks above class and below kingdom, equivalent to the division in botany.

country-specific microbial impressions. They do, however, share similar characteristics such as metabolic and protein profiles [40]. Processes in the gut are often the source of energy such as fermentation and sulphate reduction. In order for gut microbiota to survive, they should adapt to certain lifestyles over time provided by the host, and thus they are limited to phenotypical features [40]. In addition to adapting to the environment, a dynamic intestinal barrier with several components, i.e. physical, biochemical, and immunological, exists to protect the microbiota from the host immune system and maintain homeostasis [40].

Dysbiosis is the disrupted mechanism of microbiotic functions due to alterations in the composition of the microbiota. These alterations can be due to exposure to unhealthy environmental factors such as drugs, pathogens, toxins, and diet. Enteric pathogens are shown to have the greatest impact on causing dysbiosis. Foodborne viral pathogens, for example, can cause a local and systemic inflammation that alters the composition of the microbiota and disrupts the barrier function [41].

There is increasing evidence of dysbiosis contributing to several intestinal diseases, such as CRC, with high complexity in profiling these ecosystems to characterise them. The colon is found to contain the highest bacterial density within the GI tract, which can indicate the role of microbiota in the development of CRC. Studies were able to link intestinal microbiota with the development of CRC through the identification of specific bacterial species that promote tumorigenesis [42]. To find a reasonable correlation between gut microbiota and CRC and with the strong evidence displayed by ongoing research and studies, researchers are using sequencing technology aiming to recognize candidate carcino-genetic pathogens in the gut [12, 43]. In addition to the contribution of gut microbiota to CRC, it is involved with T2DM. Manipulation

of the gut microbiota is a viable treatment option for metabolic illnesses, according to an increasing body of evidence [44].

E. The mTOR Pathway

1. Overview

Through a variety of signaling pathways, changes in the gut microbiota and its metabolites affect the host's normal physiological activities, the majority of which are regulated by the Mechanistic target of rapamycin (mTOR). mTOR is a serine/threonine protein kinase of the PI3K-related kinase family (PIKK) that forms the catalytic subunit of two different protein complexes known as mTOR 1 (mTORC1) and 2 (mTORC2) based on their sensitivity to the allosteric inhibitor, Rapamycin. [45]. mTOR forms the nucleus of the two complexes with the minor protein mLST8 [46]. mTORC1 is defined by the Raptor protein, while mTORC2 is defined by the Rictor and SIN1 proteins [47]. mTORC1 manages the balance between anabolism and catabolism in response to environmental variables and regulates the creation of proteins, lipids, and nucleotides for development and cell division [45]. mTORC2 promotes cell proliferation and survival, principally by phosphorylating various members of the AGC protein kinase family (PKA / PKG / PKC) [45]. **Figure 5** summarizes the mTOR signaling effects.

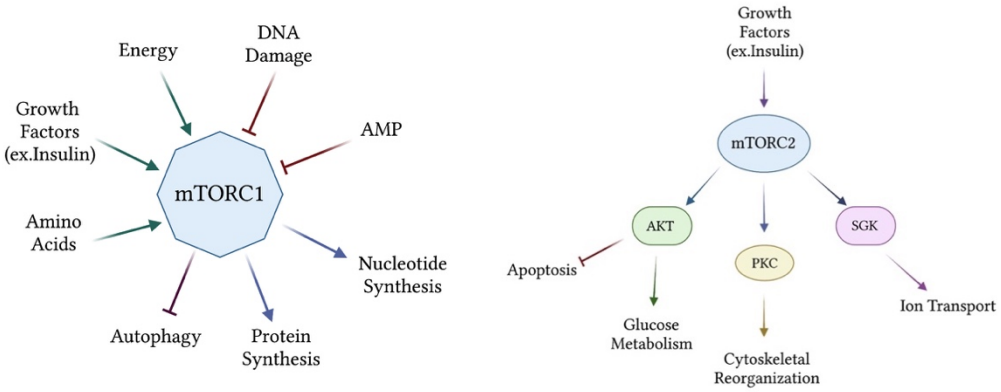

Figure 5: mTOR signaling effects [45]

2. The mTORC2 Pathway and mTOR Signaling in DM and CRC

Because most of the research has concentrated on mTORC1, the control and functions of mTORC2, as well as the precise mechanism of RICTOR's regulation of mTORC2 and other functions, are less well understood [48]. mTORC2 is the central component in the PI3K-AKT pathway, phosphorylating AKT at Ser473, causing its activation [49-51]. Other substrates of mTORC2 are AGC kinases, SGK and PKC, which have multiple functions in controlling cell survival, metabolic regulation, and cytoskeletal organization [52, 53]. RICTOR is a crucial component of mTORC2 and is necessary for its function, as evidenced by RICTOR knockdown inhibiting AKT activation significantly [51, 54].As a key regulator of the PI3K/AKT pathway, RICTOR plays a crucial role in cancers triggered by Receptor Tyrosine Kinases (RTKs) mutations. Furthermore, the RICTOR gene has recently been discovered to be increased in cancer, emphasizing its significance in cancer development and therapeutic potential. A thorough knowledge of the molecular process underlying RTK-induced carcinogenesis is required to develop therapeutic agents. Several studies have found that the RICTOR gene is amplified or that its protein is overexpressed in several cancer

types. The most common tumor types among RICTOR-amplified samples are neuroendocrine prostate cancer (18%) and lung squamous cell carcinoma (16%), followed by sarcoma (12%) and esophageal and stomach cancer (10%). (10 percent). Rictor dysregulation could have a significant influence tumor growth because it activates AKT, it may be involved in the tumorigenic potential of altered RTK and cooperates with changed RTKs to transform cells or as a crucial regulator of a major pathway downstream of RTKs.

The phosphorylation and activation of Akt, a critical effector of insulin/PI3K signaling, is arguably the most critical role of mTORC2 [51]. By phosphorylating other downstream effectors, Akt affects cellular activities such as metabolism, cytoskeletal structure, survival, death, growth, and proliferation [55]. Because of its role as a central controller of cell development, abnormal mTOR signaling is seen in a variety of illnesses. Adjustments in available energy sources necessitate changes in whole-body metabolism after fasting or dieting to maintain homeostasis, and mTOR signaling is required for optimal metabolic control [45]. Unlike mTORC1, nothing is known about mTORC2's possible role in chronic disease, and the existing evidence is contradictory. As previously proven, the ramifications of mTORC2 via an Akt-dependent pathway play a major role in diabetic kidney injury. Recently, it was discovered that mTORC2 is involved in the hypertrophy of mesangial cells in diabetic nephropathy, implying that blocking mTORC2 could be beneficial [56]. In the diabetic setting, Akt downregulation has been linked to the activation of retinal, endothelial, and neuronal cell death [57]. However, other research has suggested that an increase in the Akt signaling pathway in retinal endothelial cells may play a role in diabetic retinopathy pathogenesis [58]. Furthermore, in diabetic kidneys, the Rictor / mTORC2 pathway has been found to

cause podocyte death in vitro and in vivo, as well as to increase NADPH-dependent oxidative stress [59].

mTORC2 signaling is also involved in cancer, owing to its function in activating Akt, a protein that promotes pro-proliferative activities like glucose uptake and glycolysis while simultaneously suppressing apoptosis [45]. mTOR regulators like as RHEB, PTEN, and TSC have been found to be altered in malignancies in numerous studies [60]. In colon cancer cell lines, inhibition of mTOR using a combination of phytochemicals has been found to be pro-apoptotic through lower expression of cyclin D1 and c-Myc [61]. Despite these findings, little is known about the role of the mTORC2 pathway in colon carcinogenesis in diabetic patients.

F. Reactive Oxygen Species in Diabetes and Cancer

In 2005, Brownlee looked at the potential underlying factors that predispose diabetic problems [62]. He concluded that oxidative stress and the formation of ROS are the unifying factors in the development of all diabetic problems [62]. Reactive Oxygen Species (ROS) contribute to the pathophysiology of diabetes and its consequences through relaying intracellular signals. GI inflammation and CRC carcinogenesis are both influenced by oxidative stress [63]. Our lab and others have investigated the role of ROS production in diabetic complications, specifically diabetes-related CRC [31, 64-66]. Overproduction of ROS causes cellular aging, apoptosis, and cellular death. Low quantities of ROS, which are required to govern cell development, differentiation, death, and gene expression, have been assigned a physiological role [67]. Nutrition and the local microbiota regulate oxidative stress, and any disruption of this equilibrium can cause intestinal inflammation [68, 69]. We will focus on a specific

source of ROS production in diabetes and CRC, which is NADPH oxidase 4 (NOX4), because inhibition of total ROS production has been shown to be ineffective. There are seven members of the NADPH oxidase family (NOX1–5 and dual oxidase "DUOX"1–2) [70]. Many of these members have been linked to diabetes problems [31, 64, 66], as well as cancer progression and development [71]. NOX1 is thought to be important for oncogenic Ras transformation [72], and NOX5 has been linked to cell viability [73]. However, in the context of cancer, NOX4 is the most critical member of the NOX family. NOX4 is overexpressed in prostate cancer [74], glioblastoma [75], liver cancer [76], and melanoma [77], among other cancers. NOX4's role in cancer transformation, proliferation, apoptosis, metastasis, and therapy resistance has also been documented in this research. In CRC [78], NOX4 predicts a poor prognosis and increases cancer progression [78]. In several ways, NOX4 differs from the other members of the NOX family. It possesses unique enzymatic characteristics besides being highly expressed in cardiovascular tissue.

Because NOX4 is constitutively active, regulating its expression is the sole way to regulate ROS generation. However, recent evidence of a putative posttranslational control [79] has been discovered. NOX4 also differs from other members of the NOX family by emitting various patterns of ROS, subcellular localization, tissue distribution, and influence on signaling pathways [80, 81]. The regulation and functions of NOX4 are summarized in **Figure 8.**

Many scientists have looked at the function of ROS in diabetic complications, particularly in CRC [66]**.** Excessive amount of ROS lead to senescence, apoptosis, and death. Low amount of ROS, which are required to regulate cell growth, differentiation, apoptosis, and gene expression [67] has been given a physiological purpose. Oxidative

stress is controlled by nutrition and the local microbiota, and any disruption in this balance can cause intestinal inflammation [68].

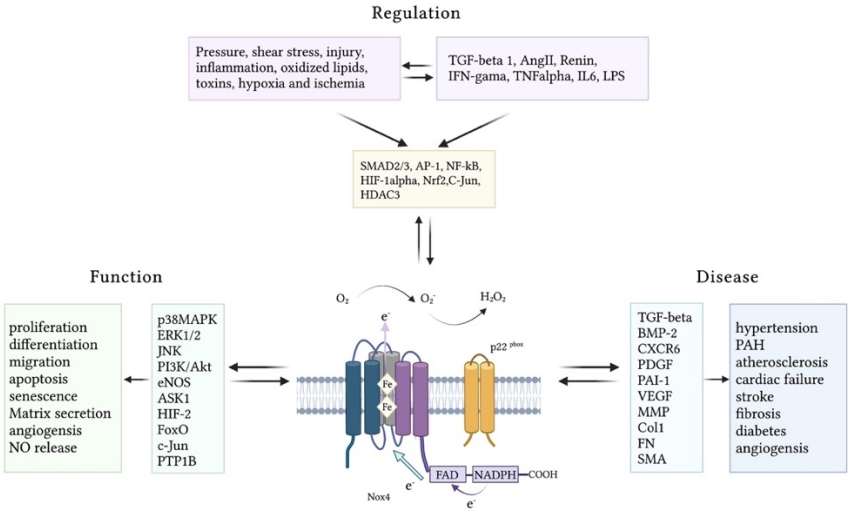

Figure 6: Regulation and Functions of NADPH oxidase 4 [81]

G. Inflammation in Diabetes and CRC

Although autoimmune cell death does not predispose T2DM, an aberrant immune response has been associated to the pathophysiology of this form of diabetes [82]. The microbiota in the gut is necessary for priming the immune system and defending against pathogenic infections. To avoid an undesirable or overwhelming immune response to the local microbiota, the immune system has developed mechanisms to prevent the microbiota from initiating inflammatory responses. Toll-like receptor (TLR) ligands released by the microbiota, such as LPS, are not recognized by the gut immune system [83, 84]. Other activities, such as mucus formation and antimicrobial peptide production from the intestines, limit the interaction between the

microbiota and the immune system, resulting in intestinal homeostasis [85]. An imbalance in the normal gut microbiota can cause colon inflammation, which can be alleviated if the microbiota is eradicated using antibiotics [86].

In the diabetic milieu, dysbiosis affects the intestinal immune system and lymphocyte-homing receptors, which may lead to the development of autoimmune diabetes, a discovery that could explain the change in inflammatory markers seen in diabetes [87, 88]. In T2DM, IL-1 plays a crucial function in cell mass loss [89]. IL-1 activates an autoinflammatory response against cells, resulting in cell death and contributing to the etiology of diabetes and its consequences [90]. Inhibition of IL-1 signaling, either by blocking the IL-1Ra receptor or neutralizing IL-1 with antibodies, has been demonstrated to be effective in T2DM [90]. It is unclear whether higher levels of IL-10 protect against the development of T2DM by reducing the production of pro-inflammatory cytokines, or if greater levels of IL-10 in T2DM trigger a compensatory response against the rise of pro-inflammatory mediators like TNF and IL-6 [89]. Furthermore, IL-17 plays a role in DST2 pathogenesis by activating the NFB pathway, which upregulates the production of pro-inflammatory cytokine genes, which are known to inhibit insulin signaling, resulting in insulin resistance and the development of T2DM [91].

Immunity has a role in the development of CRC as well. The microenvironment around cancer cells can encourage tumor formation by sending out abnormal inflammatory signals [92]. IL-1 is one of many mediators and cytokines that play a role in cancer-promoting inflammation [93]. IL-1 is produced by a variety of types, including immune cells, stromal cells, and tumor cells [93]. The presence of elevated blood IL-1 levels in the serum of CRC patients has been linked to a poor prognosis [94].

Furthermore, it has been demonstrated that interaction between immune cells in the tumor microenvironment and cancer cells causes IL-1 to release [95, 96]. Cancer growth and invasion are also thought to be aided by IL-1 [97]. This evidence supporting IL-1's central role in T2DM, colon inflammation, and CRC suggests that IL-1 is one of the missing links in diabetes' predisposition to CRC. Individual cytokines have been investigated, and the pro-inflammatory cytokines IL-23 and IL-17, as well as the anti-inflammatory cytokines IL-10, have been discovered to have a complex role in gastrointestinal carcinogenesis [98]. Increased serum IL-10 levels have been linked to advanced colorectal cancer in previous investigations. The role of IL-10 in cancer, on the other hand, is unclear. IL-17 has also been demonstrated to induce angiogenesis and regulate the production of several proangiogenic factors, such as vascular endothelial growth factor [98].

H. Use of probiotics in the Treatment of CRC Overview

1. Overview

Probiotics are living bacteria that, when given in sufficient proportions, improve the health of the host by upregulating the gut microbiota [99, 100]. As our understanding of probiotics grows, we're learning that they can do more than just mediate the microbiota; they can also cause physiological and metabolic changes in the host [18]. Competition for the adhesion site, development of microbicidal agents such as bacteriocin, improvement of intestinal permeability, release of bioactive metabolites, control of immune pathways, and stimulation of cellular protective responses are all important functions of a potent probiotic strain, all of which help prevent tumorigenesis, including colorectal cancer [100].

2. Mode of action

Probiotics that alter the gut microbiota appear to help patients with insulin resistance through processes that are both connected and unrelated to inflammation [101]. The molecular mechanisms underlying probiotics' anti-diabetic actions are unknown, however they could be connected to oxidative stress reduction, immunomodulation, inflammation reduction, and gut microbiota modification. Probiotics have also been demonstrated to improve antioxidant absorption and reduce postprandial lipid concentrations, both of which are linked to oxidative stress [101].

Even though various research has sought to identify the mechanism of probiotics' anti-carcinogenic activities, a consistent mechanism for probiotics' anti-CRC action has yet to be identified. Probiotics improve health by changing the composition of the microbiota and its metabolic activities, producing anticarcinogenic and antimicrobial compounds, improving the host's antioxidant system, degrading carcinogens, modifying the expression of genes linked to inflammation, strengthening the immune system, and preventing cancerous proliferation, according to several studies [100]. The possible mechanism underlying the anti-carcinogenic property of probiotics in summarized in **figure 7**.

Furthermore, probiotics have been proven to protect against cancer associated with diabetes. The maintenance of ROS homeostasis and the decrease of pro-inflammatory cytokines are thought to be the mechanisms behind their activity [102].

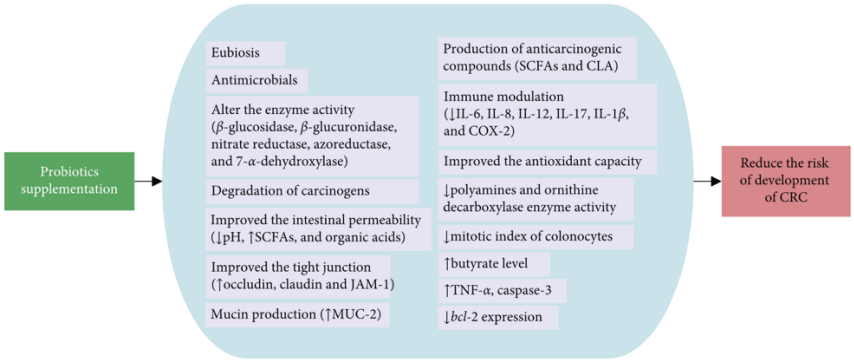

Figure 7: The possible mechanism underlying the anti-carcinogenic property of probiotics [100]

Probiotics are the most effective and safest way to change the gut flora, which leads to improved metabolic status and overall health [103]. Non-targeted techniques were used in the early research evaluating the effects of probiotics on T2DM [104, 105]. They didn't have any previous molecular hypotheses to test. Probiotics have been shown in recent studies to influence inflammatory responses and diminish endotoxemia [106, 107]. There is little doubt that lowering inflammation in T2DM, and associated consequences is beneficial. However, there was no solid evidence that probiotics target a specific route. Delzenne and Cani have proposed several hypotheses concerning probiotics' metabolic targets and how to target the diabetic microbiome [108]. The hypothesized mechanisms for the effects of probiotics therapies on the host metabolic health in diabetes are depicted in **Figure 8.**

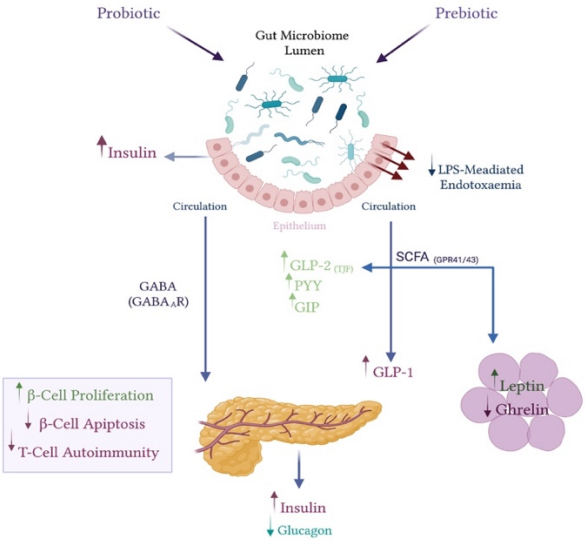

Figure 8: Putative mechanisms for the effects of probiotics treatment in diabetes. GLP: glucagon-like peptide; GABA: gama-amino butyric acid; LPS, lipopolysaccharide; SCFA: short chain fatty acid. [109]

Similarly, it is thought that manipulating the gut microbiome with probiotics can help with CRC [110]. Many clinical experiments have been done, and are currently being done, to see how probiotics affect the outcome of CRC. **Table 1** summarizes the outcomes of clinical trials that used probiotics intervention for patients with CRC [111]

Study Name	Study Type	Population	Intervention/Cohort Arms	Summary of Key Results
Studies evaluating probiotics and cancer prevention:				
Rafter 2007 [112]	RCT [1]	Colon cancer (n = 37) & polypectomized (n = 43) patients	SYN1 [2] + LGG [3] + BB12 [4] vs. placebo	Several CRC [5] biomarkers altered favorably (e.g., decreased genotoxin exposure, IL-2 [6], and IFNγ [7])
Ishikawa 2005 [113]	RCT	Tumor-free patients with history of ≥2 colorectal tumors removed (n = 398)	Wheat bran vs. *Lactobacillus casei* vs. both vs. neither	No significant difference in colorectal tumor occurrence rate with wheat bran or *L. casei*. However, atypia of tumors was lower in the *L. casei* group.
Pala 2011 [114]	Prospective cohort study	EPIC-Italy cohort (n = 45,241)	Yogurt intake by tertile [8]	CRC occurrence was significantly lower in highest vs. lowest tertile of yogurt intake. HR [9] = 0.62 (95% CI 10, 0.46–0.83).
Studies evaluating probiotics and alleviating adverse effects of cancer therapy:				
Mego 2015 [115]	RCT	CRC patients starting treatment with irinotecan-based therapy (n = 46) 11	Colon Dophilus ™ probiotic formula vs. placebo	Reduced incidence in probiotic group of severe diarrhea (0% vs. 17.4%, p = 0.11) and diarrhea overall (39.1% vs. 60.9%, p = 0.11), but not statistically significant.
Osterlund 2007 [116]	RCT	Post-resection CRC patients requiring adjuvant chemotherapy (n = 150)	Randomized to 5-FU via Mayo regimen vs. de Gramont regimen, then randomized to LGG vs. no probiotic	Less grade 3–4 diarrhea in patients receiving LGG (22% vs. 37%, p = 0.027)
Fuccio 2009 [117]	Meta-analysis	Three RCTs evaluating probiotic supplementation to prevent radiation induced diarrhea (n = 632). One RCT evaluating therapeutic role.	Probiotic supplementation vs. placebo/control	No significant difference in rates of radiation-induced diarrhea between probiotic and control arms in preventative trials (OR [12] 0.47, 95% CI 0.13–1.67) or in the single therapeutic trial
Kotzampassi 2015 [118]	RCT	Patients undergoing surgery for CRC (n = 168) [13]	Probiotic formulation [14] vs. placebo	Significant decrease in all major post-operative complications in probiotics arm (28.6% vs. 48.8%, p = 0.010, OR 0.42)
Krebs 2016 [119]	RCT	Patients undergoing surgery for CRC (n = 73)	Preoperative prebiotics [15] vs. preoperative synbiotics [16] vs. mechanical bowel cleansing	No statistical difference in systemic inflammatory response, postoperative course, or complication rate

Table 1:Clinical trials for probiotics use in colorectal cancer [111]

Furthermore, probiotics have been proven to protect against CRC[102]. Their activity is thought to be mediated by preserving ROS homeostasis and lowering proinflammatory cytokines [102]. The combination of probiotics and an antidiabetic medication like metformin is thought to protect the colon from oncogenic transformation [112].

CHAPTER II

AIMS

Increasing body of evidence, as stated earlier in the introductory background, suggests an association between diabetes and CRC. However, the mechanisms of how diabetes can contribute to gastrointestinal complications and CRC are still vague if not unknown. Diabetes changes the body metabolism, and an important site of this action is the gut. Since the gut harbors a huge diverse population of microbiota, their profile will be altered. Hence, this project is an effort to study the effects of probiotics on dysbiosis under diabetic, CRC and diabetes-associated CRC conditions. Moreover, the project tries to verify that GI complications due to diabetes and CRC independently are worsened in diabetic hosts with CRC. Our central hypothesis is that the onset of diabetes, cancer or their association induce dysbiosis that stimulates NADPH oxidases-activated ROS production by inducing the mTORC2 signaling pathway alteration. Further, we hypothesize that probiotic might regulate this dysbiosis and have therapeutic effect on the GI complications.

To explore our hypothesis, we focused on the following aims:

Aim 1: To study diabetes-associated CRC complications.

Aim 2: To investigate whether the restoration of the homeostatic balance of the intestinal microbiota by probiotics can protect against Diabetes.

Aim 3: To investigate whether the restoration of the homeostatic balance of the intestinal microbiota by probiotics can protect against CRC.

CHAPTER III

MATERIALS AND METHODS

A. Animal Studies

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut, Lebanon following the National Institutes of Health (NIH) animal care guidelines. Our present study included MKR male mice that were purchased from The Jackson Laboratory (Bar Harbor, Maine). MKR male mice have a mutation in their muscle insulin receptor that makes them susceptible for insulin resistance and the development of T2DM at adulthood 169. They were developed on FVB-NJ background, so these wildtype mice were used as controls. These mice have been shown to develop diabetic GI complications resembling those seen in humans.

Our central hypothesis that the onset of diabetes, cancer or their association induce dysbiosis that stimulates NADPH oxidases-activated ROS production by inducing the mTORC2 signaling pathway alteration.

B. Colorectal Cancer Induction Experiment (Experiment 1)

In this set of experiment, four groups of male mice (n=4) were used:

a. Control mice or FVB-NJ mice
b. Non-obese type 2 diabetic MKR mice
c. FVB-NJ mice treated with azoxymethane and Dextran Sulfate Sodium (DSS), known to induce CRC
d. Non-obese type 2 diabetic MKR mice treated with azoxymethane and DSS.

The azoxymethane-DSS protocol involves an initial intraperitoneal injection with the genotoxic azoxymethane (10 mg/kg) then after one week, mice were supplied with 2.5% DSS solution instead of drinking water for one week. Mice were allowed to rest for two weeks then the DSS cycle was repeated for two additional times. **(Figure 9)**.

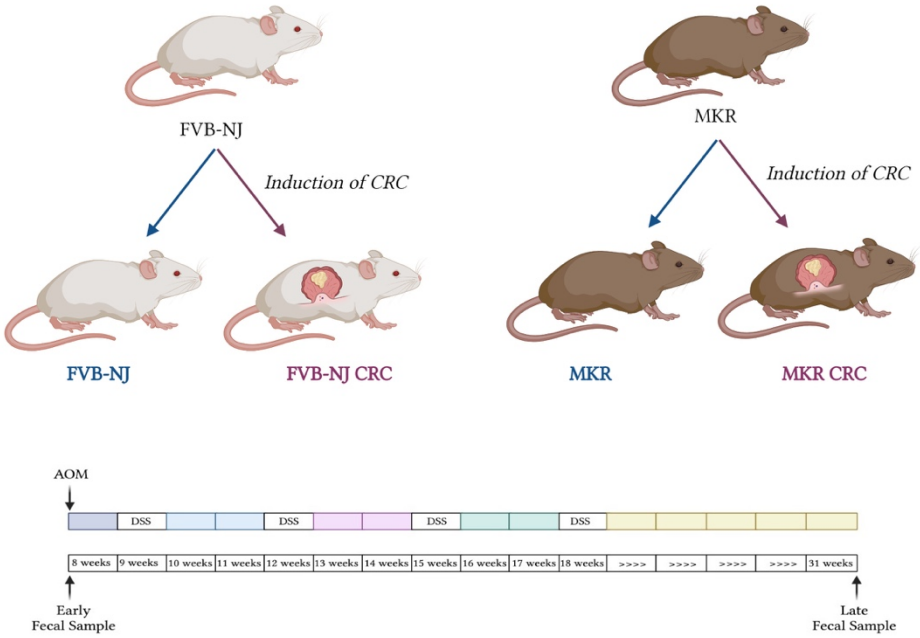

Figure 9: Experimental design of the colorectal cancer induction experiment **(Experiment 1)**

Sacrifice at 31 weeks of age was performed for organs collection.

C. Probiotics treatment (Experiments 2 and 3)

To assess if restoring the homeostatic balance of the gut microbiota can delay the onset of diabetes-associated GI complication, our different group of mice were treated with probiotics (ProbioLife; Valio Ltd., Finland). ProbioLife contains a mixture of complex probiotics including *Lactobacillus acidophilus, Bifidobacterium lactis, Lactobacillus plantarum, Bifidobacterium breve, Saccharomyces boulardi, Lactobacillus rhamnosus, Lactobacillus reuteri,* and zinc as a prebiotic. Experiment 2 included 3 groups of male mice:

a. Control FVB-NJ mice,
b. Non obese type 2 diabetic MKR mice treated with PBS (vehicle)
c. MKR mice receiving probiotics (ProbioLife) dissolved in PBS at a dose of 5 mg/kg body weight by oral gavage for 8 weeks (from week 19 of age till week 31 of age) **(Figure 10)**

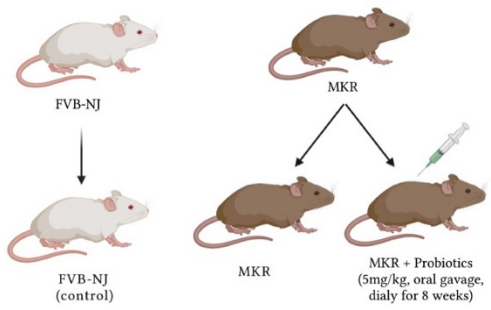

Figure 10**:** Experimental design of the probiotic's treatment in diabetic mice experiment **(Experiment 2)**

Experiment 3 included 3 groups of male mice:

a. Control FVB-NJ mice

b. FVB-NJ mice with CRC treated with PBS (vehicle)

c. FVB-NJ mice with CRC and treated with probiotics (ProbioLife) dissolved in PBS at a dose of 5 mg/kg body weight by oral gavage for 8 weeks (from week 19 of age till week 31 of age) **(Figure 11).**

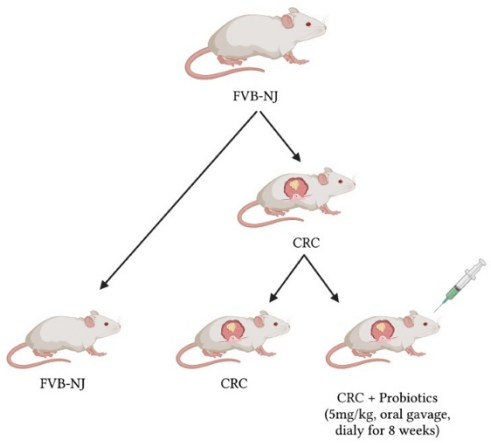

Figure 11: Experimental design of the probiotic's treatment in CRC mice **(Experiment 3)**

After the end of the treatment period, all mice were sacrificed and colon tissue extracted, and analysis performed to assess the development of CRC and polyps and the severity of CRC as well as for metabolic and biochemical analysis.

D. Sacrifice and Organ Harvesting

After 31 weeks of age, all mice were sacrificed by spinal dislocation after anaesthetizing them with isoflurane. Subsequently, distal colon segments were harvested for protein extraction. Colon lengths were recorded (from just underneath the cecum till the anus) to assess the effect of diabetes and treatments on the colon anatomy. Blood was collected via cardiac puncture under anaesthesia in heparin vacutainers for determination of inflammatory cytokines. In total, 1 ml of blood was withdrawn from the heart. Blood was centrifuged at 3000 rpm for 15 minutes at 4oC and plasma was collected and stored at -80oC for further analysis.

E. Histology

Tissue preparation for light microscopy was performed according to routine procedures and protocols already established in the laboratory [113]. Colon tissues were fixed in 10% formaldehyde and stained with **Hematoxylin and Eosin (H&E)** to provide a complete picture of tissue microanatomy and **Masson's Trichrome Staining** to visualize collagen deposition.

F. Immunohistochemistry (IHC)

Immunohistochemical detection of Rictor was performed using 1:200 concentrations of rabbit anti-Rictor ab70374 (Abcam, Cambridge, UK), and visualized using Novolink Polymer Detection Kit (Leica Biosystems, Buffalo Grove, Illinois) according to the manufacturer’s protocol. Hence, Colon tissues were collected and fixed in 4% formalin overnight, rinsed in PBS, and transferred to 70% ethanol before standard processing to obtain paraffin-embedded sections. Unstained tissue sections

were deparaffinized, and antigen retrieval was performed in a citrate buffer in a steamer at 100°C for 60 min followed by 30 min incubation at room temperature. Slides were treated with peroxidase block for 5 min and then blocking was performed with protein block from Leica biosystems (UK) for 5 min at room temperature. Primary antibody incubation was performed overnight at 4°C, followed by post primary block for 30 min. Slides were incubated then with Novolink polymer (Leica biosystems, UK) for 30 min followed by incubation with DAB chromogen prepared in Novolink DAB substrate buffer for 5 min. All slides were counterstained with hematoxylin [114].

G. Isolation of RNA from Colon Tissues

The preparation of the colon samples was done by transferring a very small piece of colon tissue into a new labeled eppindorf tube. 500 µL of trizol was added to each sample tube, then vortexed well. 100 µL of chloroform were then added and vortexed for 15 s. Incubation for 3 minutes at room temperature. Centrifugation at 12,000 xg for 15 minutes at 4 ° C. The upper phase was transferred to a new tube. 250 µL of isopropanol were added to the new tube. Incubation for 10 minutes at room temperature. Centrifugation for 10 minutes at 12,000g at 4 ° C. The supernatant was discarded. 500 µL of 75% ethanol were added. Centrifugation at 7500 xg for 5 minutes at 4 ° C. The supernatant was discarded, and the last 2 steps were repeated with 50 µL of ethanol only. The tubes were inverted in the hood for 30 minutes at 37 ° C. 30 µL of RNase-free water were added and stored at 20 ° C.

H. cDNA Synthesis

Total tissue RNA was extracted using a RNeasy mini-kit (Qiagen Ltd., Crawley, UK). The amount and purity of RNA was evaluated using the NanoDrop ND-1000 spectrophotometer (Wilmington, NC). A packet of M-MLV reverse transcriptase buffers (Promega, Lyon, France) was used for reverse transcription, according to the manufacturer's instructions.

I. Real Time PCR

Real Time PCR was conducted using Bio-Rad CFX384 RT-PCR system using 10 ng of DNA, 300 nM of each primer, SYBR Green Master Mix (Applied Biosystems) and RNase-free water to reach final volume of 10 µl as previously described [188]. Cycling conditions included an initial pre-heating step to 95^{O}C for 2 minutes followed by 40 cycles of denaturation (95^{O}C for 15 seconds), annealing (60^{O}C for 30 seconds) and extension (72^{O}C for 15 seconds) and a final extension step at 72^{O}C for 2 minutes. Each sample was performed in triplicates. Primers used are listed in **table 2.**

NOX4	F: 5’ -TCAGGACAGATGCAGATGCT- 3’ R: 5’ -CTGGAAAACCTTCCTGCTGT- 3’
RICTOR	F: 5' TGCCTCCCTCAATGAAAAAC 3' R: 5'-GCAATCTTGATGGGRGTGGT- 3'
YWHAZ	F: 5’ GGTGATGACAAGAAAGGAATTGTG 3’ R: 5’ GCATCTCCTTTTTGCTGTTTCA 3’

Table 2: List of primers

Analysis of changes in the expression of NOX4 mRNA in colon tissues between healthy controls, MKR, CRC, MKR CRC groups and MKR and CRC groups administered with 5 mg / kg of probiotics (Probiolife). The data represents the number of gene normalized to the YWHAZ gene 'housekeeping gene'. Data are presented as the mean for n = 2 by test groups.

Western Blot

Total proteins from homogenates of the distal segments of the colon were obtained using 200 µl of radioimmune precipitation assay buffer containing 20 mmol/l Tris.HCl, pH 7.5, 150 mmol/l NaCl, 5 mmol/l EDTA, 1 mmol/l Na3VO4, 1 mmol/l PMSF, 20 µg/ml aprotinin, 20 µg/ml leupeptin, and 1% NP-40 then incubated overnight on a rotator at 4 C. Subsequently, samples were centrifuged at 13,700 rpm for 30 min at 4 C. Total protein content of each sample was quantified using the Lowry Protein Assay 191. Samples (containing 30 µg of proteins) were loaded on 15% SDS-PAGE and transferred to nitrocellulose membrane. Blots were incubated with rabbit polyclonal anti-NOX4 (1:500, Santacruz, USA), rabbit polyclonal anti-IL-1b (1:500, Abcam, USA), mouse polyclonal HSC-70 (1:1000, Santacruz, USA), or mouse polyclonal bactin (1:1000, Santacruz, USA). The primary antibodies were detected using horseradish peroxidase-conjugated IgG (1:20000). Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using National Institutes of Health ImageJ software.

J. Statistical Analysis

Statistical analysis was performed using Graphpad Prism Software (Graphpad, version 6.0, CA, USA). Sample size was calculated to give 80% power and $p \leq 0.05$. All results are expressed as mean ± SD (standard deviation). We used a two-tailed student's t test or Analysis of Variances (ANOVA) to determine significance and $p \leq 0.05$ was considered statistically significant. We used Levene's test to test for differences in group variances and chose the t test calculation method accordingly.

CHAPTER IV

RESULTS

A. Histological Alterations

The colon has the typical histological structure as the digestive tube: mucosa, submucosa, muscularis and serosa/adventitia. The mucosa is lined by simple columnar epithelium (Lamina Epithelialis) with long microvilli. It is covered by a layer of mucus which aids the transport of the feces. The mucosa does not contain villi but many crypts of Lieberkuhn in which numerous goblet cells and enteroendocrine cells are found. The connective tissue layer (Lamina Propriae Mucosae) is filled with macrophages, plasma cells and other immune cells. The submucosa comprises blood vessels, lymph nodes and particularly fat tissue. The inner circular musculature of the muscularis is strongly pronounced whereas the outer longitudinal musculature is practically only found in the taeniae **(Figure 12)**

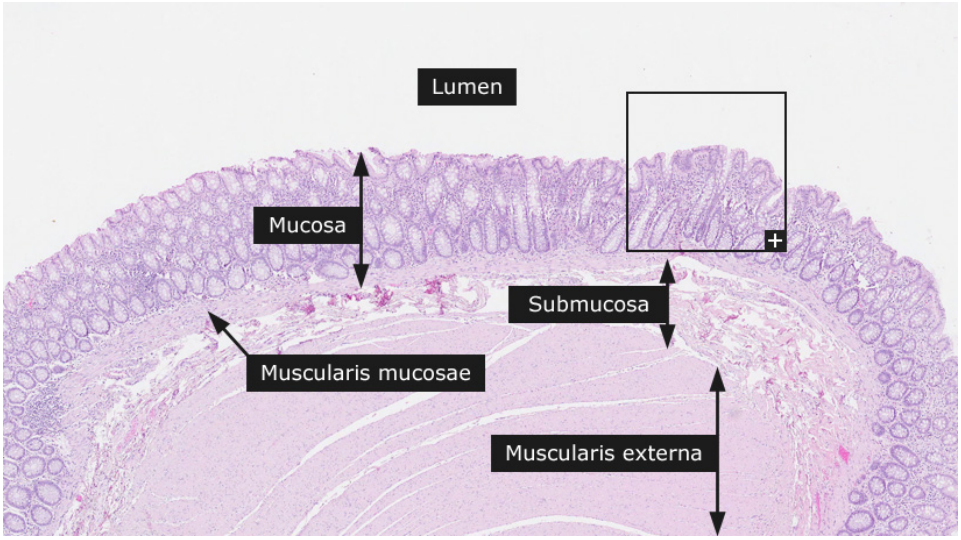

Figure 12: Normal histology of the colon

1. *Hematoxylin and Eosin (H&E) Staining:*

Experiment 1 histological results are presented in the upcoming **figures 13.**

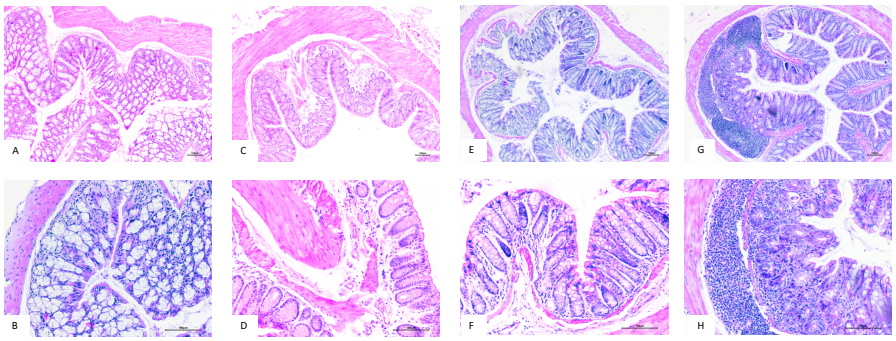

Figure 13: Colon section of FVB control, FVB CCR mice, MKR diabetic and MKR CRC mice stained with **H&E**

A: 4x magnification showing an overview of the colon of FVB-NJ control mice. **B:** 10x magnification of the colon of FVB-NJ control mice. **C:** 4x magnification of the tumor section of the colon of FVB-NJ CCR mice. **D:** 10x magnification of the tumor section of the colon of FVB-NJ CCR mice. **E:** 4x magnification showing an overview of the colon of MKR diabetic mice. **F:** 10x magnification of the colon of MKR diabetic mice. **G:** 4x magnification of MKR diabetic colon with CRC. **H:** 10x magnification of the MKR diabetic colon with CRC

In **Figure 13, images A and B** show the 4X and 10X microscopic magnification of the FVB-NJ control group respectively. These images show a normal alignment of the colonic crypts which characterizes a healthy colon. In addition to that, the mucosa of the colon shows no significance hence, a normal appearance. However, **images C and D**, show the 4X and 10X microscopic magnification of the FVB-NJ mice with CRC respectively. **Images C and D** show a colonic wall with increased crypts per surface

area which characterizes an abnormal colon. In **images E and F**, it was clearly shown that the muscle and submucosal layers of the diabetic colon slice became much thicker than those of the normal colon seen earlier in the FVB-NJ control **'images A and B'**. For the group of MKR diabetic mice with CRC, a few inconspicuous atypical cells accompanied by a dense inflammatory infiltrate are seen after H&E staining **(Figure 13, images G and H).** Also, the colonic wall displayed focal crowding of the crypts. This is along with the presence of aggregates of lymphocytes forming lymphoid follicles. **Experiment 2** histological results are presented in the upcoming **figures 14.**

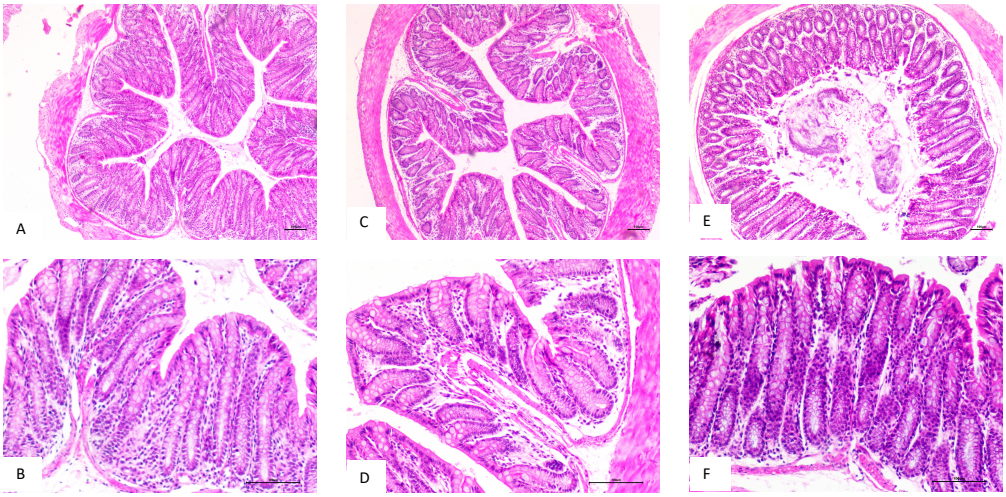

Figure 14: Colon section of FVB-NJ control mice, MKR Diabetic mice and MKR Diabetic mice with probiotics treatment stained with **H&E**

A: 4x magnification of the FVB-NJ colon control section. **B:** 10x magnification of the FVB-NJ colon control section **C:** 4x magnification of MKR diabetic colon. **D:** 10x magnification of MKR diabetic colon. **E:** 4X magnification of MKR diabetic colon with probiotics treatment. **F:** 10X magnification of MKR diabetic colon with probiotics treatment.

In **Figure 14**, we notice that the mucous and muscular layers of the diabetic colon **'images C and D'** become much thicker than those of the normal control colon **'images A and B'**. In addition to a well observed cellular atypia in the diabetic colon. Treatment of diabetic mice with probiotics 'image E and F' decreased the alterations observed in the diabetic colon slice, due to the presence of less inflammatory cell aggregates, less than disturbances of the mucosal architecture and irregularities of the epithelial mucosa as well as submucosal edema.

Experiment 3 histological results are presented in the upcoming **figures 15.**

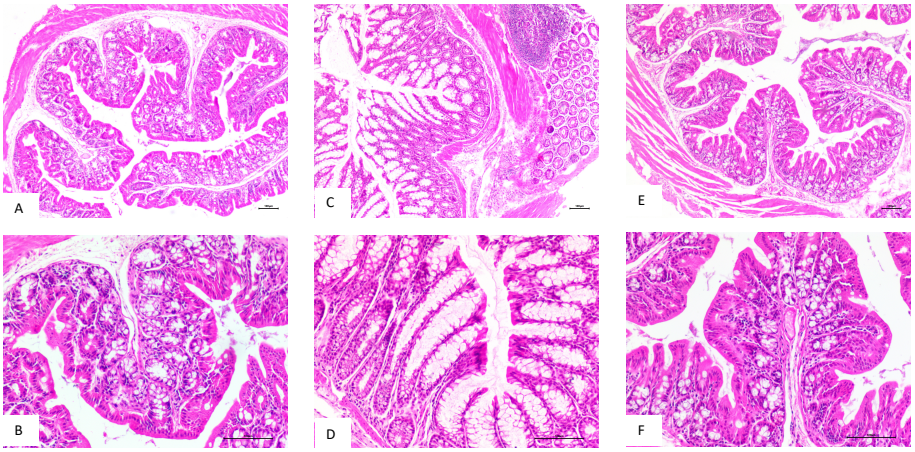

Figure 15: Colon section of FVB-NJ control mice, FVB-NJ CRC mice and FVB-NJ CRC mice with probiotics treatment stained with **H&E**

A: 4x magnification of the FVB-NJ colon control section. **B:** 10x magnification of the FVB-NJ colon control section **C:** 4x magnification of FVB-NJ CRC colon. **D:** 10x magnification of FVB-NJ CRC colon. **E:** 4X magnification of FVB-NJ CRC colon with probiotics treatment. **F:** 10X magnification of FVB-NJ CRC colon with probiotics treatment.

Experiment 3 histological results will be presented in the upcoming **figures 15.** In **Figure 15**, **images A and B** presents the FVB-NJ control colon under 4X and 10X magnification respectively. The images show a normal colon with a healthy colonic wall that has an equal distribution of the colonic crypts. However, **images C and D, presenting the 4X and 10X magnifications of the** FVB-NJ CRC colon respectively, show a prominent dysplastic change in the gland with a focal cribriform pattern. In images E and F, we have the FVB-NJ CRC colon with probiotics. The images show an improved appearance in comparison to images C and D of the CRC colon. The colonic wall shows only mild dysplastic changes upon treatment with probiotics.

2. Masson's Trichrome Staining

By use of the three stains, Masson's Trichrome staining technique is used for the detection of collagen fibres in tissues such as the skin, heart, muscles. The samples are formalin-fixed, paraffin-embedded sections, or frozen sections. **Weigert's Hematoxylin**, an iron hematoxylin dye is used to stain the nuclei. This dye is resistant to decolorization by acidic staining solutions. **Biebrich scarlet-acid fuchsin** solution stains all the acidic tissues such as the cytoplasm, muscle, and collagen. Phosphomolybdic acid is used as a decolorizing agent, making the Biebrich Scarlet-acid fuchsin to diffuse out of the collagen fibres. this leaves the muscle cells staining red. **Aniline blue** stains the collagen along which 1% acetic acid is added to show a difference in the tissue sections. The collagen fibres stain blue and the nuclei stain black, with a red background.

Since The Masson Trichrome staining procedure stains the collagen-rich fibrotic regions in blue, it is especially suited to assess and visualize the extent of fibrosis in dystrophic skeletal muscle on transverse muscle sections.

Experiment 1:

Figure 16: Colon section of FVB control mice, FVB CRC mice, MKR diabetic and MKR CRC mice stained with **Masson's Trichrome Staining**

A: 4x magnification showing an overview of the colon of FVB-NJ control mice. **B:** 10x magnification of the colon of FVB-NJ control mice. **C:** 4x magnification of the tumor section of the colon of FVB-NJ CRC mice. **D:** 10x magnification of the tumor section of the colon of FVB-NJ CRC mice. **E:** 4x magnification showing an overview of the colon of MKR diabetic mice. **F:** 10x magnification of the colon of MKR diabetic mice. **G:** 4x magnification of MKR diabetic colon with CRC. **H:** 10x magnification of the MKR diabetic colon with CRC

Experiment 2: In **Figure 16**, Collagen fibers from FVB-NJ control of normal colonic mucosa **'images A and B'** were thin and barely observed. However, for the group of MKR diabetic mice **'images C and D' and** the FVB-NJ CRC mice **'images E and F'** the collagen fibers were arranged differently; collagen formed dense clusters of fibers that might be attributed to sites of injuries in the colon. **Images G and H** presenting colon of MKR diabetic mice with CRC at 4X and 10X magnification respectively did not show an increase in collagen fiber deposition compared to MKR diabetic mice and FVB-NJ mice.

Experiment 2:

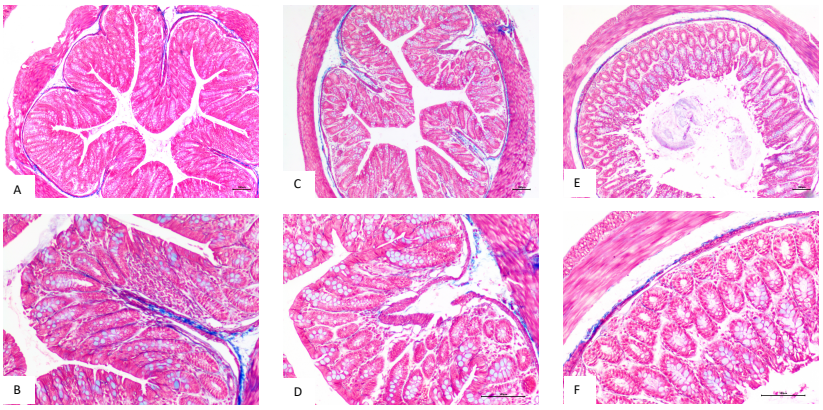

Figure 17: Colon section of FVB-NJ control mice, MKR Diabetic mice and MKR Diabetic mice with probiotics treatment stained with **Masson's Trichrome staining**

A: 4x magnification of the FVB-NJ colon control section. **B:** 10x magnification of the FVB-NJ colon control section **C:** 4x magnification of MKR diabetic colon. **D:** 10x magnification of MKR diabetic colon. **E:** 4X magnification of MKR diabetic colon with probiotics treatment. **F:** 10X magnification of MKR diabetic colon with probiotics treatment.

Collagen fibers from FVB-NJ control of normal colonic mucosa **'images A and B'** were thin, wavy, and scattered. However, for the group of diabetic MKR mice **'images C and D'** the collagen fibers were arranged differently; in fact, the fibers, showing small changes, were more linearized and denser than normal collagen fibers. A decrease in collagen density and a poorly linearized collagen fiber arrangement, clustered with extended curvature, was visualized for MKR diabetic groups treated with probiotics **'images E and F'.**

Experiment 3:

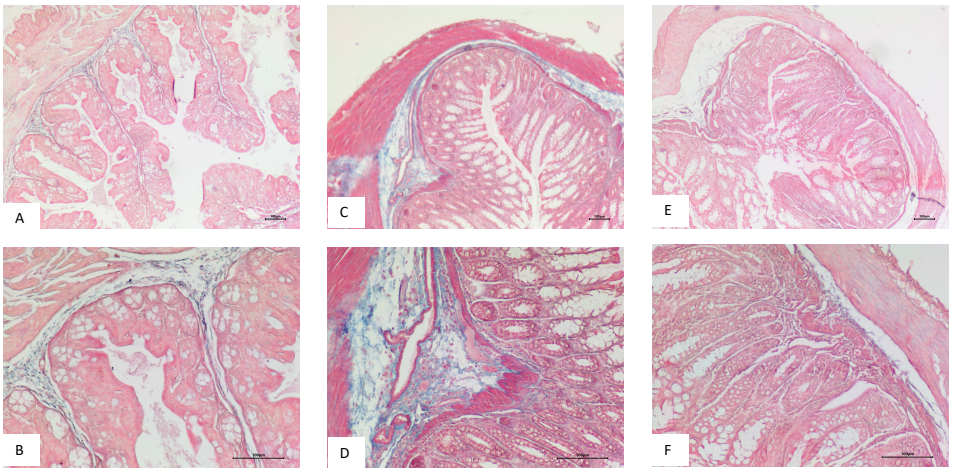

Figure 18: Colon section of FVB-NJ control mice, FVB-NJ CRC mice and FVB-NJ CRC mice with probiotics treatment stained with **Masson's Trichrome staining**

A: 4x magnification of the FVB-NJ colon control section. **B:** 10x magnification of the FVB-NJ colon control section **C:** 4x magnification of FVB-NJ CRC colon. **D:** 10x magnification of FVB-NJ CRC colon. **E:** 4X magnification of FVB-NJ CRC colon with probiotics treatment. **F:** 10X magnification of FVB-NJ CRC colon with probiotics treatment.

At the FVB-NJ CRC tumor invasion front **'images C and D'**, the density of collagen fibers shows an obvious increase in comparison to FVB-NJ control group **'images A and B'**. In addition, these fibers were crosslinked into bundles with a more uniform arrangement, compared to the control group. Alignment of collagen fibers decreases in colon carcinoma tissues treated with probiotics, associated with increased stiffness **'images E and F'**, compared to normal control tissues.

B. Immunohistochemistry (IHC)

Experiment 1:

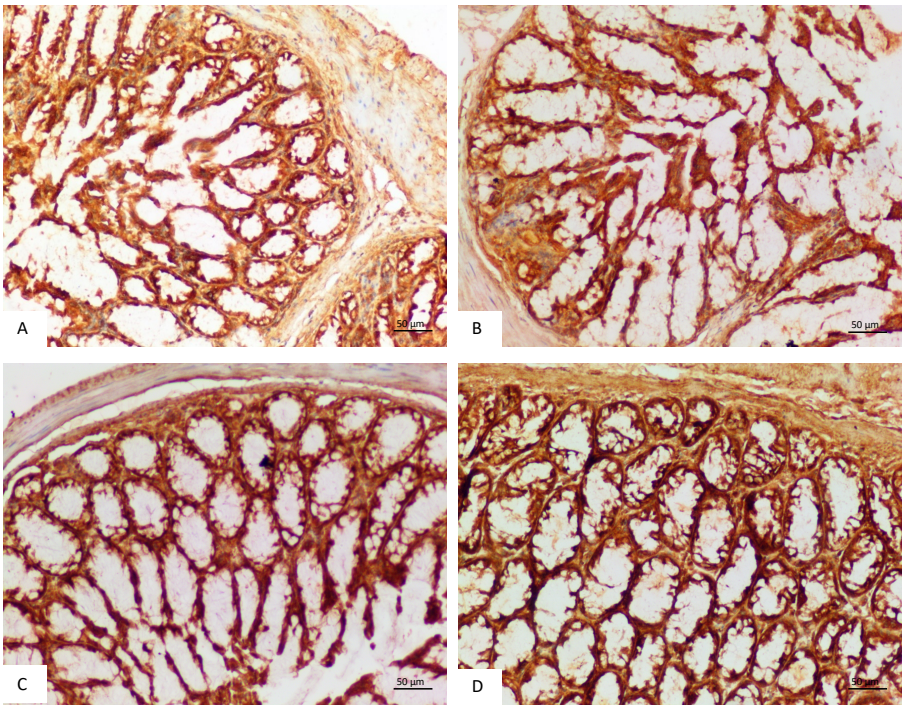

Figure 19: Colon section of FVB-NJ control mice, FVB-NJ CRC mice, MKR diabetic mice and MKR CRC mice under IHC Rictor staining

A: 10x magnification showing an overview of the colon of FVB-NJ control mice. **B:** 10x magnification of the CRC colon section. **C:** 10x magnification of the colon of MKR diabetic mice. **D:** 10x magnification of the MKR diabetic colon with CRC

Figure 19 shows the Rictor protein staining intensity in the different experimental groups of **experiment 1**. In the FVB-NJ CRC colon **'image B'** shows a greater Rictor staining intensity in comparison to the FVB-NJ control colon **'image A'**. This implies that there is a greater expression of Rictor protein in the CRC colon compared to a healthy normal colon. **Image C** presents the MKR diabetic colon which

shows an even greater Rictor staining intensity than the FVB-NJ CRC colon which means a greater expression of Rictor. This expression of Rictor was remarkably intensified in the MKR diabetic colon with CRC **'image D'.** This shows how diabetes and CRC, when present together, manifest greater complications than when present independently from each other.

Experiment 2:

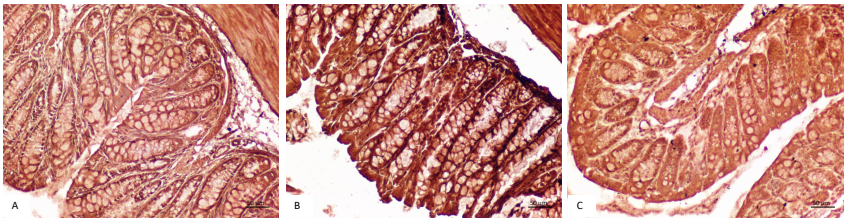

Figure 20: Colon section of FVB-NJ control mice, MKR Diabetic mice and MKR Diabetic mice with probiotics treatment under IHC Rictor staining

A: 10x magnification of the FVB-NJ colon control section. **B:** 10x magnification of MKR diabetic colon. **C:** 10X magnification of MKR diabetic colon with probiotics treatment.

Figure 20 shows the Rictor protein staining intensity in the different experimental groups of **experiment 2**. As seen earlier in experiment 1, In the MKR diabetic colon **'image B'** shows a greater Rictor staining intensity in comparison to the FVB-NJ control colon **'image A'**. This implies that there is a greater expression of Rictor protein in the MKR diabetic mice compared to a healthy normal colon. **Image C presents the MKR diabetic** colon with probiotics treatment, it shows a decreased Rictor staining intensity than the MKR diabetic colon which means decreased expression of Rictor.

Experiment 3:

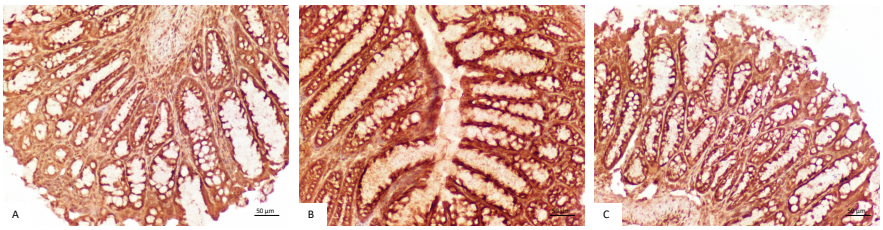

Figure 21: Colon section of FVB-NJ control mice, FVB-NJ CRC mice, and FVB-NJ CRC mice with probiotics treatment under IHC Rictor staining

A: 10x magnification of the FVB-NJ colon control section. **B:** 10x magnification of FVB-NJ CRC colon. **C:** 10X magnification of FVB-NJ CRC colon with probiotics treatment.

Figure 21 shows the Rictor protein staining intensity in the different experimental groups of **experiment 3.** As seen earlier in experiment 1, In the FVB-NJ CRC colon **'image B'** shows a greater Rictor staining intensity in comparison to the FVB-NJ control colon **'image A'**. This implies that there is a greater expression of Rictor protein in the FVB-NJ CRC colon compared to a healthy normal colon. **Image C** presents the FVB-NJ CRC colon with probiotics treatment, it shows a decreased Rictor staining intensity than the MKR diabetic colon which means decreased expression of Rictor.

C. Real Time PCR

We studied the protein expression of NOX4 and Rictor involved in the key pathways of diabetic complications and especially CRC. We found that their protein expression of NOX4 and Rictor was higher in the groups of MKR diabetic, and CRC

mice compared to the control FVB-NJ groups, as shown in the graphs of **Figures 22 and 23**.

In addition, a significant overexpression of NOX4 observed in the MKR CRC groups, which probably shows that diabetes worsens colorectal cancer **(Figure 22).** However, in the MKR CRC group, Rictor expression did increase in comparison to MKR diabetic group, but this expression didn't get higher than that of the FVB CRC group.

Experiment 1:

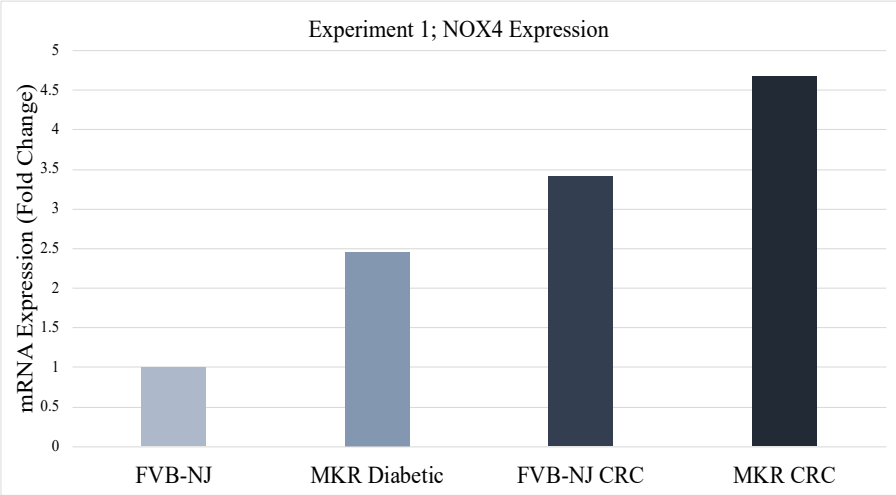

Figure 22: NOX4 expression in colon section of FVB control mice, MKR Diabetic, FVB-NJ CRC and MKR CRC mice

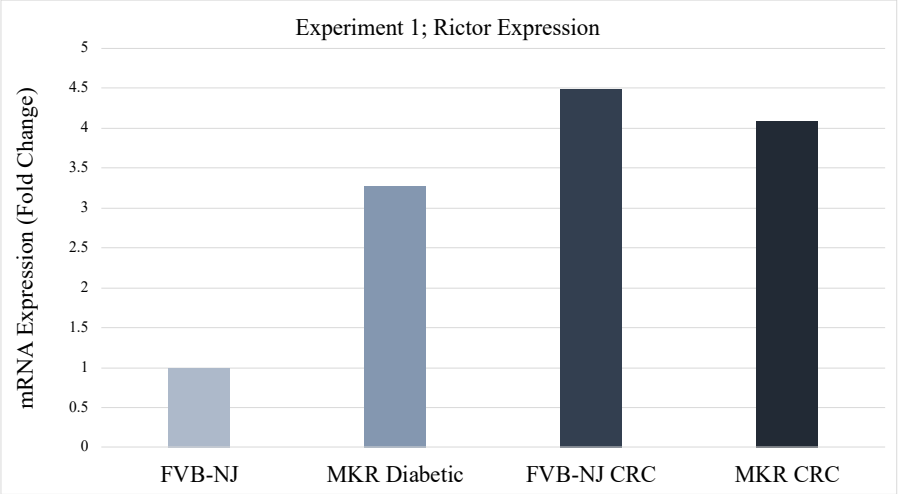

Figure 23: Rictor expression in colon section of FVB-NJ control mice, MKR diabetic, FVB-NJ CRC and MKR CRC mice

To study the beneficial effect of probiotics on reversing gastrointestinal changes seen in MKR- Diabetic mice, mice were treated with a single daily dose of 5 mg / kg of probiotics (ProbioLife) administered by oral gavage for 8 weeks. It was noted that treatment with probiotics did not lower the expression of NOX4 of the MKR diabetic mice and instead increased its expression **(Figure 24)** however, a slight decreased expression of Rictor was found in the MKR diabetic mice upon treatment with probiotics **(Figure 25).**

Experiment 2:

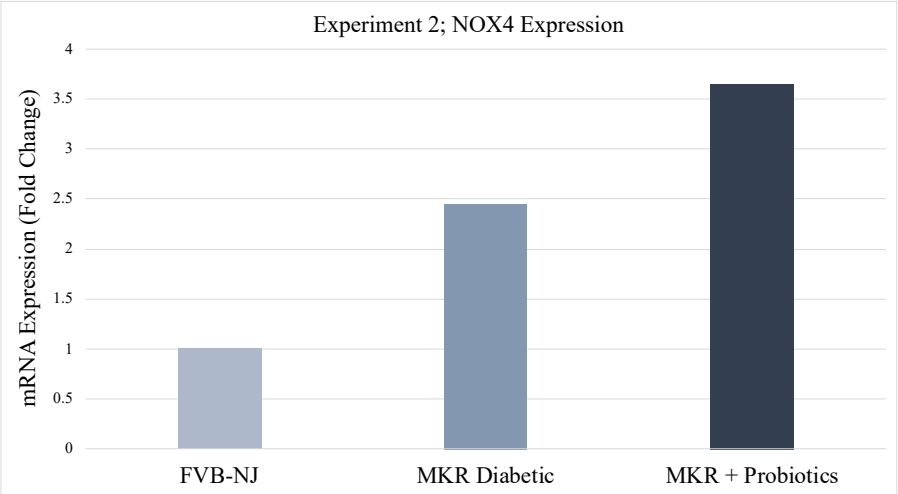

Figure 24: NOX4 expression in colon section of FVB-NJ control mice, MKR Diabetic mice and MKR Diabetic mice with probiotics treatment

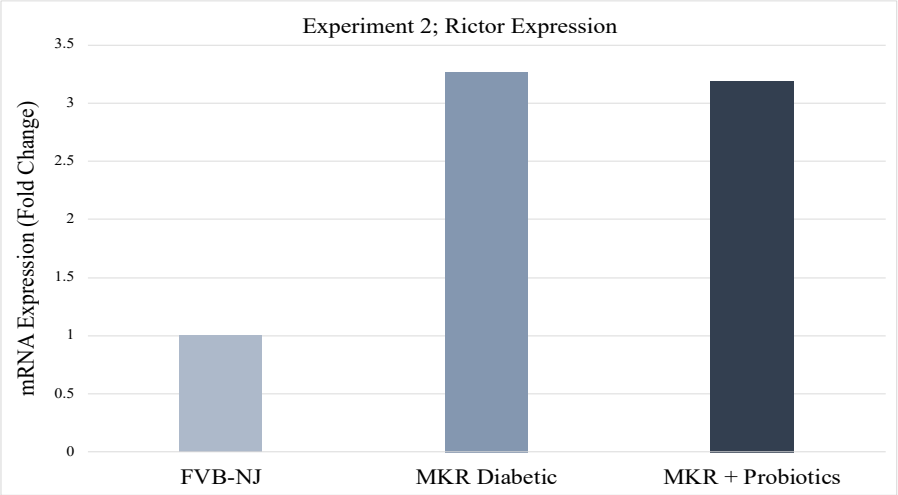

Figure 25: Rictor expression in colon section of FVB-NJ control mice, MKR Diabetic mice and MKR Diabetic mice with probiotics treatment

To study the beneficial effect of probiotics on reversing gastrointestinal changes seen in FVB-NJ CRC mice, mice were treated with a single daily dose of 5 mg / kg of probiotics (ProbioLife) administered by oral gavage for 8 weeks. It was noted that treatment with probiotics did not lower the expression of NOX4 of the FVB-NJ CRC mice and instead increased its expression **(Figure 26)** however, a decreased expression of Rictor was found in the FVB-NJ CRC mice upon treatment with probiotics **(Figure 27).**

Experiment 3:

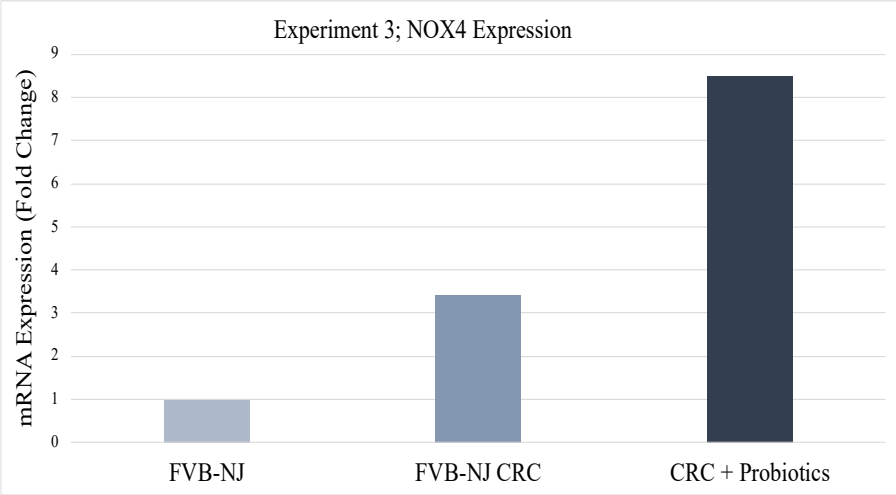

Figure 26: NOX4 expression in colon section of FVB-NJ control mice, FVB-NJ CRC mice, and FVB-NJ CRC mice with probiotics treatment

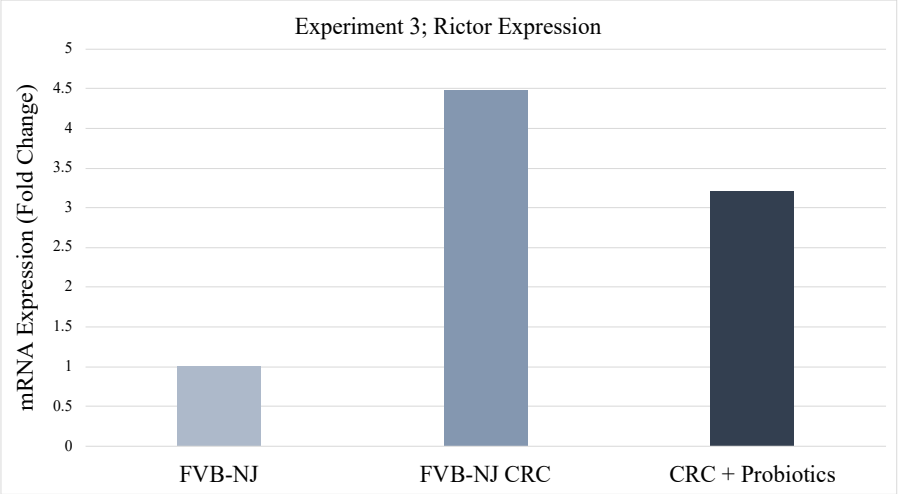

Figure 27: Rictor expression in colon section of FVB-NJ control mice, FVB-NJ CRC mice, and FVB-NJ CRC mice with probiotics treatment.

D. Western Blot

Experiment 1

We measured the protein expression NOX4 in colon tissues of control, diabetic, CRC mice and diabetic mice with CRC. We found that the expressions of NOX4 was increased due to cancer and diabetes compared to those of control mice **(Figure 28)**. Of note, there were no significant differences between the expression of both proteins between the cancer and diabetic groups.

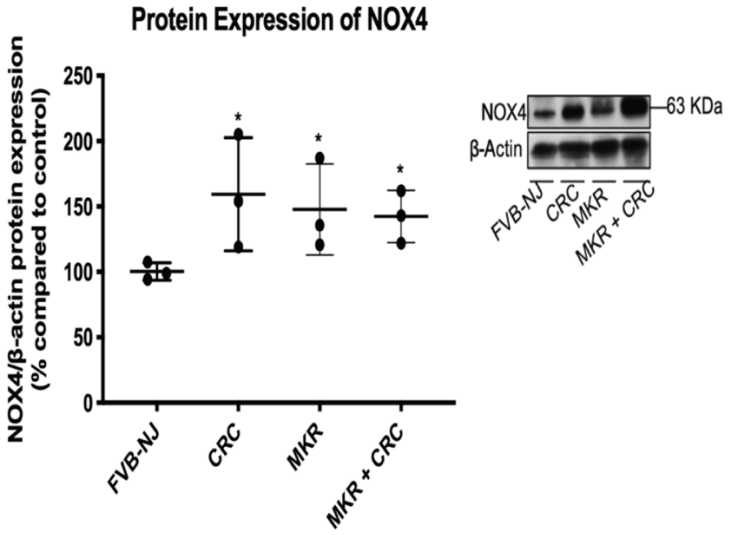

Figure 28: Protein expression of NADPH oxidase (NOX4) in colorectal cancer (CRC), MKR and control mice groups (n=3). Data are expressed as mean ± standard deviation (SD). * Statistically significant at $p<0.05$ vs control. CRC: Colorectal Cancer, NOX: NADPH oxidase

In further attempt to investigate the beneficial effect of probiotics in reversing the GI changes observed in diabetes, we treated a subset of MKR with once daily dose of 5 mg/kg of ProbioLife probiotics given by oral gavage for 8 weeks**, Experiment 2.** We measured the protein expression NOX4 in colon tissues of control, MKR diabetic, and MKR diabetic mice with probiotics treatment **(Figure 29).** MKR diabetic group showed a significant increase in protein expression of NOX4 in comparison to the FVB-NJ control mice. Also, probiotics significantly decreased this expression significantly in comparison to the MKR diabetic group.

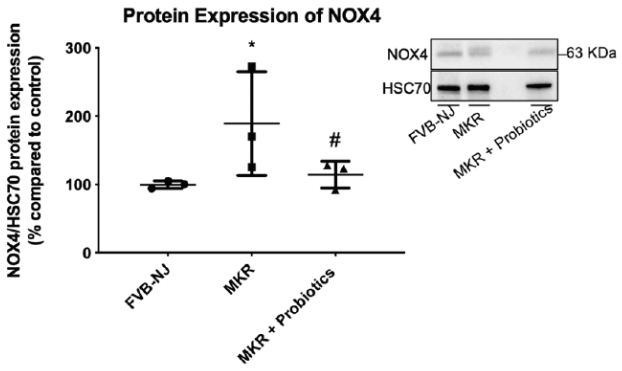

Figure 29: Probiotics protects against the overexpression of NOX4 caused by diabetes (n=3). Data are expressed as mean ± standard deviation (SD). * Statistically significant at $p<0.05$ vs control. # Statistically significant at $p<0.05$ vs MKR diabetic mice

In experiment 3 we also investigated the beneficial effect of probiotics in reversing the GI changes observed in CRC, we treated a subset of FVB-NJ mice with once daily dose of 5 mg/kg of ProbioLife probiotics given by oral gavage for 8 weeks. We measured the protein expression NOX4 in colon tissues of FVB-NJ control, CRC, and CRC mice with probiotics treatment **(Figure 30).** CRC group showed a significant increase in protein expression of NOX4 in comparison to the FVB-NJ control group. Also, probiotics significantly decreased this expression significantly in comparison to the CRC group.

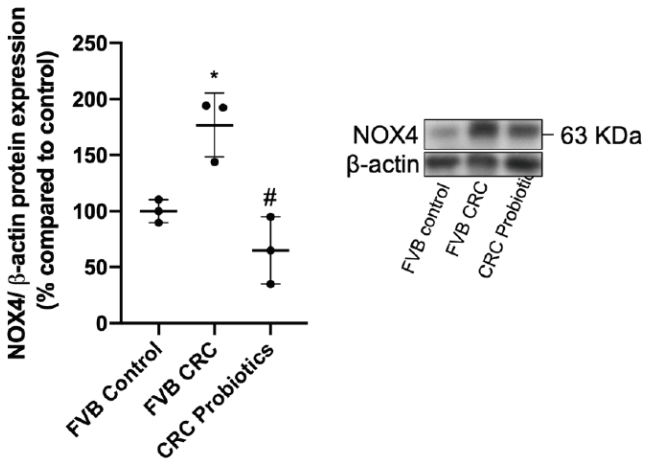

Figure 30: Probiotics protects against the overexpression of NOX4 caused by colorectal cancer (n=3). Data are expressed as mean ± standard deviation (SD). * Statistically significant at $p<0.05$ vs control. # Statistically significant at $p<0.05$ vs MKR diabetic mice

CHAPTER IV

DISSCUSSION

A growing body of evidence is elucidating the association between diabetes mellitus and colorectal cancer. The mechanisms underlying these two medical conditions are not yet fully defined; however, hyperglycaemia associated with increased oxidative stress and chronic inflammation create a favourable environment for the progression of diabetes, IBD and CRC. Above all, a modified gut microbiota is recognized as a key player in this crosstalk. Clinical observations and clinical studies indicate that the prevalence of diabetes in newly diagnosed cancer patients ranges from 8 to 18%, suggesting a bidirectional association between these 2 diseases[34] Numerous animal and human studies have shown that T2DM alone or colorectal cancer alone generally induces changes in the proliferation of the different layers of the colon[35, 39]. Therefore, in our study, we sought to examine the effect of diabetes and CRC as well as both their presence on colonic injury (as studied in **experiment 1**). In addition, in recent years, the scientific community has paid increasing attention to experimental and clinical studies supporting the role of probiotics and butyrate in the management of colorectal carcinogenesis and diabetes. For this, we also tried to examine the potential therapeutic effects of probiotics in lowering the expression of Rictor protein and NOX4 in the context of diabetes and CRC (as studied in **experiment 2** and **3**).

Diabetic animals (MKR diabetic mice) exhibited a significantly greater histological alteration than non-diabetic animals (FVB-NJ control), thus shedding light on the damage caused by diabetes alone on colonic tissue. Of note, the diabetic MKR

mice suffered from more aggressive cancer and exhibited greater alterations than the FVB-NJ CRC mice. On the other hand, diabetes mellitus is one of the most common and rapidly increasing comorbid conditions. For more than 10 years, the medical literature has shed light on the relationship between diabetes and CRC, linking the onset of diabetes to poor cancer prognosis, since comorbid diabetes worsens the course of chronic inflammatory diseases and complicates its development. This agrees with our results where the cancer was exacerbated by diabetes; a poorer clinical profile was obtained in untreated animals which underwent induction of diabetes with CRC compared to their non-diabetic counterparts.

Analysis of the different histological profiles indicated a significant effect of therapy using probiotics in improving colonic tissue, both in diabetic and non-diabetic CRC. **One possible explanation could be that the deregulated microbiota in CRC could prevent probiotics from exerting their protective effects.** Correcting dysbiosis with probiotics underlined the beneficial effects of therapy in different groups, to varying degrees, by restoring homeostatic balance and actively preventing inflammatory and carcinogenic processes. Several potential mechanisms of action of probiotics have been proposed, including improvement of the gastrointestinal mucosa, changes in the gut microbiota and its metabolic activity, modulation of immune responses, improvement of glycaemic parameters, inhibition of cell proliferation, induction of apoptosis, and exercise of anti-inflammatory and antioxidant effects, among others [115, 116]**.**

An elevated Rictor expression is a dysregulation that could have a significant influence on tumor growth. The performed IHC on tissues from **experiment 1 (Figure 19)** showed that the expression of Rictor in the MKR diabetic group and FVB-NJ CRC

is greater than that of the FVB-NJ control group. And Rictor expression was the highest in the MKR+CRC group which showed the highest staining intensity in comparison to the rest of the groups. Evidence on the role of probiotics in correcting diabetic and CRC complications including Rictor protein elevated expression was further established from **experiments 2 and 3 (Figure 20 and 21). Experiment 2** showed how the MKR diabetic group had an increased Rictor expression in comparison to the FVB-NJ control group. This expression was decreased upon probiotics treatment, MKR +Probiotics group. The same trend was observed in **experiment 3** where FVB-NJ CRC mice had greater Rictor expression than their FVB-NJ control littermates. And just as expected, expression decreased upon probiotics treatment, CRC +Probiotics.

This ongoing study was performed to elucidate whether probiotics can regulate the dysbiosis of the gut microbiota that contributes to diabetic pathogenesis and complications. To better understand the effects of probiotics, we performed an RT-PCR to study the protein expression of Rictor, which is the mammalian target subunit of the complex of rapamycin 2 (mTORC2) and NOX4 which is involved in key pathways of diabetes complications.

We found that the expression of NOX4 was higher in diabetic MKR diabetic mice compared to the control groups. NOX4 was also overexpressed in colon tissues of FVB-NJ CRC mice **(Figure 22).** The only function of NOX4 is the production of ROS which are beneficial to the cell in moderate amounts. Overexpression of NOX4 can lead to excessive production of ROS which can damage the cell or induce mutations that can contribute to the oncogenic transformation of the cell, in addition to exaggerated inflammatory immunity, by possible increase of IL-1β, which predisposes to

gastrointestinal dysfunction and contributes to the oncogenic transformation of colon epithelial cells [31].

However, NOX4 expression was not lowered upon the treatment with probiotics in our rt-pcr experiment. **Figure 24** showed that the MKR diabetic group showed increased NOX4 expression that maintained its increase in the MKR diabetic group with probiotics treatment. This was also observed in the context of CRC **(Figure 26)** where the same trend was observed. This might be due to the possibility that probiotics effect might not be inducing its effect on NOX4 production at the transcriptional level of the possible pathway.

In the case of Rictor expression, **figure 25** showed that the MKR diabetic group showed increased Rictor protein expression in comparison to the control group. This expression was decreased in the MKR diabetic group with probiotics treatment. Same pattern was observed in the context of CRC **(Figure 27)** where Rictor expression was significantly lowered upon probiotics treatment.

The western blot experiment showed remarkable significant results on the therapeutic effects of probiotics in regulating NOX4 and Rictor overexpression in diabetes and CRC **(Figure 28, 29 and 30).** These findings underscore the advantageous outcomes of probiotics treatment. Despite seeing this difference in results between the rt-pcr and western in terms of NOX4 expression, this might be due to possibility of probiotics inducing their effects at the translation level.

In general cell controls expression at 3 points. First at transcription, second at translation and third at expression therefore your treatments act on one or all of them. It is quite possible that

mRNA expression is increased while protein translation is reduced. This requires further studying to better understand the pathway in which the probiotics treatment works.

They also confirm that rectifying the microbiota or compensating for the reduced can be beneficial in diabetes. However, probiotics should be used in combination with an antidiabetic drug as they have no effect on the hyperglycemia according to studies done our lab. Short Chain fatty acids (SCFAs) are one of the proposed mechanisms for probiotic activity [117, 118]. SCFAs are thought to bind to certain G-protein coupled receptors in the gut, causing the enteroendocrine system to be manipulated. As a result, gut hormones such as proglucagon[119], glucagon-like peptide-1 (GLP-1)[120], GLP-2, gastric inhibitory peptide (GIP) [121]and leptin[122] will be upregulated, while ghrelin will be downregulated [123]. Intestinal permeability, satiety, stomach emptying, and food intake are all influenced by these hormones. These findings highlight the role of probiotics and butyrate in maintaining a healthy microbiome, which in turn supports a healthy metabolism[124].

The novelty of this work is represented in underscoring the role of microbiota in mediating GI complications of T2DM and aggravating CRC. The results obtained from this work paves the way for better understanding of the mechanisms by which T2DM is precipitating GI complications and potentiating CRC with focus on the potential therapeutic role and mechanistic pathways of probiotics.

Our study suffered from some limitations that should be tackled in future studies mainly the low sample size which prevented the deduction of significance in some experiments, n=2 for the rt-pcr and n=3 for the western blot assay. Along with the limited time due to the global pandemic COVID-19 and its consequent national lockdowns. Needless to

mention the economic and social crisis in our country Lebanon that hindered work and the ability to order and receive needed material that were needed to proceed.

CHAPTER VI

CONCLUSION

In conclusion, our study shows that diabetes and CRC are associated with dysbiosis characterized by overexpression of NOX4 and Rictor protein, thus leading to gastrointestinal complications and further colon injuries. These effects have been controlled upon treatment with probiotics.

In addition, deregulation of mTORC2 components and downstream effectors is increasingly emerging as a finding common to all tumor types. However, the oncogenic properties of mTORC2 have only recently been identified, implying the need for a more in-depth understanding of its tumor promotion mechanisms as well as its connection with other interaction pathways such as NOX4 and ROS.

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