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A NOVEL APPROACH IN COLORECTAL CANCER AND DIABETES MANAGEMENT: ROLE OF METFORMIN AND RAPAMYCIN

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A NOVEL APPROACH IN COLORECTAL CANCER AND DIABETES MANAGEMENT: ROLE OF METFORMIN AND RAPAMYCIN

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Dedication

I dedicate my Doctorate Thesis to:

My beloved husband, Imad Geagea, who always supported me, kept me going forward.

My children; Maria and Christina, the best part of me, my tomorrows, for their smiles and for being my little angels in disguise!

My parents; Abdo and Ilhame for their continuous love and support.

With love

Alice

Palermo, March 2019

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Abstract

The link between colorectal cancer (CRC), diabetes mellitus (DM) and inflammation is well established, and polytherapy, including rapamycin, has been commonly adopted. However, due to the relatively weak response of CRC to rapamycin, combination with other molecules including metformin has become a potentially promising approach. This study is a novel approach that aimed at assessing the effect of a combination therapy of metformin and rapamycin on the control or prevention of colorectal cancer in diabetic animals, in presence or absence of probiotics.

Fifty NOD/SCIDs male mice developed xenograft by inoculating HCT116 cells into the flank; they were equally divided into diabetics (induced by STZ) and non-diabetics. Metformin was given in drinking water, whereas rapamycin was administered via i.p injections. Probiotics were added to the double therapy two weeks before the sacrifice. Assessment was performed by clinical observation, gross anatomic inspection of abdominal organs, histological analysis, mast cells and ROS activities determination, as well as, by molecular analysis of pro-inflammatory cytokines (IL-3, IL-6 and TNFα), AMPK and mTOR.

A decrease in the level of tumorigenesis resulted, to various extents, with the different treatment regimens. The combination of rapamycin and metformin had no significant added effect, however, when probiotics were added to the combination, there was a marked delay in tumor formation and reduction of its size, suppression of ROS and a decrease in inflammatory cytokines as well as an inhibition of p-mTOR.

Existing evidence clearly supports the use of rapamycin and metformin especially in the presence of probiotics. There is an immunomodulatory effect of probiotics in colorectal carcinogenesis. This study confirmed some of the effects observed in several studies and clinical trials. It also described the possible mechanism of action of the 2 drugs through AMPK and mTOR signaling pathways and offered preliminary data on the significant role of probiotics in the combination. However, the application of probiotics in CRC still needs further investigation aiming to clarify its exact role and decipher in more details the involved pathways.

Key words: CRC, DM, Probiotics, Inflammatory Cytokines, AMPK, mTOR, ROS.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

AACE: American Association of Clinical Endocrinologists

ACF: Aberrant crypt foci

ADA: American Diabetes Association

AKT: Protein kinase B (PKB), also known as Akt

AMPK: AMP-activated protein Kinase

APR: Abdominoperineal resection

cAMP: Cyclic adenosine monophosphate

CD: Crohn disease

COX-2: cyclooxygenase-2

CRC: Colorectal cancer

CSF: Colony-Stimulating Factor

DAI: Disease activity index

DCCT: Diabetes control and complications trial

DM: Diabetes Mellitus

DNA: Deoxyribonucleic acid

EGFR: Epidermal growth factor receptor

ERK: Extracellular signal-regulated kinases

GAD: Glutamate decarboxylase

GLP-1: Glucagon-like peptide-1

HbA1C: Hemoglobin A1C

HIF: Hypoxia-inducible factor

HLA: Human leukocyte antigen

IACUC: Institutional Animal Care and Use Committee

IBD: Inflammatory bowel disease

IFG: Impaired fasting glucose or impaired fasting glycemia

IGF: Insulin-like growth factor

IGT: Impaired glucose tolerance

IL-1: Interleukin-1

IL-1: Interleukin-2

IL-3: Interleukin-3

IL-4: Interleukin-4

IL-6: Interleukin-6

IVC: Individually ventilated cages

KRAS: Kirsten rat Sarcoma 2 viral oncogene homolog

LKB1: liver kinase B1

MAPK: mitogen-activated protein kinase

MBP: Mechanical Bowel Preparation

mTOR: mechanistic (or mammalian) target of rapamycin

mTORC1 : mechanistic (or mammalian) target of rapamycin complex 1

mTORC2: mechanistic (or mammalian) target of rapamycin complex 2

NFKB: nuclear factor kappa-light-chain-enhancer of activated B cells

NK-cell: Natural Killer cells

NOD/SCID mouse: Non-obese diabetic/severe combined immunodeficiency mouse.

P-mTOR: Phosphorylated- mammalian target of rapamycin

P53: Tumor protein p53

PCNA: Proliferating cell nuclear antigen

PCOS: Polycystic ovary syndrome

PDGF: Platelet-derived growth factor

PKA: Protein Kinase A

RNA: Ribonucleic acid

ROS: Reactive Oxygen Species

SCFA: Short-chain fatty acids

STZ: Streptozotocin

TLR4: Toll-like receptor 4

TME: Total mesorectal excision

TNFα: Tumor necrosis factor alpha

UC: Ulcerative Colitis

Chapter One: **INTRODUCTION**

1. Background

Several investigators, including our team, reported the co-occurrence of diabetes mellitus (DM) and colorectal cancer (CRC) along with bowel inflammation and dismicrobism (Basso et al., 2014; Cannata, Fierz, Vijayakumar, & LeRoith, 2010; Cheng et al., 2011; Fischbach & Gittes, 2014; Jurjus et al., 2016; Tomasello et al., 2014). Moreover, multiple reports suggested that the gut microbiome is involved in the evolution of DM, in particular type 1 diabetes (T1DM), and that potential modulation of the intestinal microbiota could prevent or delay its progression (Karlin et al., 2018). Furthermore, data are increasing about the greater risk for CRC in patients with DM by almost 1.2% to 1.5 % (Kasznicki, Sliwinska, & Drzewoski, 2014). According to the Global Burden of Disease study data, mortality from CRC increased annually from 1990 through 2013 in line with a worldwide decrease in the age of onset of DM (De Kort et al., 2017). In addition, Type 2 diabetes mellitus (T2DM) has been reported to increase the risks of a wide spectrum of cancers including CRC, and that most colorectal cancers have a multifactorial pathogenesis. Estimates suggest that 14% of CRC patients have T2DM as a comorbid condition at diagnosis (Hardikar, Burnett-Hartman, Cohen, & Newcomb, 2017). They conferred an increased risk of CRC in T2DM patients and a higher mortality rate (De Kort et al., 2017; de Kort et al., 2016; Jurjus et al., 2016; Luo, Lin, He, & Hendryx, 2014; Mills, Bellows, Hoffman, Kelly, & Gagliardi, 2013; Zhu et al., 2017).

Published data also showed that CRC, colorectal adenoma and chronic colitis are positively associated with T2DM and hyperinsulinemia, thus representing the link between the various disease entities(Toma et al., 2013). Further studies have also shown that in human epithelial colorectal cancer cells, high glucose or insulin activates a cascade of cross reacting pathways leading to an alteration in a panoply of proteins in the signaling cascade involved in cell proliferation, survival and apoptosis(Toma et al., 2013).

It is also well documented that in diabetes and CRC, there is an increased generation of Reactive Oxygen Species (ROS). More importantly, in tumors, ROS metabolites can act as signaling molecules to promote cell survival over apoptosis. On the other hand, studies have also shown that in diabetes, there is an increased production of 20-HETE which, through a ROS dependent pathway contributes to organ damage. So we hypothesize that inhibitors of 20-HETE synthesis

might have anticancer and anti-diabetic activities. However, the upstream and downstream signaling pathways leading to injury are not yet fully studied and defined. The mechanistic pathway can be simplified by inactivating AMP-activated protein Kinase (AMPK), activating the mammalian target of rapamycin (mTOR) signaling pathway, and consequently increasing tumor development.

On the other hand, it is important to note that chronic inflammation, as a process, has been considered as forming a favorable basis and a promising environment for such a mechanistic pathway to occur. This task is achieved through a complex inflammatory response which may involve a balance between a huge panel of bioactive molecules, pro and anti-inflammatory (IL-6, NFKB, TNFα and TGFβ, among others …), provided from resident or infiltrating inflammatory cells (Jurjus et al., 2016). However, a persistent or an inadequately resolved chronic inflammation, due to the tilting of the balance in favor of pro-inflammatory agents, may increase the risk of several pathologies such as IBD, CRC and T2DM (Nie, Zhu, & Gu, 2016). Pharmacologically modulating the inflammatory process might be of value in decreasing, preventing or even managing the disease process underlying these diseases (Jurjus et al., 2016).

Metformin, an oral biguanide discovered, as a pharmacological molecule, almost a hundred years ago, is prescribed to over 120 million people worldwide for the treatment of conditions including T2DM, polycystic ovarian syndrome (PCOS), and gestational diabetes (Kinaan, Ding, & Triggle, 2015). Over the past decade, multiple epidemiologic, preclinical and clinical studies have consistently associated metformin with decreased cancer incidence and cancer-related mortality, shedding light on the anti-cancer effects of this hypoglycemic agent (Jurjus et al., 2016; Zhang et al., 2011). Although the exact mechanisms of metformin action are not entirely understood, there is robust literature that defines the hallmarks of its cellular and molecular signaling in colon cancer cell lines with regards to AMPK activation that leads to inhibition of mTOR and a reduction in translation initiation, thus providing a possible mechanism of action of metformin in the inhibition of cancer cell growth (Dowling, Zakikhani, Fantus, Pollak, & Sonenberg, 2007; Hosono et al., 2010).

Similarly, rapamycin, discovered more than thirty years ago, as an immunosuppressor has been used successfully to reduce organ rejection with kidney transplantation (Saunders, Metcalfe, & Nicholson, 2001). Furthermore, rapamycin inhibited cell growth in tumor cell lines (Seto, 2012), which involves binding to the mammalian target of rapamycin (mTOR) whose signaling pathway

is critical to cell growth, proliferation, and survival; in brief, rapamycin could inhibit most of these hallmark processes of cancer (Y. C. Chen, Lo, Lin, & Hsiue, 2013; Seto, 2012).

Exploring the possible additive effects of metformin (an AMPK activator) and rapamycin (a blocker for mTOR activation) might open a new horizon in dealing with the two co-morbid disease entities. Furthermore, modulation of the microbiota by increasing its diversity through probiotic use might hold the promise of effective protection against both DM and CRC (Knip & Honkanen, 2017). The aim of this study is to determine the roles of metformin and rapamycin alone and in combination in the management of diabetes and colorectal cancer in an ectopic xenografts mouse model, at clinical, histological and molecular levels, with an emphasis on the downstream signaling elicited by these drugs in the presence of probiotics.

2. Historical evolution of Diabetes Mellitus management

2.1. Definition

It is well established that diabetes mellitus is a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas or the ineffectiveness of the insulin produced. Such a deficiency results in increased concentrations of glucose in the blood, which in turn affects the body at a multi-organ level. The blood vessels and nerves are particularly susceptible to this damage(WHO, 2010).

2.2. Epidemiology

Worldwide in 2017, 424.9 million people aged 20–79 years or 451 million people aged 18–99 years lived with diabetes. It was also predicted that the number of people with diabetes aged 20–79 years will rise to 629 million or to 693 million among 18–99 years by 2045. The prevalence of diabetes in adults aged 18–99 years was estimated to be 8.4% in 2017 and predicted to rise to 9.9% in 2045. It was also estimated that, in 2017, approximately 5.0 million deaths were attributable to diabetes among people aged 20–99 years. Hence, diabetes accounted for 9.9% of the global all-cause mortality among people within this age range (Cho et al., 2018).

2.3. Evolution of diabetes mellitus management

2.3.1. Before the middle of the first millennium

Diabetes mellitus is well documented in ancient history and its management dates back to at least 1500 BC with multiple cornerstones of new achievements appearing over the years. About 1500 BC, it was reported that the option preferred for DM treatment by "experts" of the Pharaoh of Egypt, 3,500 years ago, was a mixture of "water from the bird pond," elderberry , oil of roses, dates, raw quince, gruel, jelly of viper's flesh and many others (Lakhtakia, 2013; White, 2014). After more than a century, in 250 B.C., the term "diabetes" was proposed and credited to Apollonius of Memphis, who referred to a disease which drains patients of more fluid than they can consume (The Global Diabetes Community, 2016). Then between 131-201 B.C., a Greek physician, Galen of Pergamon, theorized that diabetes is an affliction of the kidneys. Later on, in 400-500 A.D. an ancient Indian physician, Sushruta, and the surgeon, Charaka, were able to identify the two types, later to be named Type I and Type II diabetes (Lakhtakia, 2013).

2.3.2. After the middle of the first millennium

The Persian polymath called Avicenna (980-1037) published "The Canon of Medicine", thus providing a detailed account on diabetes mellitus. The sweet urine of people with diabetes was described, also with abnormal appetite, diabetic gangrene and sexual dysfunction. However, around the 11th century 'uroscopy' became a way of identifying the disease; it involved examining the color, sediment and odor of urine. Some physicians even tasted the urine, and this is apparently how diabetes was given its second name, mellitus, meaning 'honey' in Latin(The Global Diabetes Community, 2016).

In the 17th century, even opium ('syrup of poppies') was prescribed for treatment of diabetes mellitus for over two hundred years (1675-1898); one can assume that it was used to treat complications like gangrene (Lakhtakia, 2013). In the second half of the eighteenth century, Matthew Dobson 1770-1800 identified the reason behind the sweet taste in the urine of people with diabetes, namely, the presence of excess sugar in the urine and the blood. He also observed that diabetes could be fatal for some, leading to death within five weeks, while others live much

longer. This was another indication of two different types of diabetes: type 1 and type 2. During the same period, John Rollo treated a patient using a high-fat and protein diet, the first significant dietary approach to the treatment of diabetes (The Global Diabetes Community, 2016). By the early 19th Century, chemical tests have been devised to detect excess sugar in the urine. Besides, it was not until the Franco-Prussian War, when the French physician Bouchardat noticed that restricted diets and calorie intake helped his patients. Diet and exercise advocacy was the hallmark of treatment of that time(Lakhtakia, 2013; The Global Diabetes Community, 2016).

2.3.3. The twentieth century

About the same period, in the 19th century, Claude Bernard coined the term "glycogen" after discovering that a substance was formed by the liver related to the same sugar found in the urine of people with diabetes. This was the first link between diabetes, glycogen and metabolism. In addition Johann Peter Frank was credited as being the first physician to distinguish clinical differences between diabetes mellitus and diabetes insipidus (Sajid, Shakir, Ansari, & Zulkifle). Moreover, in 1869, a medical student Paul Langerhans described two types of cells, forming islands in the pancreas referred to later as the "Islets of Langerhans". Almost 20 years later, in 1889, Joseph von Mering and Oskar Minkowski's experiments produced an extract of pancreas that reduced the hyperglycemia and glycosuria in dogs made diabetic by the removal of their pancreases. Next, they developed a procedure for extraction from the entire pancreas without the need for duct ligation. This new extract, was made from whole beef pancreas and it was successful for treating humans with diabetes (Rosenfeld, 2002; The Global Diabetes Community, 2016).The name "insulin" came later on from the Latin insula, meaning island, in reference to the insulin-producing islets of Langerhans in the pancreas (American Diabetes Assosiation, 2014).

Early in the 20th century, Stanley Rossiter Benedict devised a new method to measure glucose in urine, called later as Benedict's Solution. About the same period, in 1916, Elliott Joslin, MD, a clinician and educator published the first edition of "The Treatment of Diabetes Mellitus" (American Diabetes Assosiation, 2014). In addition, Dr. Frederick Allen, in 1919, published a book, "Total Dietary Restriction in the Treatment of Diabetes", it described diabetes patients treated with the 'starvation diet'. The treatment helped extend the lives of diabetes patients, but many of his patients died as a result of starvation. In the next year, 1920, an American called Moses Barron links the Langerhans cells with the basis of diabetes mellitus. Picking up on the research

of Barron, a doctor called Frederick Banting conducted critical experiments linking the pancreas and diabetes (The Global Diabetes Community, 2016). Frederick Banting, MD, and his then student assistant, Charles Best, MD, extracted insulin from pancreases of dogs, they were working in a laboratory space at the University of Toronto provided by Professor J.J.R. Macleod. They injected the insulin into dogs whose pancreases have been removed, and the animals' blood sugar levels went down. James Collip purified the extract and used it in humans for the first time on a 14-year-old Leonard Thompson. Even though he was put on a strict diet of 450 cal/day, his blood glucose easily reached 28 mmol/L, after two insulin injections his blood glucose dropped to 6.7mmol/L (Québec, 2016). The work was considered a great success. The average life expectancy for a child with type 1 diabetes at the beginning of the 20th Century was roughly a year; Leonard lived until the age of 27, when he eventually died of pneumonia (American Diabetes Assosiation, 2014; The Global Diabetes Community, 2016). By July 1922, the first bottles of Lilly's Iletin (insulin) arrived in Banting's office and by the year 1923, Eli Lilly and Company began commercial production of insulin. In the decades that followed, the manufacturers developed a variety of slower-acting insulins, the first being protamine insulin introduced by Novo Nordisk in 1936 (American Diabetes Assosiation, 2014; White, 2014). The next major advancement in insulin was its crystallization in 1926, and 10 years later, in 1936 Sir Harold Percival differentiated again between 2 types of diabetes based on the degree of insulin sensitivity in patients (The Global Diabetes Community, 2016; White, 2014). The same year, the first commercially available, extended-action insulin, PZI (protamine zinc insulin), was released. This formulation was composed of an amorphous combination of protamine, zinc, and insulin. PZI continues to be used today in the management of cats with diabetes. In the 1940s, the American Diabetes Association was founded to address the increasing incidence of diabetes and the complications that develop from the disease. Insulin treatments continued to develop and by 1945 the life expectancy of someone with diabetes was increasing (The Global Diabetes Community, 2016; White, 2014). The next major development in insulin formulation came in 1946, when the Nordisk Insulin Laboratory in Denmark released the second extended-action insulin, NPH (neutral protamine Hagedorn). This insulin contained ~ 10% of the protamine found in PZI along with zinc insulin crystals. This insulin was shorter acting than PZI and could be combined with regular insulin (The Global Diabetes Community, 2016). One year later, in 1947, Joslin also sets up "The Victory Medal Award" to celebrate patients who lived with diabetes for 25 years and had no health complications regarding

their kidneys, eyes and blood vessels (The Global Diabetes Community, 2016). In 1949, Rachmiel Levine, MD, discovered that insulin worked like a key, transporting glucose into cells. In the same year, Becton Dickinson and Company began production of a standardized insulin syringe designed and approved by the American Diabetes Association. One year later, the American Dietetic Association (ADA) and the U.S. Public Health Service devised a meal planner that divided foods into six groups, or "exchanges", based on the calories, carbohydrate, protein, and fat in each serving of food.

At the turn of the second half of the 20th Century, in 1952, the ADA funded its first direct research grants (American Diabetes Assosiation, 2014).The following year, in 1953, Dr. Elliot Proctor Joslin and his staff developed the first hospital blood glucose monitoring system and Helen Free developed the Clinistix "dip-and-read" urine test which allowed instant monitoring of blood glucose levels. About the same time, the first oral drug carbutamide was developed. Carbutamide helped to lower blood glucose levels. Two years later, by 1955, sulfonylureas, oral medications that stimulate the pancreas to release more insulin, were available. Moreover, in 1959, Solomon Berson, MD and Rosalyn Yalow, PhD, developed a radioimmunoassay technique, a method for measuring insulin in the blood. They noticed that some people with diabetes still make their own insulin, and they identified "insulin-dependent" (type 1) and "non-insulin-dependent" (type 2) diabetes (American Diabetes Assosiation, 2014; The Global Diabetes Community, 2016).Urine strips were made available in the 1960's for home testing; helping people with diabetes get faster readings. Moreover, Miles Laboratories released Dextrostix, testing strips which required a drop of blood for a minute. The blood was then washed off and an indication of blood sugar levels was revealed on a color chart. At the same time, doctors at the University of Minnesota attempted the first pancreas transplantation in an attempt to cure type 1 diabetes (The Global Diabetes Community, 2016). On the other hand, glucagon, a hormone produced by the pancreas that raises glucose levels, was introduced by Eli Lilly and Company as a treatment for severe hypoglycemia, and in 1964, the Ames Company introduced the first strips for testing blood glucose by color code. Two years later, in 1966, the first successful pancreas transplant was performed at the University of Minnesota Hospital. Besides, in the 1970s, the Ames Company introduced the first glucose meter and in the same period, the first synthetic human insulin was produced using recombinant DNA techniques. Prior to this development, insulin manufacturers have had to stockpile pancreatic tissue from animals and during this period insulin receptors were discovered on cell membranes

(American Diabetes Assosiation, 2014; Lakhtakia, 2013; The Global Diabetes Community, 2016). This discovery raised the possibility that missing or defective insulin receptors may prevent glucose from entering the cells, thus contributing to the insulin resistance of type 2 diabetes. About the same time, the relationship between blood vessel disease and hyperglycemia was reported. In 1972, U100 insulin was introduced along with insulin syringes marked with only a U100 scale; consequently, the frequency of dosing errors was reduced. Then, in 1974, the development of the Biostator enabled continuous glucose monitoring and closed loop insulin infusion. In addition, human Leukocyte Antigens (HLAs) were discovered on cell surfaces and people with type 1 diabetes had specific patterns of HLA that were associated with varying levels of risk for diabetes. In the last quarter of the 20th century, in 1976, the first insulin pumps were invented by Dean Kamen and Rosalyn Yalow, PhD, was awarded the Nobel Prize in Physiology and Medicine for her work in measuring insulin in the body. Moreover, researchers from Boston developed a test to measure glycosylated hemoglobin (HbA1c) which became the gold standard for measuring long-term diabetes control. Two years later, in 1978, researchers at the City of Hope National Medical Center in Duarte, California, and Genentech, Inc., in San Francisco, induced E. coli bacteria to produce insulin identical to human insulin. After some time, portable insulin pumps were introduced and researchers, using them, achieved normal blood glucose levels in patients, however, due to their large size, they were impractical at that time. In addition, The National Diabetes Information Clearing House was created by the federal government to gather and document all diabetes literature. Furthermore, in 1979, The National Diabetes Data Group developed a new diabetes classification system: 1) insulin-dependent or type 1 diabetes, 2) non-insulin-dependent or type 2 diabetes, 3) gestational diabetes, and 4) diabetes associated with other syndromes or conditions. One year later, in 1980, a new animal model of type 1 diabetes in the non-obese diabetic (NOD) strain of mice was described in Japan (American Diabetes Assosiation, 2014). Humulin, the first biosynthetic human insulin, insulin produced by genetically altered bacteria, was FDA approved in 1982 for distribution in several countries. It is identical to the structure of human insulin and has the advantage of being less likely to lead to allergic reactions than animal insulin. In the same year, a 64K autoantibody was discovered and was found to be associated with type 1 diabetes. A year later, in 1983, a link between hypoglycemia and brain metabolism was established and the second-generation sulfonylureas entered the market allowing patients to take smaller doses with reduced side effects. In 1984, the insulin molecule

was identified to be a target of autoimmune response in individuals with type 1 diabetes and scientists discovered a relationship between pregnancy and the worsening of diabetic retinopathy. About the same time the first insulin pen delivery system, called the NovoPen, was introduced by Novo Nordisk (American Diabetes Assosiation, 2014; Lakhtakia, 2013; The Global Diabetes Community, 2016) .In 1986, the National Diabetes Data Group reported that type 2 diabetes was more common among African Americans, Mexican Americans, and Native Americans than among Caucasians. Fifty percent of all Pima Indians in Arizona, over the age of 35, had diabetes – the highest rate in the world. In 1987, the 64K autoantibody originally discovered in 1982 was found to be predictive of type 1 diabetes. In addition, researchers determined that tight control of glucose levels during pregnancy is important for the health of the baby, and continued to study how diabetes increases the risk for birth defects. In 1989, the American Diabetes Association released its first Standards of Care to guide physicians in the treatment of diabetes. At the same time, glucose was discovered to be distributed into muscle and fat cells via a transporter known as GLUT-4. Understanding how glucose was transported from the bloodstream into cells to be used as fuel was important to locating different drug targets that could improve insulin sensitivity. In 1990, the protein glutamate decarboxylase (GAD), an important enzyme involved in cellular communication in the brain and pancreas was identified and the immune system's attack on GAD triggering a progressive autoimmune response that leads to diabetes was described(American Diabetes Assosiation, 2014). In 1991, the World Health Organization launched World Diabetes Day in response to the rapid rise of diabetes around the world, held on November 14, the birthday of Frederick Banting. In 1992, Medtronic released the MiniMed 506 insulin pump, which delivers meal bolus memory and daily insulin totals (The Global Diabetes Community, 2016). One year later in 1993, the Diabetes Control and Complications Trial (DCCT) showed that keeping blood glucose levels as close to normal as possible slowed the onset and progression of eye, kidney, and nerve diseases caused by diabetes. In fact, it demonstrated that any sustained lowering of blood glucose helps, even if the person has a history of poor control (American Diabetes Assosiation, 2014). Their report demonstrated that regular activity and good nutrition help to improve diabetes control and stave off the risk of long-term health complications (The Global Diabetes Community, 2016). In mid 1900s the incretin hormone GLP-1 was discovered. Incretin hormones are secreted from the gut in response to food and encourage the body to produce insulin. Its discovery led to a new class of diabetes drugs that can increase insulin secretion in response to glucose, and even

increase the amount of beta cells in the pancreas. About the same time, the drug metformin, the focus of this study, first discovered in 1918, rediscovered in the 1940's and reported for the first time to treat diabetes in 1957, became available in the U.S. in 1995. Metformin is a biguanide that prevents glucose production in the liver. In 1996, the drug acarbose, brand name Precose (Bayer Corporation) became available in the U.S. Acarbose is an alpha-glucosidase inhibitor that slows digestion of some carbohydrates. In the same year, Lispro (a lysine-proline analog) was introduced by Eli Lilly and Company as the world's fastest acting insulin. In 1997, Troglitazone, brand name Rezulin (Parke-Davis), was approved by the FDA. It was the first in a class of drugs known as thiazolidinediones, it improved insulin sensitivity in muscle cells, however, it was eventually removed from the market due to liver toxicity. Rosiglitazone and pioglitazone, also in this drug class, were later brought on to the market. At the same time, the fasting glucose level for diagnosing diabetes was lowered from 140 mg/dl to 126 mg/dl. In 1998, Repaglinide, brand name Prandin (Novo Nordisk) was developed. Repaglinide belongs to a class of drugs known as meglitinides. They stimulate insulin secretion in the presence of glucose. In the same year, the United Kingdom Prospective Diabetes Study (UKPDS) showed that people with type 2 diabetes who practice tight control of blood sugar levels and blood pressure levels reduce their risk of complications, similar to the results of the DCCT in people with type 1 diabetes. Together these two studies transformed the nature of diabetes care around the world (American Diabetes Assosiation, 2014).

2.3.4. The twenty first century

At the turn of the twenty first century, there was a growing interest in islet cell transplantation as Shapiro et al published findings from seven patients with type 1 diabetes who underwent the procedure as a means of helping them achieve insulin independence (Shapiro, 2012). In 2002, a more targeted therapy was reported with the anti-CD3 monoclonal antibody, hOKT3gamma1(Ala-Ala), which slowed the deterioration of insulin production and improved metabolic control during the first year of type 1 diabetes in the majority of patients (American Diabetes Assosiation, 2014; The Global Diabetes Community, 2016). During 2002, the American Diabetes Association defined prediabetes as impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT). IFG was defined as fasting blood glucose of 100-125 mg/dl, and IGT was defined as a glucose level from 140 mg/dl – 199 mg/dl two hours after consuming a glucose-rich drink. Later, A1C levels of 5.7%

to 6.4% were also used to identify individuals with prediabetes. In 2005, Exenatide, brand name Byetta, was approved in the U.S. as a first-in-class incretin mimetic (GLP-1) drug to treat type 2 diabetes. An injectable drug, exenatide works by increasing insulin production in response to blood glucose levels. In addition, pramlintide, brand name Symlin, was approved in the U.S. as an injectable adjunct treatment for people who use insulin at mealtimes but still fail to achieve desirable blood glucose levels. Besides, in 2006, the FDA approved JANUVIA (sitagliptin phosphate), the first in a new class of drugs known as DPP-4 inhibitors that enhance the body's ability to lower elevated blood sugar. DPP-4 is an enzyme that naturally blocks GLP-1 from working, so by inhibiting this enzyme, GLP-1 works in the gut to promote insulin secretion (American Diabetes Assosiation, 2014). Moreover, in 2008, Suzanna M. de la Monte proposed the term "type 3 diabetes" to describe insulin resistance in the brain. Five years later, in 2013 the University of Cambridge reported trials of an artificial pancreas which combines the technology of an insulin pump with a continuous glucose monitor. In the same year, the FDA approved Invokana (Canagliflozin), the first in a new class of drugs known as the SGLT-2 inhibitors, for lowering elevated blood sugar in patients with type 2 diabetes. SGLT-2 inhibitors block the activity of sodium glucose transport proteins in the kidney, reducing glucose re-uptake and increasing secretion of glucose in the urine (American Diabetes Assosiation, 2014; The Global Diabetes Community, 2016). At the same time, the FDA declined to approve Degludec, an ultra-long-acting insulin (duration of 42 hours). However, this compound is available in Europe and will probably be resubmitted for approval in the United States. Lately, in 2015, Dr. Edward Damiano introduced the "ILet", a bionic pancreas that delivers both insulin and glucagon every five minutes, he described the device as a "bridge to a cure" (The Global Diabetes Community, 2016; White, 2014). In conclusion, The management of diabetes is a long term commitment and the goal of the current therapies is to improve the quality of life of the patients as well as to lower the risk and delay the onset of diabetic complications such as blindness, end-stage renal disease, neuropathy, cardiovascular diseases and cancer (The Global Diabetes Community, 2016).

The main target of management is to lower glycaemia and maintain sugar levels in the blood within an acceptable margin. Table 1 below shows the current glycemic targets in the treatment of diabetes according to the American Diabetes Association (ADA) and the American Association of Clinical Endocrinologists (AACE) **(Association, 2017; Garber et al., 2017; Handelsman et al., 2015) .**

Table 1: Current glycemic targets in the treatment of diabetes for non-pregnant adults (Association, 2017; Garber et al., 2017; Handelsman et al., 2015) .

	Glycemic Targets	
Glycemic Measure	**ADA**	**AACE**
Fasting glucose (mg/dl)	80-130	< 110
2-h post-meal (mg/dl)	< 180	< 140
Hemoglobin A1c (%)	< 7.0	< 6.5

Insulin is the Best treatment for Type 1 Diabetes since it is replacing the missing hormone. Treatment of type 2 diabetes however is more complex since insulin levels in the blood are normal to high and the goal is to improve insulin action in the peripheral tissues.

The management of type 2 diabetes includes a healthy diet, weight loss, oral medications and sometimes a combination of oral and injectable drugs. Insulin is added to the therapy at any time when glycemic control is not achieved by oral drugs alone and when insulin secretion becomes impaired to the point that replacement therapy becomes a must (Stang, Wysowski, & Butler-Jones, 1999). Over the years, an armamentarium of drugs has been used including metformin or biguanides.

2.3.4.1. Biguanides

Metformin or N, N-dimethylbiguanide (Figure 1) is the first line medication for treating type 2 diabetes. It was first discovered in 1917 and described in 1922 by Emil Werner and James Bell as a product in the synthesis of N, N-dimethylguanidine.

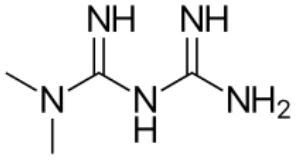


Figure 1: Molecular aspect of metformin (Song, 2016).

The origin of metformin trace back to a folk remedy derived from a toxic plant Galega officinalis or French lilac. Galega officinalis contains the phytochemicals galegine and guanidine both of

which decrease blood sugar. The plant was used in medieval times to treat the symptoms of diabetes. Clinical trials have proven that galegine and guanidine were too toxic for humans. However, when two guanidine molecules were joined together with slight modification, the result gave the biguanides: phenformin, buformin and metformin. Of this family of drugs only metformin remains, others were discontinued for their high risk of lactic acidosis and mortality.

Metformin is generally well tolerated and it decreases high blood sugar mainly by suppressing hepatic gluconeogenesis. Metformin's mechanism of action is not definitively known but its major effects on glucose lowering are linked to its action on mitochondrial metabolism and cellular pathways which lead to a reduction in glucogenesis. Many potential mechanism of actions have been suggested, including; activation of AMP-activated protein kinase (AMPK), inhibition of the mitochondrial respiratory chain, inhibition of the glucagon-induced elevation of cyclic adenosine monophosphate (cAMP) with reduced activation of protein kinase A (PKA), inhibition of mitochondrial glycerophosphate dehydrogenase and a positive effect on the gut microbiota (May & Schindler, 2016).

AMPK also known as Adenosine Mono Phospho Kinase is an enzyme that plays a major role in insulin signaling, the metabolism of glucose and fat and whole body energy balance. AMPK activation increases the expression of small heterodimer partner which in turn inhibits the expression of the hepatic gluconeogenic genes phosphoenolpyruvate carboxykinase and glucose 6-phosphatase AMPK activation is important for metformin's inhibitory action on liver glucose production (Rena, Pearson, & Sakamoto, 2013). Metformin can safely be prescribed for pregnant women with no evidence of obvious side effects on the offspring. Other characteristics of metformin, including cancer prevention, have also been described (Rowan et al., 2011).

In brief, among all the important managing and therapeutic modalities for the treatment of diabetes, metformin stood the test of time and is considered as the preferred first-line oral blood glucose lowering agent to manage T2DM. Up till now, metformin is the most used drug either alone or in combination with other molecule. Moreover, other medical benefits were described for metformin. It is for this reason and many others that this study is investigating other possible uses and exploring more into its mechanisms of action alone or in combination with rapamycin in presence or absence of probiotics.

3. Colorectal Cancer

3.1. What is colorectal cancer?

Colorectal Cancer (CRC) is a disease characterized by the unchecked division and survival of abnormal cells occurring in the colon or rectum. CRC usually begins as a noncancerous growth called a polyp that develops on the inner lining of the colon or rectum and grows slowly, over a period of 10 to 20 years. An adenomatous polyp, or adenoma, is the most common type. Adenomas arise from glandular cells, which produce mucus to lubricate the colorectum. About one-third to one half of all individuals will eventually develop one or more adenomas. Although all adenomas have the potential to become cancerous, fewer than 10% are estimated to progress to invasive cancer. The likelihood that an adenoma will become cancerous increases at is becomes larger. Cancer arising from the inner lining of the colorectum is called adenocarcinoma and accounts for approximately 96% of all CRCs.

Early CRC often has no symptoms, which is why screening is so important. As the tumor grows, it may bleed or obstruct the intestine. In some cases, blood loss from the cancer leads to anemia (low number of red blood cells), causing symptoms such as weakness, excessive fatigue and sometimes shortness of breath. Additional warning signs include:

- Bleeding from the rectum
- Blood in the stool or in the toilet after having a bowel movement
- Dark or black stools
- A change in bowel habits or the shape of the stool (e.g. more narrow than usual)
- Cramping or discomfort in the lower abdomen
- An urge to have a bowel movement when the bowel is empty

3.2. Epidemiology

Colorectal cancer (CRC) is a common and lethal disease. It is estimated that approximately 140,250 new cases of large bowel cancer are diagnosed annually in the United States, including approximately 97,220 colon and 43,030 rectal cancers. Approximately, 50,630 Americans are expected to die of large bowel cancer each year. Although CRC mortality has been progressively declining since 1990, at a current rate of approximately 1.7 to 1.9 percent per year, it still remains

the third most common cause of cancer death in the United States in women, and the second leading cause of death in men (Negoita et al., 2018; Siegel, Miller, & Jemal, 2018).

Global incidence of colorectal cancer for all ages is 9.7% (1360602) and mortality is 8.5% (693933). Moreover, the 5- year prevalence for adult population is 10.9% (3543582)(Organization, 2012).

For years, colorectal cancer has been well established as being one of the most frequent solid tumors with median age of about 70 years at the time of diagnosis. Studies have shown that CRC is rather a spectrum of diseases with molecular complexities. Accordingly, a spectrum of treatment options have evolved over the years and proved to be dependent on the stage of the disease, its location, the performance status of the patient, and increasingly the molecular make-up of the tumor to define subgroups and design targeted therapies accordingly. Such treatment protocols emerged from a single agent treatment to combination regimens, to targeted substances with surgery being as a constant option.

3.3. Surgical treatment of CRC

It was not until the eighteenth century when Giovanni Morgagni first considered resection of the rectum in treating rectal cancer. In 1739, Jean Faget of France was credited with the first attempted rectal resection and in 1776; Henry Pillore of Rouen, France performed the first colostomy on an adult for an annular "scirrhous carcinoma" that had completely obstructed the lumen of the rectum (Corman, 2000; Galler, Petrelli, & Shakamuri, 2011). Moreover Aristide Verneuil modified LisFranc's perineal resection and removed the coccyx to allow for better exposure and more radical excision. Furthermore, in 1874, Kocher closed the anus to reduce spillage and infection. Krocher, however, divided the rectum at least ''half an inch'' on either side of the tumor and removed in a similar manner to Kocher's technique. Kraske presented his technique to the Congress of the German Society of Surgery in 1885. It was received with great eagerness and quickly adopted. Carl Gussenbauer, an assistant to Billroth, performed the first abdominal resection of a rectal tumor with intraperitoneal closure of the distal rectum. J. Hochenegg developed a ''pull-through'' (duerchzug) technique by everting the anus and rectum, then excising the tumor and completing a recto-anal anastamosis. Besides, in England, William Ernest Miles defined better the nature of perirectal lymphatic spread and challenged the traditional anatomy of rectal lymphatics previously described by Dimitri Gerota in 1895. He published his findings in the Lancet in 1908 and

recommended a more extensive mesenteric lymphadenectomy in order to prevent recurrence. In his landmark article, he identified three zones of spread – downward, lateral, and upward – with upward, in his opinion, being most important (Galler et al., 2011). Miles' procedure followed five principles: (1) Creation of an abdominal colostomy, (2) resection of the rectum, sigmoid, and its blood supply, (3) resection of the mesorectum, (4) removal of the lymph nodes over the bifurcation of the common iliac artery, and (5) wide perineal resection with removal of the levator ani muscle. Despite the improved oncologic outcomes, many surgeons felt that the Miles procedure was too radical and morbid accompanied with permanent colostomy, genitourinary dysfunction, and psychosocial implications (Lange, Rutten, & van de Velde, 2009). Early in the twentieth century, Donald Balfour, an associate of Charles Mayo, described a ''tube support'' for anastamosis between the rectum and sigmoid colon. He first performed it after accidently injuring the sigmoid during an abdominal procedure. He published his technique, suggesting that it had a place in oncologic surgery (Galler et al., 2011). Surgeons were at this time pushing the limits of less radical and more sphincter-sparing procedures (Galler et al., 2011).

In the second part of 20th century anterior resection became the standard of care for middle and upper rectal tumors (Galler et al., 2011). In the 1970s, Alan Parks began restoring continuity following rectal cancer resection. Publishing his technique in 1982, ''peranal anastamosis'' of the colon and anus permitted lower rectal tumors to be completely excised without the need of a permanent colostomy (Low anterior resection) (Ruo & Guillem, 1999). Moreover, first reported in 1986, colonic J-pouch reconstruction showed short and long-term improvements over straight anastamoses with decreased frequency, urgency, and incontinence. Also, with improved perfusion of the J-pouch, fewer anastomotic leaks were seen (Galler et al., 2011). The first sophisticated stapling device was used in Budapest in 1908 by Humer Hultl for a gastrectomy. By 1977, the US Surgical Corporation reported successful use of the reusable end-to-end anastomosis (EEA) stapler (Moran, 1996). On the other hand, the 1980s brought renewed interest in circumferential margins. Previously, the pelvic dissection had been excised bluntly and total mesorectal excision (TME) relegated the radical abdominoperineal resection (APR) to a minority of patients, again revolutionizing rectal cancer surgery (Galler et al., 2011; Quirke, Durdey, Dixon, & Williams, 1986).

As recurrence rates decreased and disease-free survival increased, quality of life after rectal cancer surgery became more important. Japanese surgeons Drs. Tsuchiya, Hojo, and Moriya championed

‘‘autonomic nerve preservation’’, thus decreasing postoperative sexual and urinary dysfunction. The reproducible techniques reduced urogenital dysfunction from 50% to 10% (Galler et al., 2011). On the other hand warren Enker, an American surgeon, combined the Japanese nerve-preserving technique with TME, resulting in almost 90% preservation of urogenital function without compromising oncologic outcome (Lange et al., 2009).

3.4. Combined modality therapy

Since 1914, radiation has played an important role in treating locally advanced rectal cancer. Post-operative radiation therapy combined with chemotherapy was the standard of care for patients with locally advanced rectal cancer into the 1990s (Fisher et al., 1988). Besides, pelvic exclusion with an absorbable mesh sling emerged as a valuable technique to decrease the incidence of small bowel in the radiation treatment field after APR (Beitler et al., 1997).

Since that, several large trials have since shown the benefit of pre-operative radiotherapy combined with chemotherapy. The Dutch Colorectal Cancer Group prospectively compared pre-operative radiation therapy followed by TME to TME alone for rectal cancer. Though overall survival at two years was no different, local recurrence rates were respectively 2.4% and 8.2% for TME plus radiation versus TME alone (Kapiteijn et al., 2001). Moreover, the German Rectal Cancer Study Group compared pre-operative and post-operative chemoradiation in locally advanced rectal cancer. The pre-operative group displayed improved local control with less toxicity and without any survival difference (Sauer et al., 2004). Neoadjuvant chemoradiation treatment has improved sphincter preservation, and is now the standard of care (Galler et al., 2011).

Though the incidence of locoregional recurrence after primary resection has been drastically reduced with improved surgical technique and use of neoadjuvant therapy, failure rates were still significant and isolated anastomotic recurrence can be managed with re-resection. In some centers, intraoperative radiation has been shown to have improved local control (Wanebo, Gaker, Whitehill, Morgan, & Constable, 1987; Willett et al., 1989).

The future of rectal surgery is still emerging. Minimally invasive techniques with laparoscopic and robotic technologies are resulting in comparable outcomes to open procedures with decreased perioperative blood loss and shorter recovery times (Galler et al., 2011). Furthermore, less morbid procedures, especially for patients unfit for major surgery, have been investigated. Local excision (LE) utilizing a transanal approach has been promising. Patients with T1 or T2 lesions without

evidence of nodal involvement, low grade histology, less than 40% rectal wall circumference, and lesions less than 10 cm from the anal verge are optimal candidates (KAHLENBERG, ROUSSEAU JR, STRASSER, RABEN, & PETRELLI, 2007). With new techniques, such as transanal endoscopic microsurgery (TEMS), the trans-anal approach can be used for lesions up to 24 cm from the anal verge (Buess, Mentges, Manncke, Starlinger, & Becker, 1992). Still, without long-term follow-up data, surgery is the gold standard and the best chance for a cure for patients with rectal cancer (Galler et al., 2011). Once considered incurable, rectal cancer mortality has been reduced significantly in the last 250 years.

3.5. Chemotherapy

In early 1900, the German chemist Paul Ehrlich was the first person to use the term 'chemotherapy'. However, it can be said that the evolution of chemotherapy for CRC has begun with the development of 5-fluorouracil (5-FU) in 1957 by Charles Heidelberger. After the 5-fluorouracil, Oxaliplatin was discovered in Japan at Nagoya City University by Yoshinori Kidani in 1976. They tested the antitumor activity of various platinum (II) complexes of 1,2-diaminocyclohexane isomers (Gustavsson et al., 2015). After Oxaliplatin, Irinotecan was discovered and synthesized also in Japan by Yakult Honsha Ltd in 1983 (Gustavsson et al., 2015). On the other hand, in 1983 and 1984, John Mendelsohn, Gordon Sato and colleagues proposed epidermal growth factor receptor (EGFR) as a novel target for cancer therapy, based on observations that EGFR was frequently overexpressed in epithelial tumors and that monoclonal antibodies directed against EGFR inhibited the growth of cancer cells. The anti-EGFR monoclonal antibodies, cetuximab and panitumumab, were the first therapeutic agents targeted at a specific molecular pathology: EGFR-positive tumors expressing wild type Kirsten rat sarcoma viral oncogene homolog (KRAS) (Gustavsson et al., 2015) (figure 2).

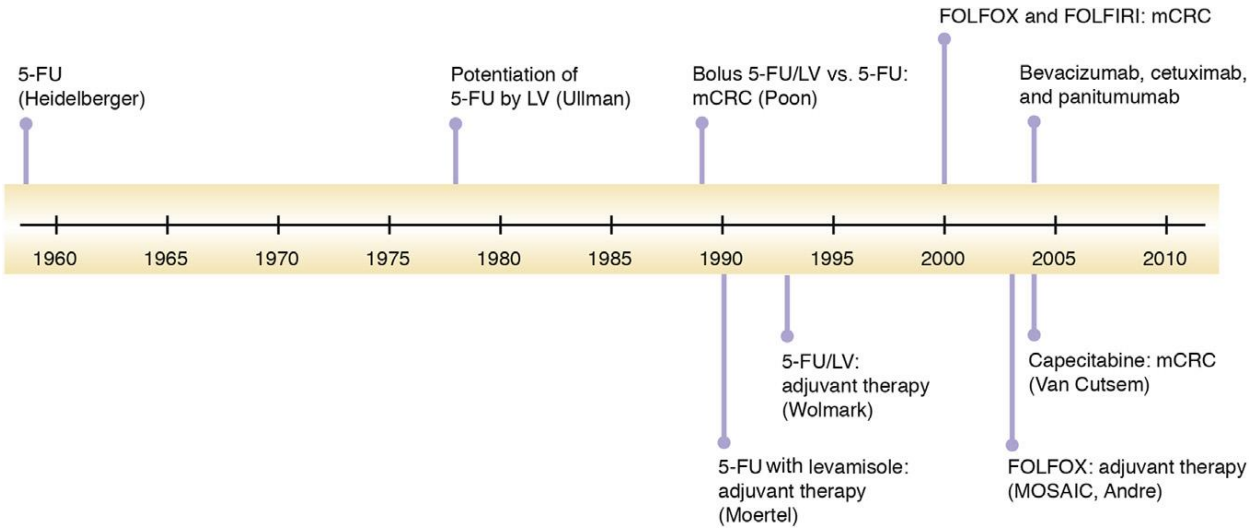


Figure 2: Flow chart of chemotherapy evolution (Gustavsson et al., 2015).

3.6. Adjuvant treatment of CRC

Investigators began to use combination chemotherapy in advanced breast cancer in the late 1960s with some encouraging results. Two programs were designed and field tested at the Clinical Center of the National Cancer Institute, L-phenylalanine mustard (L-PAM) used alone and the CMF program, a combination of cyclophosphamide, methotrexate, and 5 flurouracil, specifically designed for use as adjuvant chemotherapy (DeVita & Chu, 2008).

Within 5 years, both studies were complete and the L-PAM study was reported with too much fanfare when published in the New England Journal of Medicine in 1975, simultaneous with the announcement that the wives of the President, Betty Ford, and the Vice President, Happy Rockefeller, were diagnosed with breast cancer (Fisher et al., 1975) .The Bonadonna CMF study was published a year later. Both studies were positive, and the results set off a cascade of adjuvant studies in breast cancer and other tumor types, including colorectal cancer, with exciting results that have contributed to the significant decline in national mortality for breast and colorectal cancer, which witnessed later (DeVita & Chu, 2008).

Since development of 5-fluorouracil (5-FU) in 1957 by Charles Heidelberger and his colleagues at the University of Wisconsin, they started to work on trying to mitigate side effects without lowering the anti-tumor effect of the cytotoxic. They observed that tumor tissues preferentially used uracil for nucleic acid biosynthesis, and correctly postulated that a fluorouracil analogue would inhibit tumor cell division by blocking the conversion of deoxyuridine monophosphate

(dUMP) to deoxythymidine monophosphate (thymidylate). In 1978, two young scientists working for Heidelberger discovered that inhibition of thymidylate synthase by 5-FU could be potentiated by increased intracellular levels of reduced folates called leucovorin. By leucovirin with 5-FU the adverse events of 5-FU could be decreased and at the same time the tumor-reducing effect of 5-FU could be increased (Gustavsson et al., 2015).

On the other hand, in the 1970s and 1980s, the antihelminthic drug levamisole attracted interest as a possible chemotherapeutic agent because of its putative immunomodulatory activity. In 1989, the North Central Cancer Treatment Group (NCCTG) reported that treatment with levamisole and 5-FU led to a significant reduction in cancer recurrence and a significant increase in overall survival (OS) when compared with no adjuvant therapy. In 1990, Charles Moertel and colleagues published the results of their seminal study on the efficacy of 5-FU with levamisole versus no adjuvant therapy in patients with stage II or III CRC. 5-FU with levamisole reduced the risk of cancer recurrence by 41% and the overall death rate by 33% compared with 5-FU alone. Interestingly, treatment with levamisole alone had no effect. These findings led to the acceptance of 5-FU with levamisole as the standard adjuvant therapy in the 1990s (Gustavsson et al., 2015). However, clinical studies showed that adjuvant chemotherapy for CRC with 5-FU plus Leucovirin is significantly more effective than 5-FU plus Levomisole in reducing tumor relapse, improving survival and is less toxic (Arkenau, Bermann, Rettig, Strohmeyer, & Porschen, 2003; Tsavaris et al., 2004).

Another adjuvant chemotherapy was developed the IFL, a chemotherapy regimen for treatment of certain cancers, consisting of concurrent treatment with irinotecan, leucovorin, and fluorouracil (K. Chen, Gong, Zhang, Shen, & Zhou, 2016). The unfavorable toxicity profile of the IFL regimen led to the development of a regimen comprised of infusional IFL (FOLFIRI) (Gustavsson et al., 2015). The combination of infusional 5-FU/leucovorin (FOLFIRI), oxaliplatin, and irinotecan gives us FOLFOXIRI and is nowadays used as a treatment of advanced CRC (Nipp & Ryan, 2015). Studies reported that treatment with FOLFOXIRI has a significantly greater relative risk (RR) for patients than treatment with FOLIFIRI, but no significant differences was reported in overall survival (OS) (Falcone et al., 2007; Souglakos et al., 2006).

One of the key developments in the early 2000s included the introduction of the topoisomerase I inhibitor irinotecan and the platinum containing agent oxaliplatin as components of a cytotoxic combination therapy for metastatic CRC (Gustavsson et al., 2015).

Hybridomas were also described in 1975, and monoclonal antibodies were proven as clinically useful starting in the mid- 1990s. Although they are not chemotherapy per se, they seem to work best when they are used in conjunction with chemotherapy, as is the case for trastuzumab in breast cancer, cetuximab and bevacizumab in colorectal cancer, and rituximab in non–Hodgkin's lymphoma (DeVita & Chu, 2008).

Bevacizumab (RhumabVEGF), the first angiogenesis inhibitor that targets vascular endothelial growth factor (VEGF), was approved by the US Food and Drug Administration (FDA) in February 2004 for use as part of combination therapy with fluorouracil based regimens for metastatic colorectal cancer (mCRC) (Shih & Lindley, 2006). Clinical data have shown that bevacizumab improved the survival rate of patients with mCRC, when combined with different fluorouracil regimens (infusions and bolus), such as irinotecan, bolus followed by infusional5-fluorouracil, and leucovorin (FOLFIRI) and irinotecan, bolus 5-fluorouracil, leucovorin (IFL) (K. Chen et al., 2016) (Figure 2).

In the context of chemotherapy using a combination of different pharmacological compounds, Rapamycin has been suggested.

3.7. Rapamycin

Rapamycin (also known as Sirolimus and later marketed under the trade name Rapamune by Pfizer) is a macrocyclic lactone isolated from Streptomyces hygroscopicus, a bacterium extracted from a soil sample on Easter Island (known as 'Rapa-Nui')(S. N. Sehgal, Baker, & Vezina, 1975). This natural antibiotic was subsequently isolated in Montreal by Ayerst Research laboratories in 1972. It is a white crystalline solid insoluble in aqueous solutions, but soluble in organic solvents. Rapamycin was initially developed as an anti-fungal drug directed against Candida albicans, Cryptococcus neoformans and Aspergillus fumigatus(Vignot, Faivre, Aguirre, & Raymond, 2005). It is currently used alone or in combination with cyclosporine as an immunosuppressive drug to prevent renal graft rejection. However, the development program of

rapamycin as an anticancer agent was halted in 1982 and only resumed in 1988 after demonstration of a safe toxicological profile in animals (Vignot et al., 2005).

In 2005, rapamycin has been tested by the Developmental Therapeutic Branch, National Cancer Institute (NCI) and identified as a non-cytotoxic agent that delays tumor proliferation, finding evidence of a cytostatic activity against several human cancers in vitro and in vivo.

There are observations indicating that high doses of rapamycin block the proliferative responses to cytokines by vascular and smooth muscle cells after mechanical injury, such as balloon angioplasty or allo-rejection (Ochiai et al., 1993; Qi et al., 2000). IC_{50} values of rapamycin as an immunosuppressor are in the range of 0.1–300 nM (Vignot et al., 2005). Over the last decade, rapamycin has undergone clinical trials as an immunosuppressive agent, progressing from phase I to phase II and the completion of phase III trials which led to approval of rapamycin by the Food and Drug Administration (FDA) of the USA to prevent acute rejection in combination with cyclosporin and steroids. At the same time, the drug was approved by the European Agency as an alternative to calcineurin antagonists for long-term maintenance therapy to avoid graft rejection. Interestingly, rapamycin, unlike cyclosporin, does not seem to increase the risk of malignancy but rather to decrease the risk of post-transplant lymphoproliferative disorders (Ashrafi, Shahidi, Ebrahimi, & Mortazavi, 2015). Apart from its immunosuppressive capability, rapamycin was also recently shown to be able of preventing coronary artery re-stenosis (Gallo et al., 1999).

Rapamycin has a complex mechanism of action, it binds FKBP-12 (FK506 binding protein), and the rapamycin–FKBP12 complex can inhibit mTOR, thus preventing further phosphorylation of P70S6K, 4E-BP1 and, indirectly, other proteins involved in transcription and translation and cell cycle control ("Global guideline for type 2 diabetes," 2014; Humar, KIEFER, BERNS, RESINK, & BATTEGAY, 2002; Vezina, Kudelski, & Sehgal, 1975). Rapamycin is currently used alone or in combination with cyclosporine as an immunosuppressive drug to prevent renal graft rejection (Vignot et al., 2005).

Rapamycin inhibits T-cell proliferation induced by antigen, mitogenic lectins, alloantigen and crosslinking of T-cell surface markers with monoclonal antibodies. It also inhibits the proliferative responses induced by cytokines, including IL-1, IL-2, IL-3, IL-4 and IL-6, IGF, platelet derived growth factor (PDGF) and Colony-Stimulating Factors (CSFs) (Humar et al., 2002; S. Sehgal, 2003).

The systemic bio-availability of rapamycin is approximately 15%; it has a maximal concentration at about 1 h and is widely distributed in tissues when compared with plasma. More than 90% of the drug is recovered in the feces. On the other hand, urine represents only 2% of the drug elimination. The average elimination half-life is variable, ranging from 10 h in children to 110 h in patients with hepatic impairment(S. Sehgal, 2003).

3.7.1. Rapamycin as an anticancer drug

Rapamycin was also shown to inhibit the growth of several murine and human cancer cell lines in a concentration-dependent manner, both in tissue culture and xenograft models: B16 melanoma, P388 leukemia, MiaPaCa-2 and Panc-1 human pancreatic carcinomas (Hosoi et al., 1999). It also enhances the apoptosis induced in vitro by cisplatin in murine T-cell and human HL-60 promyelocytic leukemias and human ovarian SKOV3 carcinoma(Shi et al., 1995). Rapamycin inhibits the oncogenic transformation of human cells induced by either PI3K or AKT and has shown metastatic tumour growth inhibition and anti-angiogenic effect in in vivo mouse models (Humar et al., 2002). Based on these pre-clinical results, studies with rapamycin as an anticancer drug begun and rapamycin analogues were developed with more favorable pharmaceutical properties (Humar et al., 2002; S. Sehgal, 2003).

Following activation of membrane receptors by a variety of growth factors, secondary molecular signals are generated to transmit the stimulus toward the nucleus and activate a number of events. Many of these signals involve the phosphorylation of proteins known as kinases. Among those kinases, PI3K and PI3K-related kinases (PIKK) belong to a family of high molecular mass kinases whose catalytic domains show a strong resemblance. This family and the ribosomal protein P70S6K, mTOR, and key molecules involved in checkpoint regulation of cell cycle, DNA repair, telomere length and cell death (S. Sehgal, 2003).

3.7.2. Rapamycin and Mammalian related protein

mTOR is a serine/threonine kinase of 289 kDa, highly related to yeast TORs that belong to the PIKK family with a dual regulation by amino acid availability and by mitogen activated

PI3K/AKT. TOR proteins in Sacharomyces cerevisae and the mammalian related proteins (mTOR) are required for signaling translational initiation and therefore cell cycle progression from the G0/G1 to S phase (Wiederrecht et al., 1995).

In humans, mTOR primarily appears to be a nutrient-sensing protein: mTOR is constitutively activated in the presence of growth factor and nutrients and acts as a master switch of cellular catabolism and anabolism .Moreover, mTOR is found to be regulated by hypoxia and by AMP levels. Upregulation of mTOR can be associated to loss of the tumor suppressor gene PTEN and activation of AKT (Shaw & Cantley, 2006; Wiederrecht et al., 1995).

The mTOR kinase is an integrator of growth-factor and nutrient signals. Growth-factor signaling through Ras–ERK and PI(3)K–AKT activates mTORC1, whereas low nutrient availability (for example, low glucose or hypoxia) inhibits mTORC1, in part through the AMP-activated protein kinase pathway.

Upstream components of the raptor–mTORC1 pathway were initially discovered through classical cancer genetics. Signals that inhibit the tumor suppressor TSC2, and thus activate mTORC1, include the kinases ERK, RSK and AKT, all of which directly phosphorylate TSC2 in vivo. AKT directly phosphorylates TSC2 on a number of sites, several of which are conserved between mammals and Drosophila, although the requirement of these sites for AKT-mediated regulation of mTOR remains an area of vigorous investigation. Conversely, AMPK phosphorylation of TSC2 activates its ability to inhibit mTORC1, but the underlying mechanism is unknown. Furthermore, each of these kinases may have additional substrates in the mTORC1 pathway, and the relative importance of each of the conserved TSC2 phosphorylation sites is being investigated at present. Finally, AKT has been reported to crosstalk and inhibit AMPK, leading to further stimulating mTOR activation.

Moreover, mTORC1-dependent translation is known to control a number of specific cell-growth regulators, including the HIF-1α (hypoxia-inducible factor-1α) transcription factor, which in turn drive diverse processes including cell growth, glycolysis and angiogenesis, all contributing to enhanced tumorigenesis(Shaw & Cantley, 2006).

3.7.3. Inhibition of mTORC1 by the AMPK pathway

In addition to growth-factor-mediated stimulation, mTORC1 activity depends on the availability of nutrients such as glucose, oxygen and amino acids. Recently, a number of proteins that regulate mTOR in response to nutrient availability have been discovered. When intracellular ATP levels drop and AMP levels rise, such as under conditions of hypoxia or glucose deprivation, AMP directly binds a subunit of AMPK, causing a conformational shift that exposes the activation-loop threonine, which is then phosphorylated by LKB1. These findings suggest that the central role of AMPK in the inhibition of mTOR under normal physiological conditions has been underestimated because tissue-culture cells are grown in conditions of supraphysiological levels of glucose, oxygen and growth factors.

AMPK inhibits mTOR at least in part by the direct phosphorylation of tuberin and cells that lack tuberin retain activated mTORC1 under conditions of low glucose as well as hypoxia(Shaw & Cantley, 2006).

3.8. Use of probiotics in treatment of CRC

Probiotics are defined as microorganisms which provide, when consumed, many health benefits that are strain-specific. The concept of using probiotics to treat health conditions was first introduced by the scientist and Nobel Laureate Elie Metchinkoff. He stated in 1907 that: "the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace harmful microbes by useful microbes"(Metchnikoff, 2004).

The mechanism of action of probiotics in the hosting intestines is not yet clearly understood and in a simplified way, four propositions have been suggested, see figure 3.

1. Competition for nutrients: Probiotics may be competing with pathogens for the same essential nutrients, therefore, making less food available for the pathogen to use.
2. Blocking of adhesion sites: By binding to adhesion sites, probiotics reduce pathogen colonization by preventing pathogens attachment.
3. Immune stimulation: Probiotics can trigger an immune response against the pathogens leading to their destruction.
4. Direct antagonism: Probiotics can release bacteriocins which kill the pathogens directly(Bermudez-Brito, Plaza-Díaz, Muñoz-Quezada, Gómez-Llorente, & Gil, 2012). Figure 3 best resumes how probiotics work.

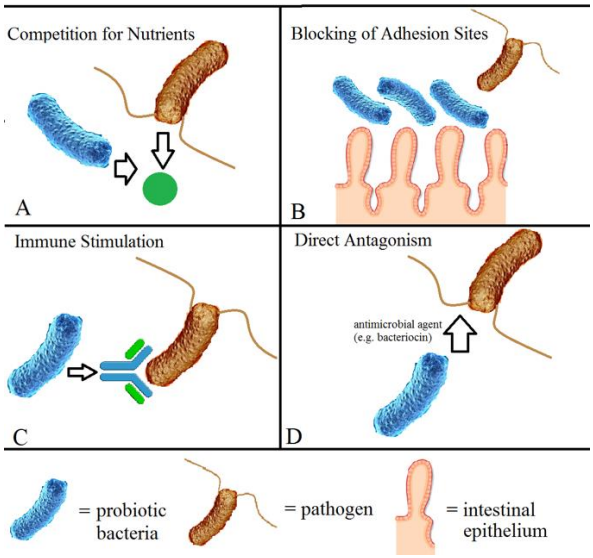


Figure 3: Mechanisms of action of probiotics (Vijayaram & Kannan, 2018).

Probiotics should be administered in adequate numbers. The dose given has to be able to trigger the targeted effect on the host. Usually an intake of around 10^7 to 10^8 probiotic cell/gram with a serving size close to 100 to 200mg per day is considered as the optimal dose (Rijkers et al., 2010). The most wildly used probiotics are: lactobacillus acidophilus, Lactobacillus rhamnosus GG, *Saccharomyces boulardii*, *Bifidobacterium bifidum* and *Bacillus coagulans* (Fijan, 2014). Live probiotic cultures are found in fermented dairy products such as yogurt and also in some probiotic fortified food. Tablets and capsules as well as powders and sachets containing the bacteria in freeze-dried form are also available to acquire (Islam, Yun, Choi, & Cho, 2010).

The oral intake of prebiotics, probiotics, and symbiotics has been shown to reduce intestinal inflammation and promote immune response by altering the intestinal microflora composition and competition. An increased infection risk may be the result of immunosuppression caused by chemotherapy or the disease process. In addition, postoperative infections in patients undergoing colorectal cancer surgery may play a pertinent role in the overall outcomes because an infection may delay additional cancer treatment, affect overall prognosis and increase the risk of morbidity and mortality (Daniluk, 2018). Kotzampassi et al. reported similar findings in a 2015 study in which they randomized 164 patients undergoing colorectal cancer surgery to one of two groups: a combination of four probiotics and preoperative mechanical bowel preparation (MBP) or a placebo and MBP. The rate of any infectious complications (i.e., pneumonia, SSI, urinary tract infection,

bacteremia, severe sepsis) was significantly lower in the probiotic group (11.9%) compared with the control group (28.7%) (P = .009). It was noted that the overall rates of infectious complications were lower in the probiotic group, but only pneumonia and surgical site *infections* (SSI) were significantly lower (P = .029 and .02, respectively)(Kotzampassi et al., 2015). However, the incidence of wound infections (3.33%) and urinary tract infections (6.67%) were the same in both the probiotic group and the placebo group (Daniluk, 2018). In addition, Sadahiro et al. compared the use of preoperative oral antibiotics with the use of a single-agent probiotic in 294 participants. The overall rates of postoperative infection (ie, incisional SSI, organ or body space SSI, remote infection) were lower in the antibiotic group (11.1%) as compared with the probiotic group (24%) and the control group (25.3%) that received the standard of care (Sadahiro et al., 2014).

It is well documented that *Lactobacillus rhamnosus* or *Bifidobacterium lactis* or commensal bacteria–*Escherichia coli* and *Atopobium minutum* can induce apoptosis in human colonic carcinoma cells (Caco-2) (Altonsy, Andrews, & Tuohy, 2010). In addition, *Lactobacillus acidophilus* and *Lactobacillus casei* were able to increase the apoptosis-induction capacity of 5-fluorouracil in colorectal carcinoma cell line LS513, suggesting that these probiotics may be used as adjuvants in anticancer chemotherapy (Baldwin et al., 2010).

Moreover, *Propionibacterium freudenreichii* was shown to induce cell death of different human colon and gastric cancer cell lines through secretion of short-chain fatty acids to culture media (Daniluk, 2018). However, it's worth mentioning that the intraoperative intestinal cleanliness is important. Findings implied that perioperative probiotics treatment could likely be of tremendous clinical benefit as a supplement during bowel preparation in patients prepared for confined CRC surgery (Daniluk, 2018). Yang et al. showed no significant difference between the placebo *vs.* probiotics groups in terms of days to first fluid, days to first solid diet, duration of pyrexia, average heart rate in a week after surgery, length of intraperitoneal drainage, length of antibiotic therapy, and postoperative hospital stay . However, the days to first flatus and the days to first defecation were significantly improved in the probiotics group (Yang et al., 2014). These findings suggest that the probiotics treatment improves recovery of bowel function for patients with CRC surgery. Moreover, the incidence of diarrhea was significantly lower in the probiotics group compared to the placebo group, whereas other non-infectious complications including anastomotic leakage, and abdominal distension were essentially quite comparable. In addition, the incidence of bacteremia was slightly lower in the probiotics group than in the placebo group; however, the difference didn't

reach statistical significance. These findings showed evidence that perioperative probiotic administration may help those patients undergoing confined CRC resection surgery in obtaining short-term clinical benefit considering faster recovery of bowel function, lower incidences of diarrhea, and slightly lower rate of bacteremia (Yang et al., 2016).

Roller *et al.* demonstrated that symbiotic treatment prevented azoxymethane-induced suppression of NK-cell activity in Peyer's patches, an effect not observed in the individual pro- and prebiotic treatments. These studies suggest that symbiotics may have a role in CRC treatment (Roller, Pietro Femia, Caderni, Rechkemmer, & Watzl, 2004).

Inulin-type fructans present in foods such as garlic, onion, artichoke and asparagus have been demonstrated to elevate the levels of bifidobacteria and to increase SCFA concentrations in the intestinal lumen. Inulin and oligofructose have been demonstrated to reduce the severity of 1, 2-dimethylhydrazine induced colon cancer in rats. A further study, by Bauer-Marinovic et al demonstrated the capacity for the prebiotic resistant starch type-3 Novelose 330 to reduce the incidence of colon carcinogenesis *via* induced apoptosis of damaged cells in rats. This effect was attributed to the increased production of butyrate (Bauer-Marinovic, Florian, Muller-Schmehl, Glatt, & Jacobasch, 2006).

In another study by Fotiadis and coworkers reported the consumption of modified arabinoxylan rice bran was able to enhance the activity of NK cells and the binding of NK cells to tumor cells. This demonstrates the ability of prebiotics to enhance the hosts' immune response (Fotiadis, Stoidis, Spyropoulos, & Zografos, 2008).

Further studies have revealed a positive correlation between CRC and certain commensal bacteria, including specific E. coli types, enterotoxigenic Bacteroides fragilis and Streptococcus bovis. Therefore, dietary components such as probiotics, prebiotics, or combination of both (symbiotics), may protect against CRC partly by preventing intestinal dysbiosis (changes in the normal microbiota). On the contrary, obesity could increase the risk of CRC, possibly by causing an imbalance of the intestinal microbiota. Specifically, a decrease in bifidobacteria and an increase in Firmicutes have been associated with obesity. Moreover, age is another risk factor for CRC. A large cohort study, involving 35,292 adults aged 18–96 years, reported that bifidobacteria significantly decreased, while E. coli and enterococci increased with age (Enck et al., 2009).

Certain microorganisms are responsible for inducing and maintaining the inflammatory disease. For examples, microorganisms including B. fragilis, Citrobacter rodentium, adherent E. coli and

Clostridium difficile have been shown to disrupt intestinal barrier, and subsequently inducing IBD and CRC in some cases(Chong, 2014; Hussein et al., 2008) Probiotics may reduce the risk of CRC by competitive exclusion of pathogenic bacteria involved in carcinogenesis. Animal studies have shown that ingestion of lactobacilli and bifidobacteria are able to increase lactic acid bacteria and reduce the faecal putrefactive microorganisms (e.g. coliforms) that have been implicated with synthesis of putative carcinogens in the colon (Chong, 2014).

The chemopreventive role of probiotics for CRC is also supported by their ability to reduce intestinal microflora enzyme activity. The human body detoxifies foreign compounds and drugs via hepatic synthesis of glucuronides prior to their entering into the intestines. A bacterial enzyme, b-glucuronidase, with broad substrate specificity can hydrolyze a number of glucuronides, causing the release of carcinogens into the colon, including PAH (e.g. benzo[a]pyrene), an important risk determinant for CRC (Rafter, 2003).

Combination of prebiotic (resistant starch, RS) and *Bifidobacterium lactis* significantly facilitated the apoptotic response to a genotoxic carcinogen in the distal colon of rats in a short time after carcinogen exposure. Moreover, it was reported that combination of RS and *B. lactis* significantly protects against the development of colorectal cancer in the rat (Daniluk, 2018).

Studies also showed that probiotics could prevent CRC by inhibiting DNA damage. In a recent clinical study, genotoxicity of fecal water from atopic patients, measured using comet assay, was found be to higher than in healthy subjects, indicating a higher risk for CRC. A dietary intervention trial demonstrated daily consumption of 300 g probiotic yogurt (containing L. acidophilus 145 and B. longum 913) for 6 weeks resulted in reduced fecal water-induced genotoxicity in human colon cancer cells HT29clone19A, i.e. less DNA strand break-inducing agents in feces (Chong, 2014).

In addition, probiotics may also reduce the risk of CRC by suppressing the promotion phase of CRC by two key mechanisms: (1) preventing formation of aberrant crypt foci (ACF) and (2) improving colonic barrier functions. Aberrant crypt foci are recognized as precursors of colorectal adenomas. ACF are characterized by hyperproliferation and lack of cell differentiation. CRC patients were found to have more ACF compared to patients with non-malignant lesions, and the majority of the ACF had K-Ras mutation, one of the key genetic events in colonic carcinogenesis. K-Ras mutations were linked to increased expression and activity of DNA methyltransferase, cyclin D1 and gastrin, all of which were involved in etiology of CRC. An assessment found that

as the number of ACF increased, the risk of a patient having colonic advanced neoplasms increased (Chong, 2014).

The delay in the onset of Colorectal Cancer in patients and these findings could be explained by the following points:

- Modifying the composition of the intestinal microflora thus favoring the presence of the "good" bacteria.
- Inactivation of oncogenic and mutagenic compounds.
- Competition with pathogenic and putrefactive microbiota.
- enhancement of the host's immune response
- Anti-proliferative effects through regulation of apoptosis and cell differentiation.
- Fermentation of undigested food.
- Inhibition of tyrosine kinase signaling pathways (Uccello et al., 2012).

Probiotics have also been linked to improving clinical signs and symptoms of Type 2 Diabetes. Studies have shown that one of the features common to metabolic diseases such as T2D is a mild chronic inflammatory state and probiotics have proven to reduce oxidative stress and inflammation (Gomes, Bueno, de Souza, & Mota, 2014).

The table below (table2) shows the positive effects of administering probiotics on diabetic patients (Gomes et al., 2014). The human intestinal microbiota presents a vast set of antigens which may participate in the modulation of immunological diseases (Gomes et al., 2014). An intestinal barrier presenting full integrity ensures specific interactions between the luminal antigens and the host. Functional disruption of this barrier such as an increase in permeability may contribute to an increased expression of inflammatory cytokines which may lead to insulin resistance and T2D (Gomes et al., 2014). Although their beneficial effect on diabetes has been proven in experimental and clinical research, the molecular mechanism on how probiotics delay the onset of type 2 diabetes and improve its clinical symptoms are not yet fully understood. No doubt that promoting the growth of the good bacteria, strengthening the gut's immunity and lowering inflammation are all contributing factors to the positive effects of probiotics on chronic diseases but the molecular mechanism behind their action is not yet clearly elucidated. On this basis, the novel trend in the management of CRC in diabetics is to use combination therapy including Rapamycin and probiotics, which forms a main objective of this study.

References	Probiotic	Study design/subjects	Sample Size	Quantity	Study period	Results
[8]	*Lactobacillus acidophilus* and *Bifidobacterium bifidum*	Double-blinded, placebo-controlled, randomized study, T2D females aged 50–65 years	Placebo group: n = 10; Probiotic group: n = 10	2 daily doses of 100 mL symbiotic shake containing 4×10^8 CFU/100 mL *Lactobacillus acidophillus*, 4×10^8 CFU/100 mL *Bifidobacterium bifidum*	45 days	↓ Glycemia
[9]	*Lactobacillus acidophilus* La5 and *Bifidobacterium lactis* Bb12	Double-blinded, randomized controlled clinical trial, T2D patients aged 30–60 years	Placebo group: n = 32; Probiotic group: n = 32	300 g/day of probiotic and conventional yogurt day 1: $7{,}23 \times 10^6$ of *L. acidophilus* La5 and 6.04×10^6 cfu/g of *B. lactis* Bb12	6 weeks	↓ Fasting blood glucose and HbA1c ↑ Erythrocyte SOD and GPx ↑ Total antioxidant capacity
[10]	*L. acidophilus* NCFM	Double-blinded, placebo-controlled, randomized study, T2D males	Placebo group: n = 24; Probiotic group: n = 24	-	4 weeks	Preserved insulin sensitivity No effect on systemic inflammatory response
[117]	*Lactobacillus rhamnosus* GG (ATCC 53 103) and *Bifidobacterium lactis* Bb12	Prospective, randomized study, mother–baby pairs	Dietetic Intervention + probiotics: n = 85; Dietetic Intervention + placebo: n = 86; Control + placebo: n = 85	Lactobacillus rhamnosus GG: 10^{10} CFU/day; Bifidobacterium lactis Bb12: 10^{10} CFU/day	33 months	↓ Risk of GDM
[118]	*Lactobacillus rhamnosus* GG, ATCC 53 103 and *Bifidobacterium lactis* Bb12	Randomized, prospective, parallel-group, combined dietary counselling, pregnant women	Diet + probiotics: n = 85; Diet + placebo: n = 86; Control + placebo: n = 85	Lactobacillus rhamnosus: 10^{10} CFU/day; Bifidobacterium lactis Bb12: 10^{10} CFU/day	18 months	↓ Blood glucose ↓ Insulin ↓ Insulin sensitivity
[119]	*L. plantarum* WCFS1	Double-blinded, randomized crossover study, healthy subjects	n = 14	10^{12} CFU	6 hours	↓ Degradation of transepithelial electrical resistance ↑ ZO-1 in tight junctions

Table 2: Effects of probiotic administration on diabetes mellitus –clinical studies (Gomes et al., 2014).

4. Hypothesis

Based on the documented co-occurrence of DM and CRC and the potential interaction of their different signaling pathways, we are hypothesizing that the combination therapy of metformin and rapamycin will prevent or delay the progression of colorectal cancer in Type 2 diabetic patients.
On this basis, we aimed to determine the role of Metformin and Rapamycin alone and in combination in the management of diabetes and colorectal cancer in an ectopic xenograft mouse model, at clinical, histological and molecular levels, with an emphasis on the downstream signaling elicited by these drugs. Moreover, this study aims also to shed light on the possible role of probiotics in this crosstalk since dismicrobism and gut microbiota have been reported to affect the outcome.

Knowing the fact that tumor development and burden are increased in diabetic patients due to a disruption in several signaling pathways, in this study, our aims targeted the following levels:

1- Reverse the inactivation of AMPK by activating AMPK through **Metformin.**
2- Block the activation of mTORC1 by giving **Rapamycin**
3- Inhibit the inflammatory process (TNFα, IL-3 and IL-6) by adding **probiotics**.

Chapter Two: **MATERIALS AND METHODS**

1. Animals

Fifty male NOD/SCIDs mice 6-8 weeks old, weighing 25–30 g, were housed in Individually Ventilated Cages (IVC) at the transgenic unit of the Animal Care Facility of the American

University of Beirut, in a controlled temperature (21°C±2°C) and humidity, with an alternating 12-hour light/dark cycle. Standard Laboratory pellet formula and tap water were provided ad libitum. All animal treatments adhered strictly to institutional and international ethical guidelines of the care and use of laboratory animals. The experimental protocol was approved by the Institutional Animal Care and Use Committee, American University of Beirut, Lebanon.

2. Experimental design

The animals were divided into 2 main groups, (1) diabetic and (2) non-diabetic. They were all subject to a subcutaneous injection of 3×10^6 HCT116 cells suspended in 200 µl normal physiological saline, in the flank which produced xenograft tumors after 9 days.

Diabetes was induced using Streptozocin (STZ) (S0130-50MG-Sigma Aldrich), a N-nitroso-containing compound that acts as a nitric oxide donor in the pancreatic islets of Langerhans; induces death of insulin-secreting cells, and thus producing an animal model of diabetes. Two Streptozotocin intra-peritoneal injections at day 1 and 8 were able to induce diabetes (glycemia >150 mg/dl).

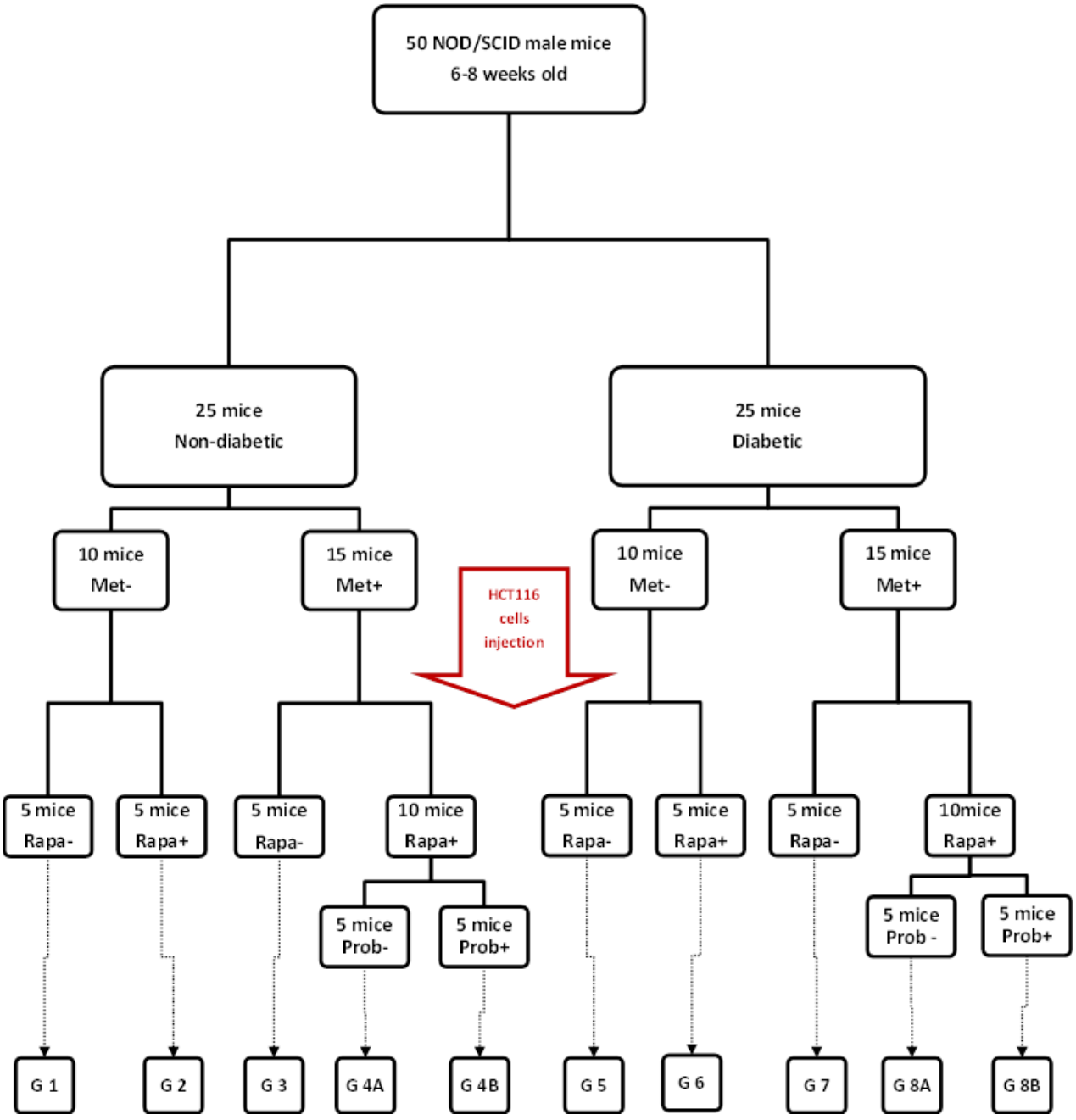


Figure 4: Schematic presentation of the various groups (G's) in the experiment.

For metformin (Glucophage) treatment, it was dissolved in drinking water to attain the dosage of 150 mg/kg body weight. The water was changed daily and measured for water intake. Metformin daily treatment was initiated 7 days before inoculation of the tumor cells and was continued until sacrifice.

As for rapamycin, it was purchased from sigma (37094-10mg) and stored at -20° C; diluted with DMSO and administered via 100 µl i.p injections (3 injections per week) at a dose of 0.5 mg/kg. The first injection of rapamycin was administered to the respective groups, 1 week after the onset of tumors, i.e. when tumor size reached $50mm^3$.

Probiotics (Probiolife®), a symbiotic mixture, combining the most studied strains of probiotics such as lactobacillus rhamnosus, Saccharomyces boulardii, Bifidobacterium breve, bifidobacterium lactis, lactobacillus acidophilus, lactobacillus plantarum, lactobacillus reuteri, in addition to prebiotics and zinc, were administered to mice in their drinking water 2 weeks before sacrifice. One capsule was dissolved in 1.75 L of autoclaved tap water with a concentration of 10^8 CFU/ml. Fresh solution was given to the animals every 2 days.

Each of the 2 main groups of 25 mice was subdivided into two subgroups, 15 mice treated with metformin, 150 mg/kg body weight administered in drinking water, and 10 mice not treated. A total of 10 groups of 5 animals each were reached: group 1 received no treatment and was considered as control; group 2 received rapamycin only; group 3 was treated with metformin alone; group 4A was treated with both metformin and rapamycin; and group 4B received Probiotics in addition to metformin and rapamycin. On the other hand, the diabetic mice, treated with STZ were divided similarly: group 5 received nothing; group 6 rapamycin; group 7 metformin; group 8A metformin and rapamycin; and lastly group 8B received probiotics in addition to metformin and rapamycin.

3. Monitoring

Mice were monitored for glycemia pre and post STZ injections and weekly afterwards. Weight changes (weight loss), stool aspect (loose or bloody), and fur shape and activity were daily checked. Mice were also regularly monitored for any signs of discomfort. All animal experiments were performed according to the American University of Beirut's Institutional Animal Care and Use Committee (IACUC) guidelines. Tumors were measured once a week with a caliper square and tumor volumes were calculated using the formula: *tumor volume=length×width×width/2.* In addition, Mice were monitored daily and checked for any signs of sickness, water intake, stool consistency, and bleeding. The scores were recorded to calculate disease activity index (DAI) based on a scale of zero to 4 for any parameter; normal status should remain as zero and highest activity as 9 (Hussein et al., 2008) (table 3).

Table 3 Criteria for scoring the Disease Activity Index (DAI)

Score	Weight loss (%)	Stool consistency[2]	Rectal bleeding
0	None	Normal	Negative
1	1-5		
2	6-10	Loose	Gross bleeding
3	11-15		Gross bleeding >1d
4	>15	Diarrhea	Gross bleeding >2d

1 Disease activity index (DAI) =combined score of weight loss, stool consistency;and bleeding/3

2 Normal stool=well formed pellets; loose=pasty stools that does not stick to the anus; diarhhea=liquid stool that stick to the anus and fur.

4. Sacrifice

Dissection and tumor excision were done when tumor size reached $1cm^3$. The animals were anesthetized by an overdose of Forane (Isoflurane), the abdominal cavity was exposed was exposed and a macroscopic assessment of the inflammatory status was performed according to an already published scale (Hussein et al., 2008).

Biopsies of the descending colon (DC), small intestine, liver and kidneys were collected. The tissues obtained were either transferred into labeled aliquots, snap-frozen in liquid nitrogen, and kept at -80°C for further molecular analysis or were kept in 10% formaldehyde to be processed

with paraffin for routine light microscopy and histology analysis according to previously reported procedures(Hussein et al., 2008).

5. Real time RT-PCR

The total RNA of the tissues was extracted using an RNeasy mini-kit (Qiagen Ltd., Crawley, United Kingdom). RNA quantity and purity were assessed using NanoDrop ND-1000 spectrophotometer (Wilmington, NC). M-MLV Reverse Transcriptase buffer pack (Promega, Lyon, France) was used for reverse transcription. Primers were designed for the determination of the following gene expression: mTORC1, AMPK, IL-6, IL-3, and TNFα. GAPDH was used as an internal control. The amplification was monitored with StepOnePlus PCR System (AB Applied Biosystems, Villebon-sur-Yvette, France) using GoTaq qPCR Master Mix (Promega, Charbonnieres Les Bains, France) according to manufacturer's instructions. Samples were run in triplicate, relative abundance of each target was normalized to GAPDH expression and gene regulation was determined by the quantitation-comparative ΔΔCT method (M. T. J. Johnson, 2012).

6. Western Blot

Protein extraction and quantification were performed using previously established protocols (Hussein et al., 2008). The extracted proteins were separated by gel electrophoresis and were transferred onto nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline and probed with primary antibodies specific for phospho-mTOR, and mTOR (all from Cell Signaling Technology) and GAPDH. Horseradish peroxidase-conjugated secondary antibodies and the ECL detection kit (Bio-Rad) were used for the detection of specific proteins. Bands were quantified and normalized to the signal generated from GAPDH.

7. ROS detection by DHE staining

Frozen sections, from frozen tissue stored at (-80), were prepared. The tissue was demarcated with a solvent resistant pen. DHE solution was prepared and dispensed over the tissue and the slides

placed for 30 min at 37°C. Then the DHE residues were removed, slides counterstained with DAPI, colversliped and stored at 4°C (light sensitive) until microscopic evaluation and quantification using Zen software. One way ANOVA: to compare between all of the groups and T-test: to compare between two groups were done (Barry-Lane et al., 2001).

8. Histology

Tissue preparation for light microscopy was performed according to routine procedures and protocols already established in the laboratory (Hussein et al., 2008). The histological alterations were assessed using a previously published scale (Hussein et al., 2008). Fields at 200 x magnification were photographed, evaluated and scored by 2 independent researchers. The scores of two independent observers were averaged. The histological grades (from "0 to 21") indicating the numerical sum of scoring criteria were divided by 7 (the number of Criteria), averaged to obtain a maximum average of 3, computed and represented with matching standard error of the mean (Hussein et al., 2008).

Table 4: Criteria for histologic assessment of inflammatory reaction

Severity of Changes				
Structural Change	0	1	2	3
Mucosal architecture	Normal	Focal surface destruction	Zonal surface destruction	Diffuse destruction
Glandular crypt architecture	Absent	Mild atrophy	Atrophy + Branching	Atrophy + Branching + Crypt abscess
Loss of Goblet cells	Absent	Mild	Moderate	Extensive
Edema	Absent	Mild	Moderate	Extensive
Crypt abscesses	Absent	Focal	Zonal	Extensive
Inflammatory cells infiltration	Absent	Mild (only Mucosa)	Moderate(to muscularis mucosa)	Extensive (to submucosa and musculosa)
Dysplasia	Absent	Focal	Zonal	Diffuse

9. Mast cells count

The evaluation of mast cell count was performed by two different observers according to previously reported criteria on slides stained with Toluidine Blue (TB) (Hussein et al., 2008).

10. Statistical analysis

Statistics were conducted using the analysis of t-test and ANOVA to compare each experimental group to the corresponding controls using the STAT3 software. Significance was determined as probability (p) <0.05.

Chapter Three: **RESULTS**

1. Clinical Profile and weight changes

Mice in group 1 (G1) (Controls, non-treated, having the HCT116 cells xenograft) had the worst clinical profile. Two mice had diarrhea and rectal bleeding as well as weakness and low alertness. In addition, one mouse died 2 weeks before the sacrifice time.

On the other hand, groups treated with metformin, with or without Rapamycin, had a better clinical profile when compared to the non-treated ones in; however, there were no significant changes in stools, activity and alertness. Besides, animals treated with Probiotics in addition to rapamycin and metformin had the best clinical profile. However, there was a trend of decreased body weight in non-treated mice with xenografts G1, but the variations were not significant. All mice in the other group showed a gradual increase in body weight without any significant differences (figure 5, table 5).

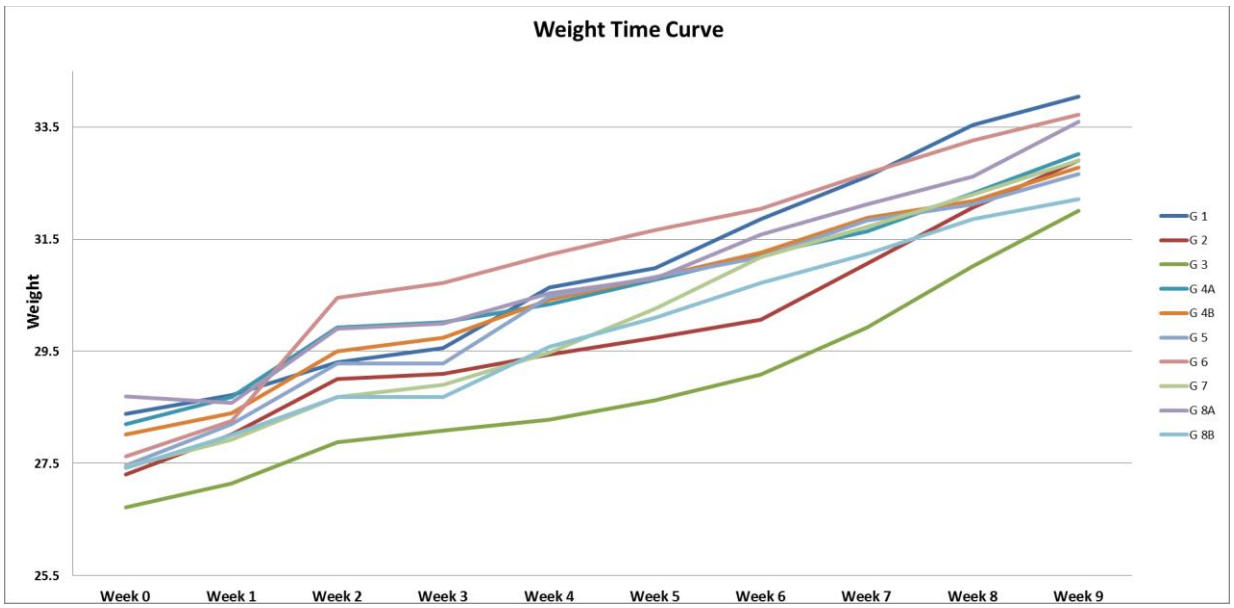


Figure 5: Weight/time curve

Table 5: Weight variations during the experiment

Group	Treatment	Weight											
		Week 0	Week1	Week 2	Week 3	Week4	Week5	Week6	Week7	Week8	Week9	Average	SEM
1	Non-Diabetic Non-treated	28.4	28.7	29.3	29.6	30.6	31.0	31.9	32.6	33.5	34.0	31.0	0.63
2	Non-Diabetic Rapa	27.3	28.0	29.0	29.1	29.4	29.7	30.1	31.1	32.1	32.9	29.9	0.55
3	Non-Diabetic Met	25.72	26.14	26.88	27.08	27.28	27.62	28.08	28.92	29.82	31.08	28.9	0.53
4A	Non Diabetic Met+Rapa	28.2	28.7	29.9	30.0	30.3	30.8	31.2	31.6	32.3	33.0	30.6	0.48
4B	Non-Diabetic Met+Rapa+ Prob	28.0	28.4	29.5	29.7	30.4	30.8	31.3	31.9	32.2	32.8	30.5	0.50
5	Diabetic Non-treated	27.5	28.2	29.3	29.3	30.5	30.8	31.2	31.8	32.1	32.7	30.3	0.55
6	Diabetic Rapa	27.6	28.3	30.5	30.7	31.2	31.7	32.0	32.7	33.3	33.7	31.2	0.63
7	Diabetic Met	27.4	27.9	28.7	28.9	29.5	30.3	31.2	31.7	32.3	32.9	30.1	0.60
8A	Diabetic Met+Rapa	28.7	28.6	29.9	30.0	30.5	30.8	31.6	32.1	32.6	33.6	30.8	0.52
8B	Diabetic Met+Rapa+Prob	27.4	28.0	28.7	28.7	29.6	30.1	30.7	31.2	31.9	32.2	29.9	0.52

Diabetes induction was successful in all animals injected with STZ; they had glycemia levels higher than 150 mg/dl even 10 days after injection (e.g. 128.52 in non-diabetics (ND) G1 and 163.72 in diabetics (D) G5. Treatment with metformin was able to reduce glycemia levels at all time points, e.g. G3 and G7 had average glycemia levels of 126.6 vs 136.6 mg/dl, respectively.

As expected, rapamycin did not show any glucose lowering effect in both ND and D animals (ND-G2 124.96 vs ND-G1 128.52, $p>0.05$ and D-G6 175.88 vs D-G5 163.72 $p>0.05$). Moreover, adding a combination of metformin and rapamycin did not produce any significant added effect in the lowering of glucose below the metformin level alone (e.g. ND-G4A 124.38 vs ND-G3 126.18, $p>0.05$ and D-G8A 146.90 vs D-G7 136.68, $p>0.05$), Figure 6.

Moreover, Probiotics added to metformin and rapamycin did not exhibit any additive effect in decreasing the glucose levels in the sera of animals.

Table 6: Glycemia variations during the experiment

Group	Treatment	Blood glucose levels											
		Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Average	SEM
1	Non-Diabetic Non-treated	124.6	129.2	131.8	124.2	126.8	133.4	134.4	127.0	129.2	124.6	128.52	1.24
2	Non-Diabetic Rapa	123.2	128.6	123.0	125.2	124.8	126.4	121.0	123.8	127.2	126.4	124.96	0.76
3	Non-Diabetic Met	124.4	126.2	128.6	130.2	127.4	123.6	122.8	122.8	124.2	131.6	126.18	1.05
4A	Non Diabetic Met+Rapa	127.8	126.6	124.4	125.0	123.4	122.2	129.2	129.8	128.8	130.0	126.72	0.94
4B	Non-Diabetic Met+Rapa+ Prob	124.4	129.4	120.0	113.8	120.2	128.0	127.6	125.8	126.6	122.6	123.84	1.59
5	Diabetic Non-treated	125.0	120.6	120.8	170.8	174.0	183.2	196.2	182.8	183.2	180.6	163.72	9.83
6	Diabetic Rapa	127	129.2	124.6	194.8	180.2	200	208.6	207	198.4	189	175.88	11.59
7	Diabetic Met	124.4	122.0	128.8	173.2	134.8	139.0	136.0	136.8	134.8	137.0	136.68	4.68
8A	Diabetic Met+Rapa	126.0	123.8	119.8	178.8	163.2	165.8	161.0	142.0	143.8	144.8	146.90	6.65
8B	Diabetic Met+Rapa+Prob	123.8	126.6	127.8	187.4	158.4	162.8	161.6	139.2	133.8	138.4	145.98	6.89

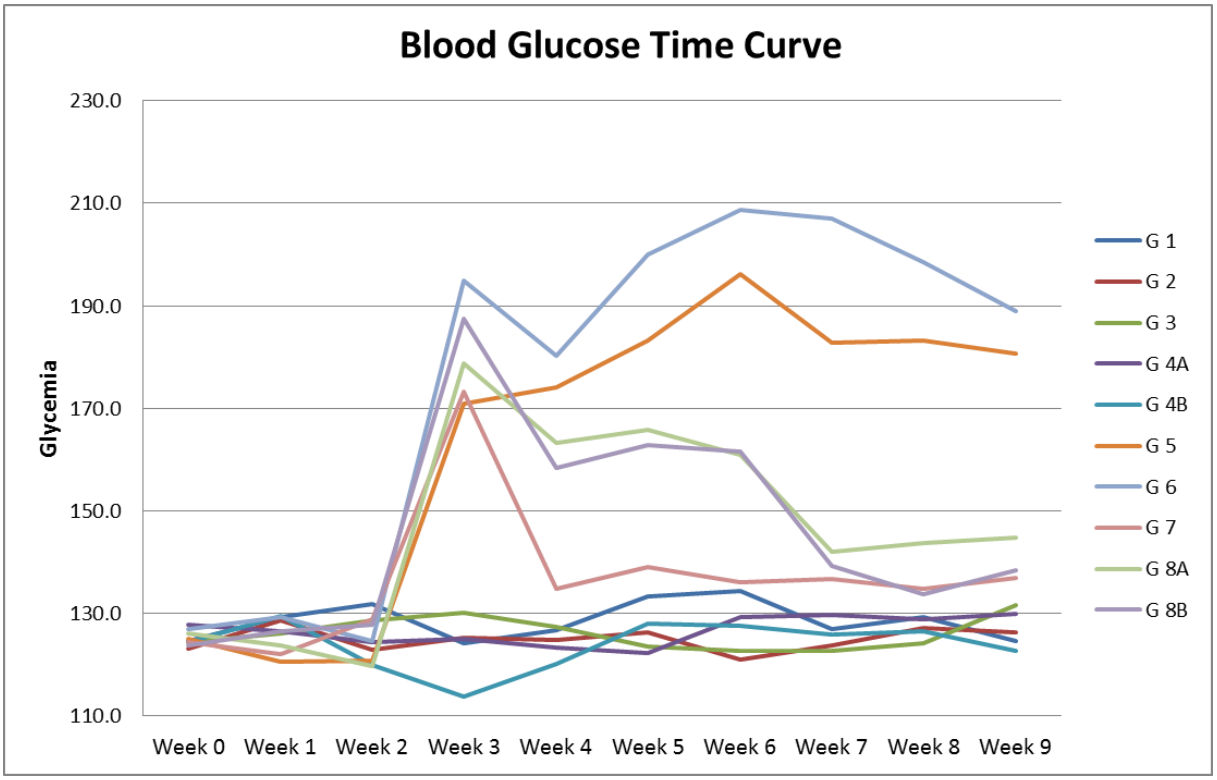


Figure 6: Blood glucose time curve

Note the difference in Glycemia levels between diabetic and non-diabetic groups, as well as the drop in glycemia in diabetic animals in groups 7, 8A and 8B treated respectively with Metformin alone, Metformin and Rapamycin, Probiotics with Metformin and Rapamycin.

Disease Activity Index (DAI) was assessed on a regular basis, as described before, and 9 for the highest disease activity. As expected, the highest indices were encountered in the non-treated groups in both D G5 (6.4) and ND G1 (5.4). However, ND animals treated with rapamycin alone G2 (3.6) or metformin alone G3 (4.4) had a lower DAI. As for the combination treatment, there was a limited additive effect in the ND G4A (2) compared to a lack of such an effect in the diabetics G8A (3).

On the other hand, when the combination of rapamycin and metformin was supplemented with probiotics, the DAI decreased drastically and significantly in both ND 4B (0.2) and D G8B (0.8) (figure 7).

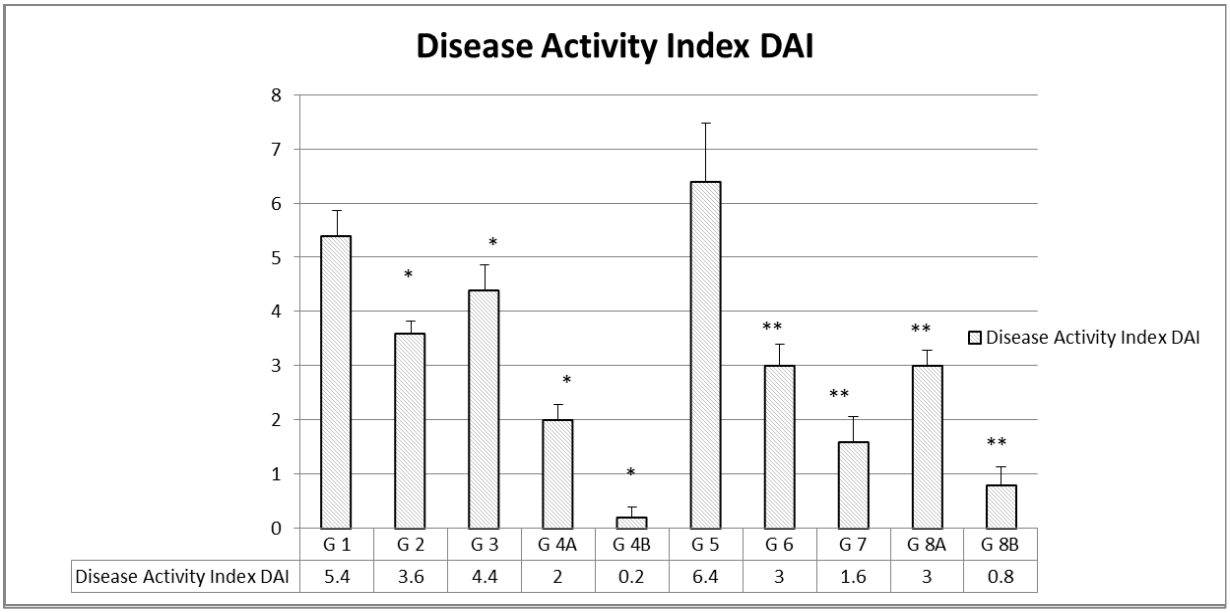


Figure 7: Disease activity index (DAI) in the different groups.

The values represent mean ± SEM (n = 6). Significance of $p<0.05$ was indicated by (*) and (**) when compared to diabetic control and Non-diabetic control respectively.

2. Tumor frequency and volume

All mice injected with the HCTT116 cells developed tumors in their right flank (site of HCT116 injection), except for 3 groups; group 4A treated with metformin and rapamycin where 4 only out of 5 mice had tumors, and in Groups 4B and 8B, where probiotics were added, tumor formation decreased by 40% as it occurred in only 3 out of 5 animals with a significantly smaller size.

Concerning tumor onset, a delay in tumor formation was observed groups treated with metformin and rapamycin plus or minus probiotics when compared to non-treated ones; in G1 (non-treated) tumor appeared only 7 days after HCT116 injection; however, in G 8B treated with rapamycin, metformin and probiotics, tumor formation was delayed till day 15 by 88% (day 15) and in 8A till day 14 respectively (Table 7), with significantly smaller size (Figure 8).

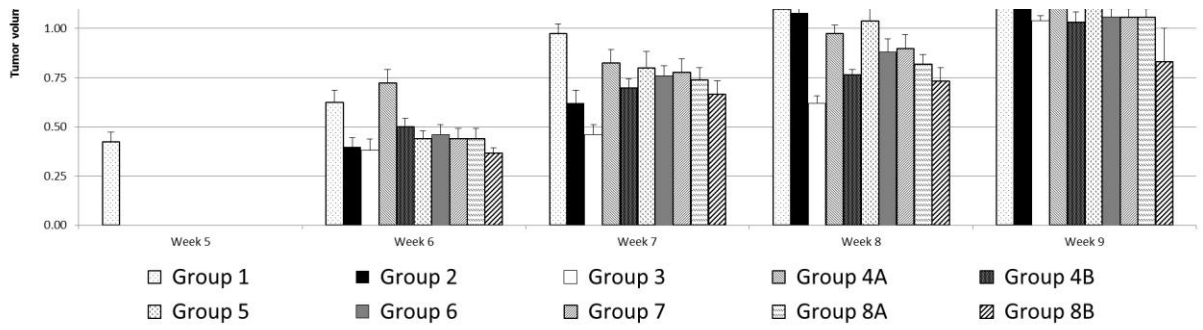
Table 7: Frequency and date of tumor formation

Group	Treatment	Tumors appeared after	Number of animals
1	Non-Diabetic Non-treated	7 days	5 out of 5
2	Non-Diabetic Rapa	9 days	5 out of 5
3	Non-Diabetic Met	9 days	5 out of 5
4A	Non Diabetic Met+Rapa	14 days	4 out of 5
4B	Non Diabetic Met+Rapa+ Prob+	14 days	3 out of 5
5	Diabetic Non-treated	9 days	5 out of 5
6	Diabetic Rapa	10 days	5 out of 5
7	Diabetic Met	10days	5 out of 5
8A	Diabetic Met+Rapa	14 days	5 out of 5
8B	Diabetic Met+Rapa+Prob	15 days	3 out of 5

The results showed that the highest tumor volumes were obtained in non-treated mice (Groups 1 and 5, 1.6 and 1.45 cm^3, respectively). In groups taking rapamycin alone or metformin alone, there was a reduction in tumor volume of 20% and 35%, respectively (G2 with rapamycin 1.28 and G3 with metformin 1.04 cm^3). For groups taking the combined therapy metformin and rapamycin, G 4A and G8A had also significantly small tumor volumes (G4A 1.15 and G8A 1.06 cm^3) close to metformin alone or rapamycin alone; obviously there was no added effect of the 2 drugs. However, groups taking probiotics, G4B and G8B, had significantly the lowest tumor volumes (1.03 and 0.83 cm^3 respectively) at all time points with a decrease in tumor volume of about 36% and 43 %, respectively (figures 8,9 and 10).

Figure 8 : Tumor volumes obtained during the experimental period.

Note that the lowest tumor volumes were seen in groups 3, 4B and 8B treated respectively with metformin or the combination with probiotics.



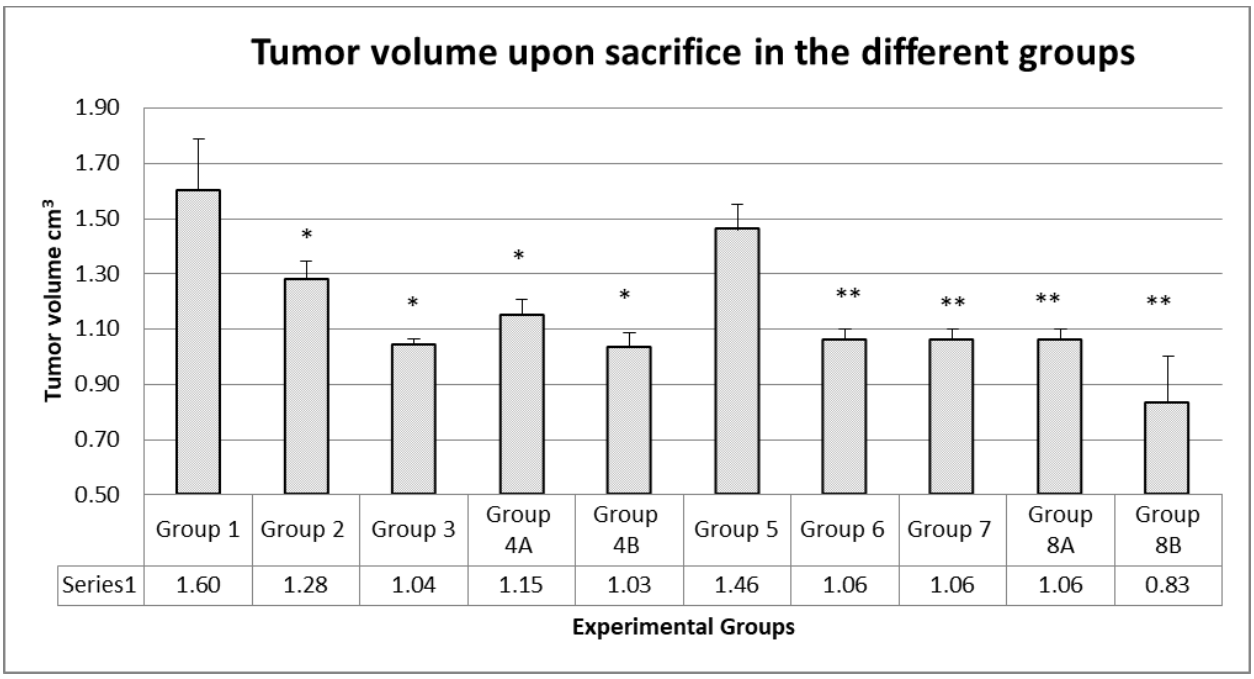


Figure 9: Tumor volumes upon sacrifice.

There was no additive effect of the combination therapy. In diabetics, group 8B, the probiotics with the combination had a significant antitumor effect. The values represent mean ± SEM (n = 6). Significance of $p<0.05$ was indicated by (*) and (**) when compared to diabetic control and Non-diabetic control respectively.

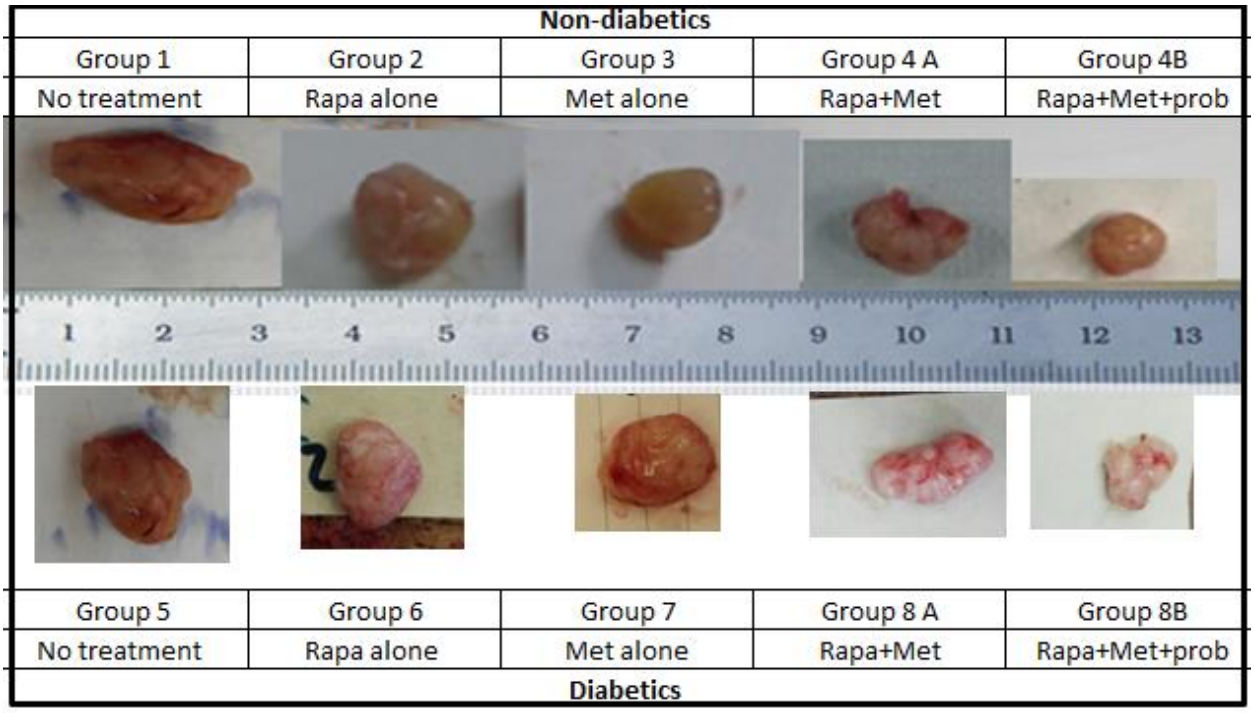


Figure 10: Prototype of tumors upon sacrifice, formed in non-diabetic and diabetic mice treated with Rapamycin, Metformin and their combination with Probiotics.

Note the difference in tumor size in the different groups; animals from groups 4B and 8B treated with Rapamycin and metformin in combination with probiotics had significantly smaller tumor size when compared to groups treated with Met alone, Rapamycin alone or untreated animals.

3. Histological alterations

Histological studies performed on the liver and kidneys showed no signs of toxicity. On the other hand, the histopathology of the xenograft was evaluated and it showed a wide range of alterations in the Xenograft growth and morphology in the various groups. Treatment of ND mice with metformin alone, rapamycin alone or with the combination plus probiotics, led to various degrees of necrosis in the tumor xenograft, the most pronounced growth decrease and necrosis were in the presence of probiotics. Compared to the non-treated mice, those treated had a smaller size tumor, much less of inflammatory cells, and a lower density of tumor cells. The non-treated showed also some ascites fluid within the well circumscribed tumor and less vascularity.

Moreover, in diabetic animals, the same picture and trend prevailed with a much lower density of cells and more of necrosis in the combination treated mice especially with probiotics. However, it is worth noting that metformin and rapamycin did not exhibit an additive inhibitor effect, yet, the density of the tumor cells was relatively lower, and the ascites fluid was also less. The same findings were consistent in all the animals of a given group, (Figures 11-15).

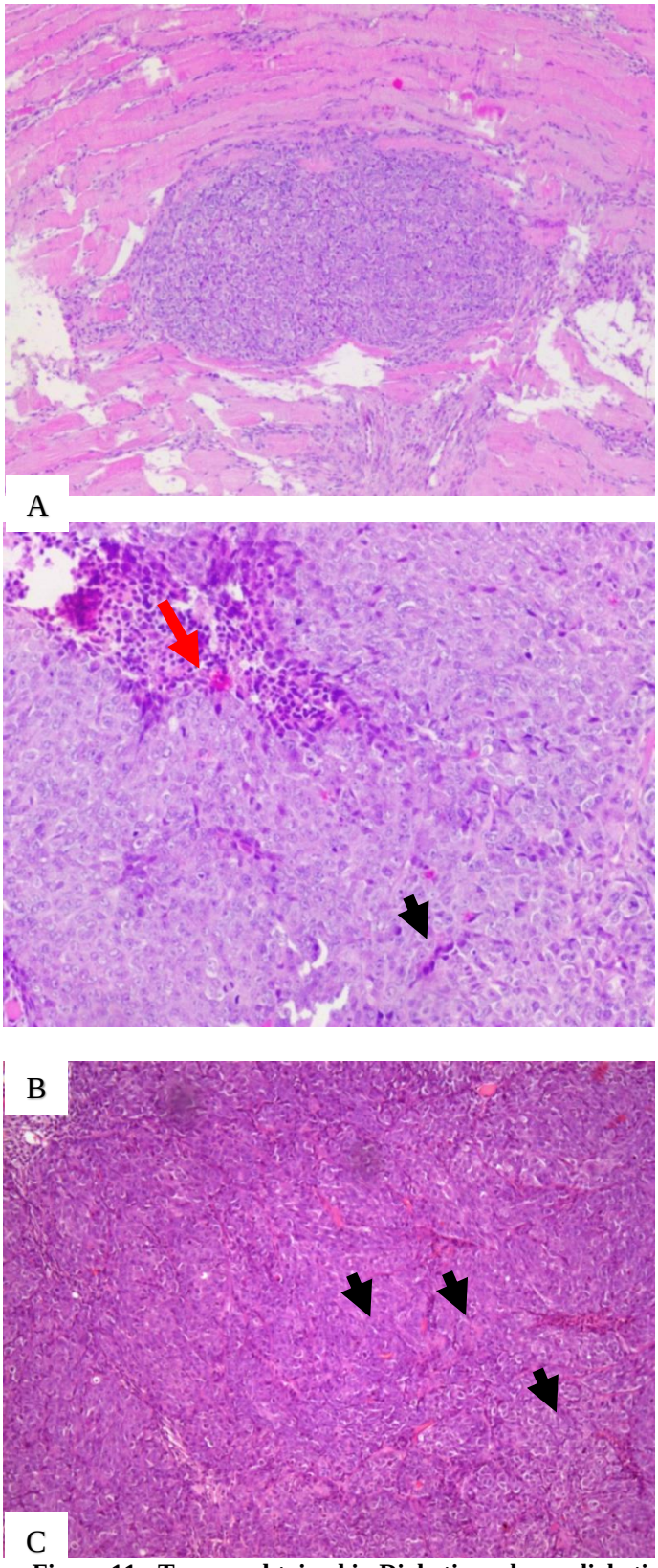


Figure 11: Tumors obtained in Diabetic and non-diabetic non-treated animals.

A: 10x magnification showing a whole view of a well-demarcated tumor formed with a scanty fibrous capsule and a moderately produced connective tissue in diabetic non-treated animals. Note the sheet-like proliferation showing growth of solid tumor cells. B: a 200x magnification of a tumor section in non-diabetic, injected with HCT116 cells and non-treated showing high cellular density, vascularization (black arrows), and tumor cells surrounded by a remarkable infiltration of inflammatory cells (red arrows). C: a 40x magnification of the tumor section in G5, note the high density of the cells along with increase in vascularity (black arrows)

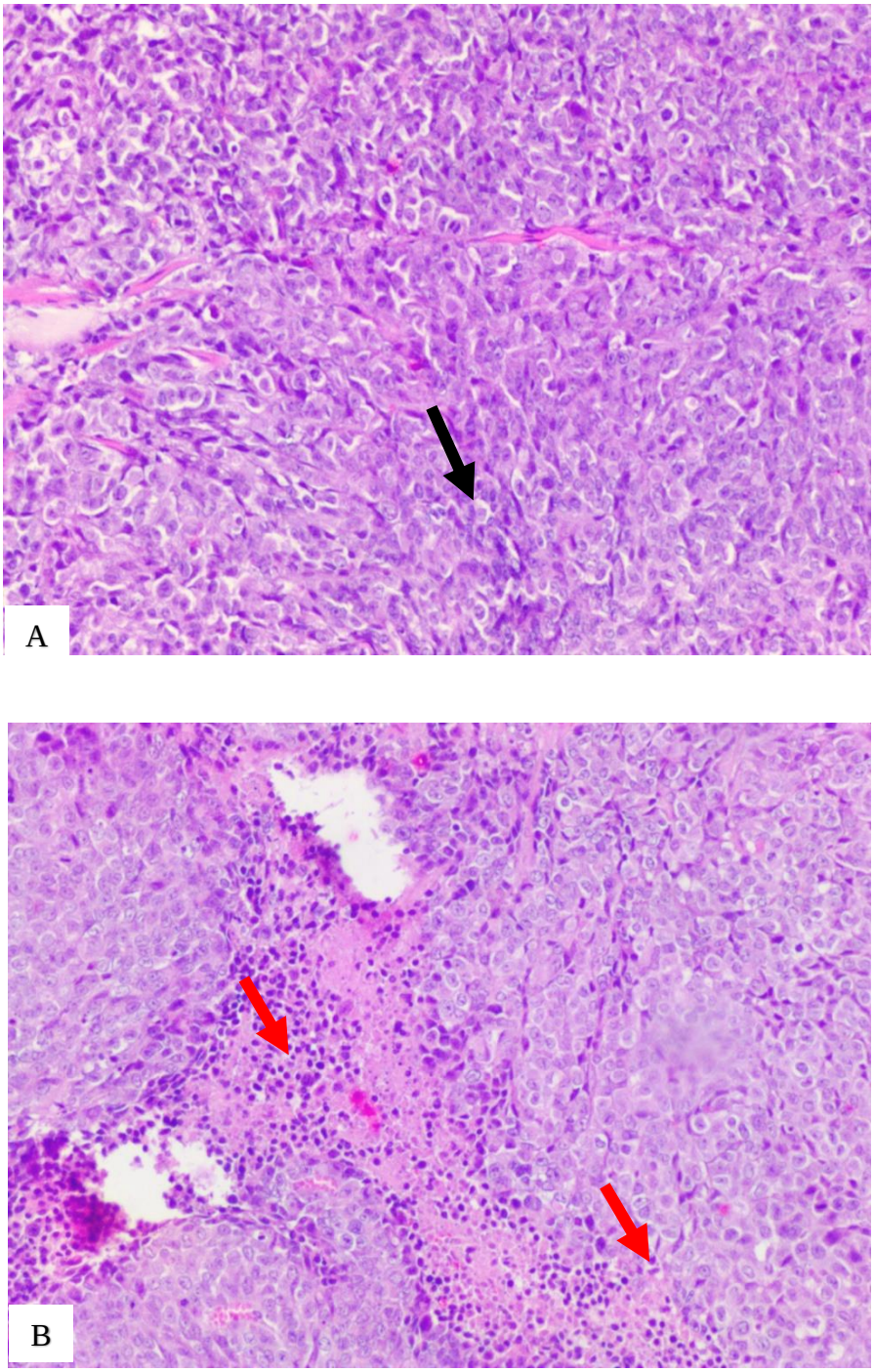


Figure 12 : Tumors obtained in animals treated with Rapamycin alone

A: 200x magnification showing some necrotic areas in the tumor (black arrows) in non-diabetics, rapamycin alone (G2)

B: 200x magnification of a tumor section, showing a moderate cells density and necrotic areas (red arrows) in diabetics treated with rapamycin alone (G6).

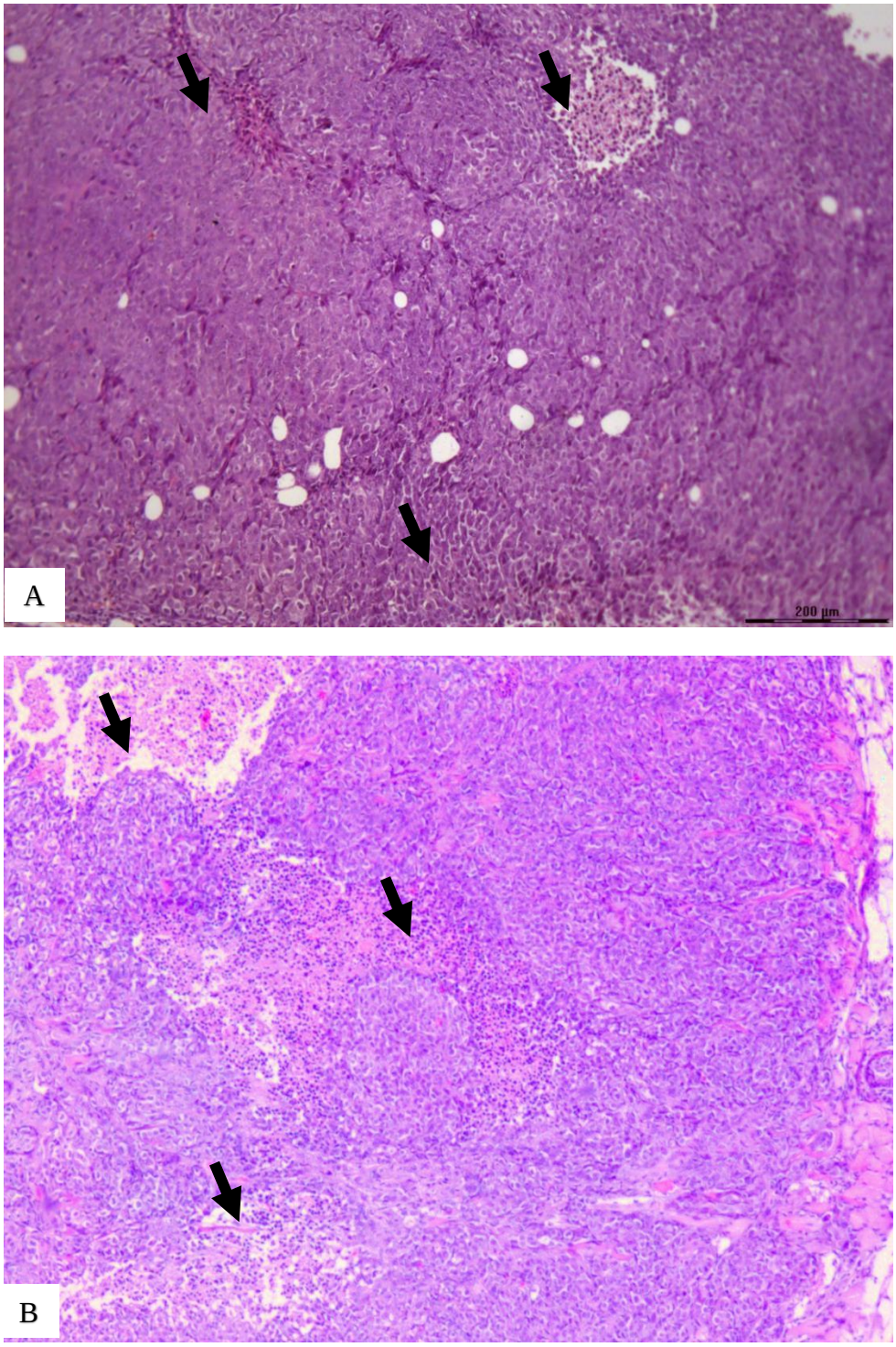


Figure 13: Tumors in animals treated with Metformin alone

A: 200x magnification showing some necrotic areas in the tumor (Black arrows) in non-diabetics metformin alone B: 200x magnification of a tumor section, note the moderate density of the cells in diabetics treated with metformin alone (G7).

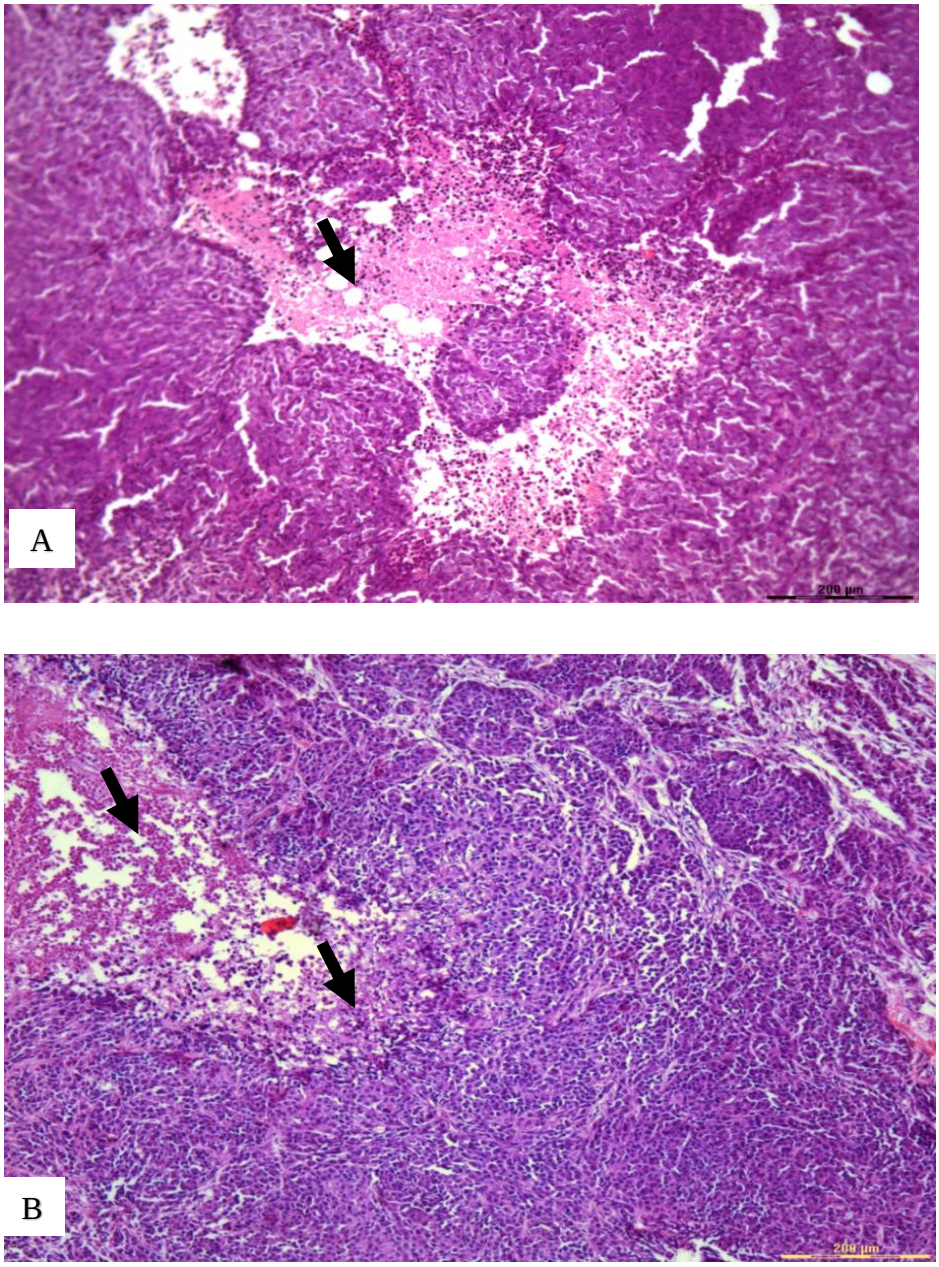


Figure 14 : Tumors obtained in animals treated with Rapamycin and metformin.

A: 200x magnification show large necrotic areas in the tumor section with low cell density (black arrows) in non-diabetics treated with metformin combined to rapamycin. B: 200x magnification of tumor section from diabetic mice treated with metformin and rapamycin showing a lesser density of the cells than either alone as well as necrotic areas (black arrows).

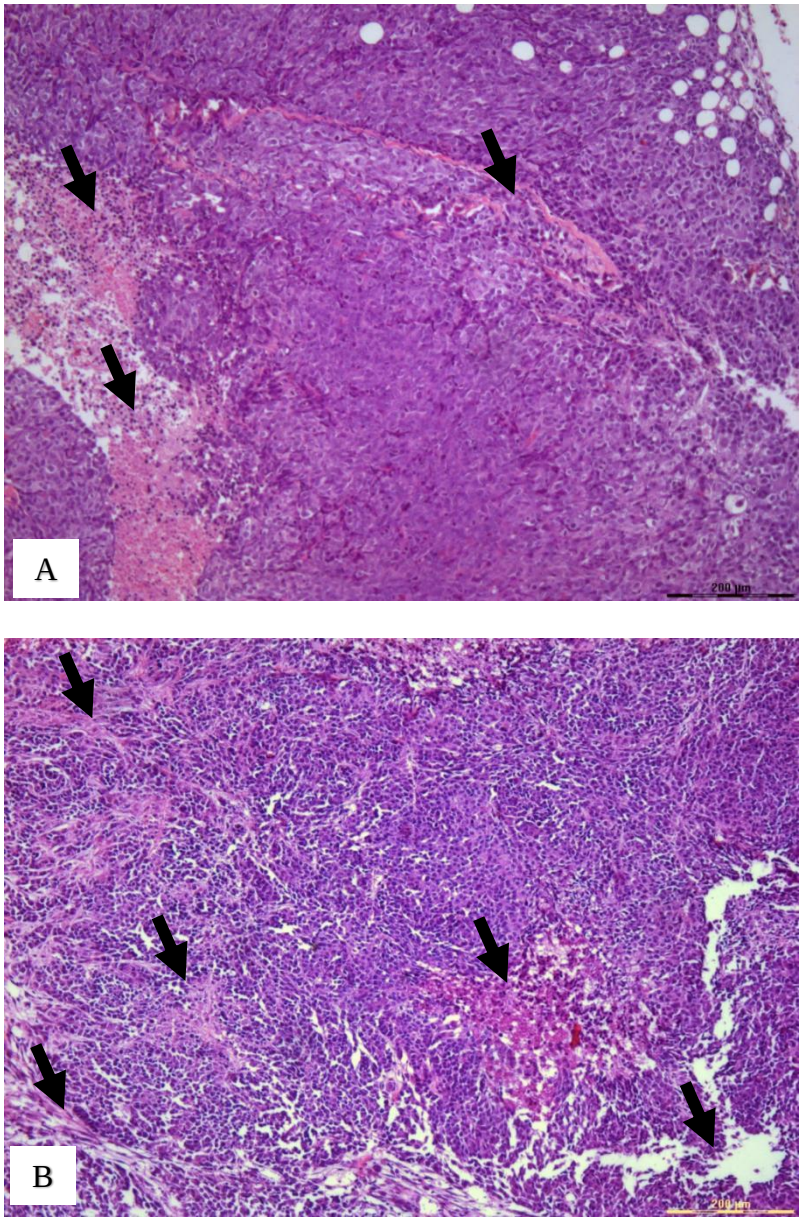


Figure 15: Tumors obtained in groups taking the tri-therapy

A: 200x magnification showing necrotic areas (Black arrows), along with a lower density of the cells in diabetic treated with metformin, rapamycin combined with probiotics (G4B). Note that all tumors from 5 animals in the same group showed similar morphology. B: 200x magnification of tumor section from diabetic mice with the triple therapy showing necrotic areas (Black arrows), along with a significant decrease in cellular density.

The microscopic findings in the descending colon were scored according to the aforementioned criteria by 2 different observers (Figures 16-20). Concerning the descending colon, most of the alterations were recorded in groups 1 and 5, not treated controls, non-diabetic and diabetic, (Figure 16 a b), respectively.

In group 1, there was a marked hyperplasia with a major loss of goblet cells (black circles), polyp formation, and inflammatory cells infiltration (black arrows). The average score of histopathological changes in G1 was 2.4 out of 3. Similar but relatively more severe alterations were encountered in group 5, the diabetic mice with a score of 2.6. Treatment with rapamycin decreased the alterations in G2 and G6 with a score of 1.3 in both based on the presence of less inflammatory cell aggregates (black arrows), less disruption in mucosal architecture and irregularities in the epithelial lining as well as submucosal edema (star). Similarly, treatment with metformin in G3 and G7 improved the alterations seen in G1 and G5 with more improvement in G3 the non-diabetic (score of 0.9) compared to diabetics (score of 1.3). However, the inflammatory reaction was more persistent in G1 (black arrow). On the other hand, the combination (Met+ Rapamycin) did show more decrease in the morphological alterations especially in the non-diabetics G4A (score 0.6) compared to G8A (score of 1.0), close to normal with little submucosal edema and inflammatory cells.

Concerning the use of probiotics plus the combination in G4B and G8B, the tissues of the colon were almost normal with scores of 0.1 both in G4B and G8B.

In brief, there was amelioration to various degrees in the colonic tissues with more effect in the presence of the combination therapy with or without probiotics; on the other hand, the histology was close to normal in presence of probiotics (Figures 16 and 21).

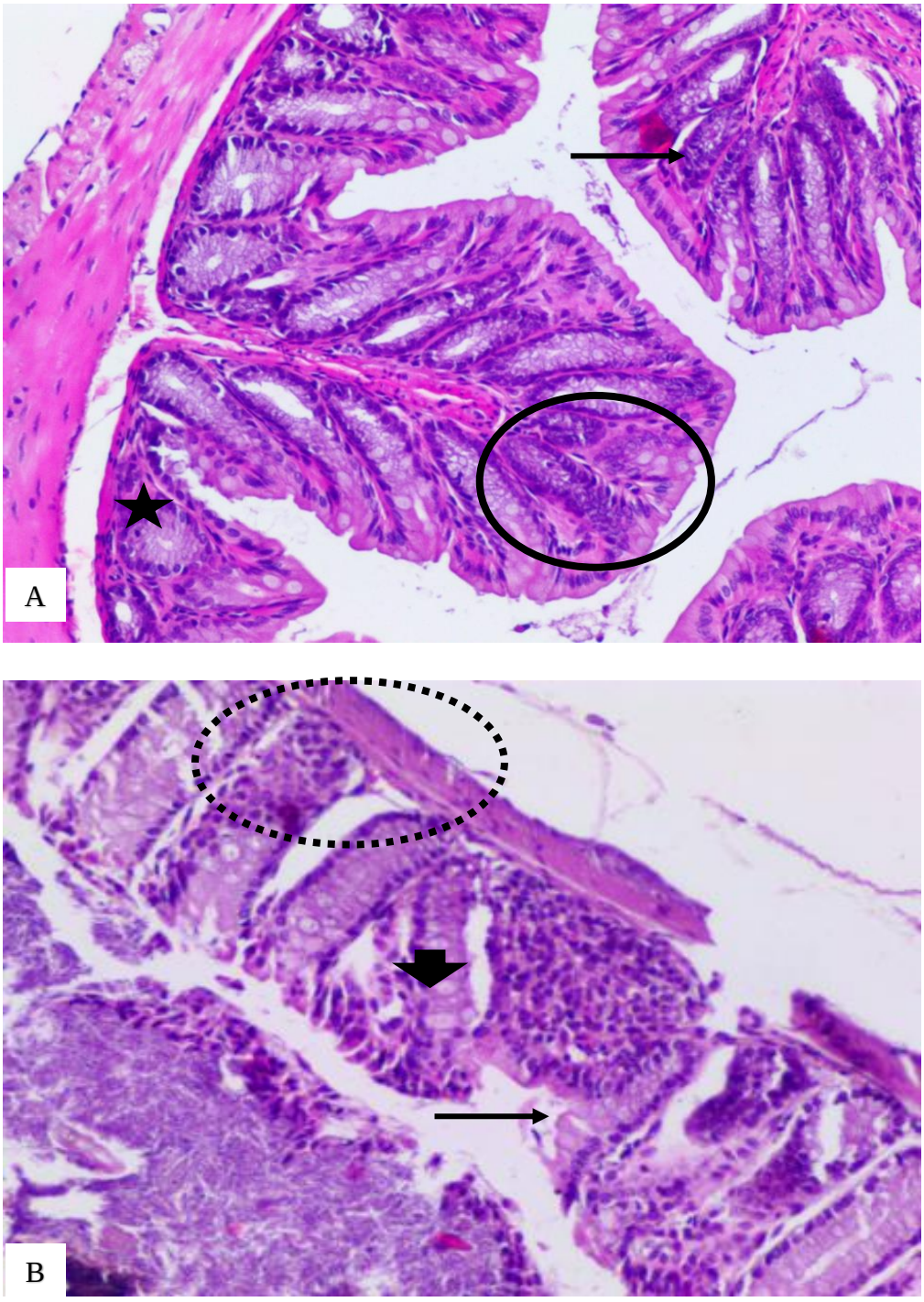


Figure 16 : Colon sections from non-treated animals

Figures 16A and 16B (x200)represent Colon sections from non-treated animals , respectively non-diabetic and diabetic showing a marked hyperplasia with loss of goblet cells (black circles), polyp formation (star shape) and inflammatory cells infiltration (black arrows) seen in animals as well as thinning of the colonic layers (dotted circle) and extensive crypt dysregulation (arrowhead).

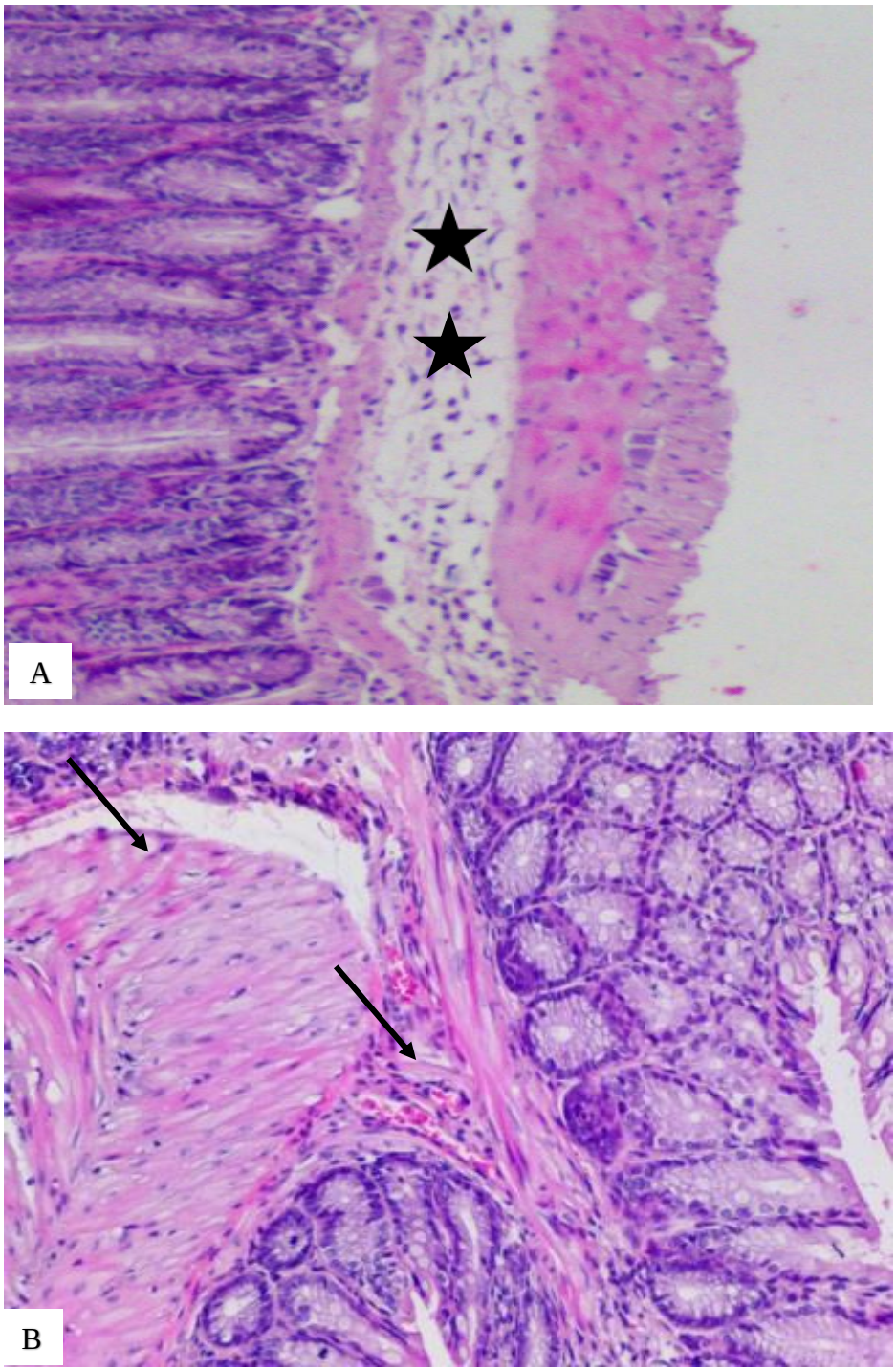


Figure 17: animals treated with Rapamycin alone.

Figures A and B(x200), in non-diabetic (G2) and diabetic (G7), respectively show few inflammatory cell aggregates (black arrows), in addition to some dysregulation in epithelial cell lining and the sub-mucosal edema (star shape).

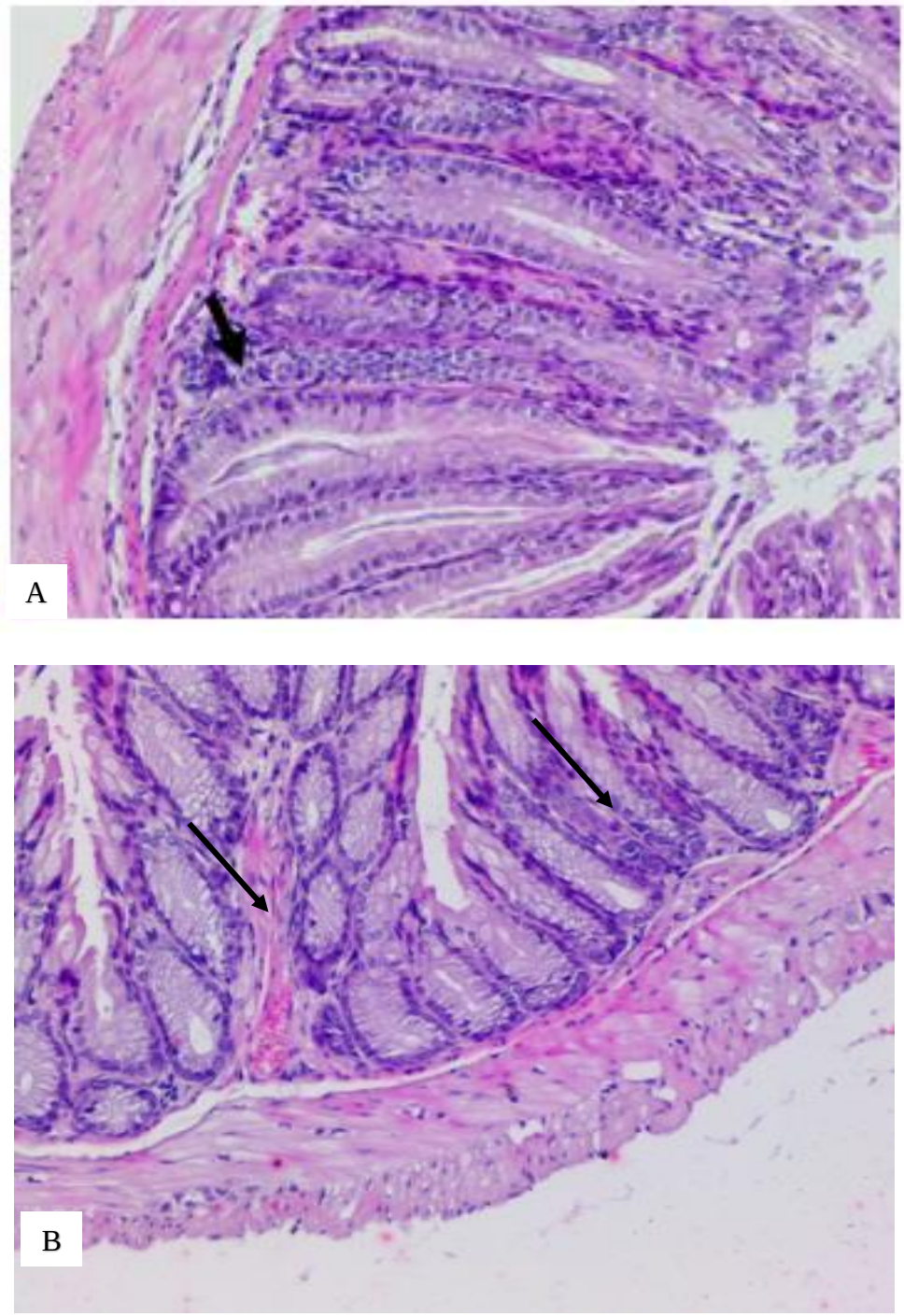


Figure 18: Colon sections from animals treated with Metformin alone

18A and 18B(x200): colon of non-diabetics and diabetics, respectively, showing few inflammatory cell aggregates (black arrows) and a close to normal colonic structure.

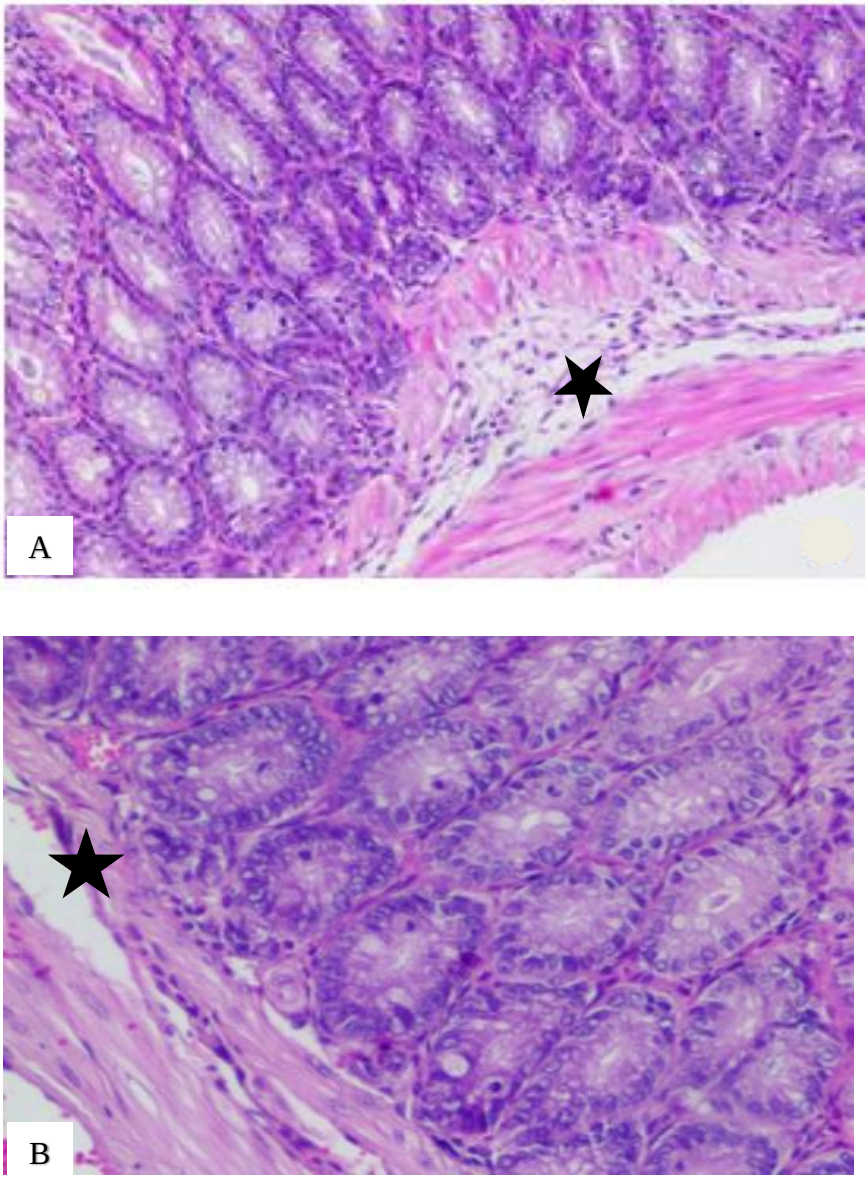


Figure 19: Colon sections from animals treated with animals treated with metformin and rapamycin.

Figures 19A and 19B(x200) show normal colonic structure and normal goblet cell distribution, in addition to a moderate and sub-mucosal edema (star shape) in non-diabetic and diabetic animals treated with metformin and rapamycin.

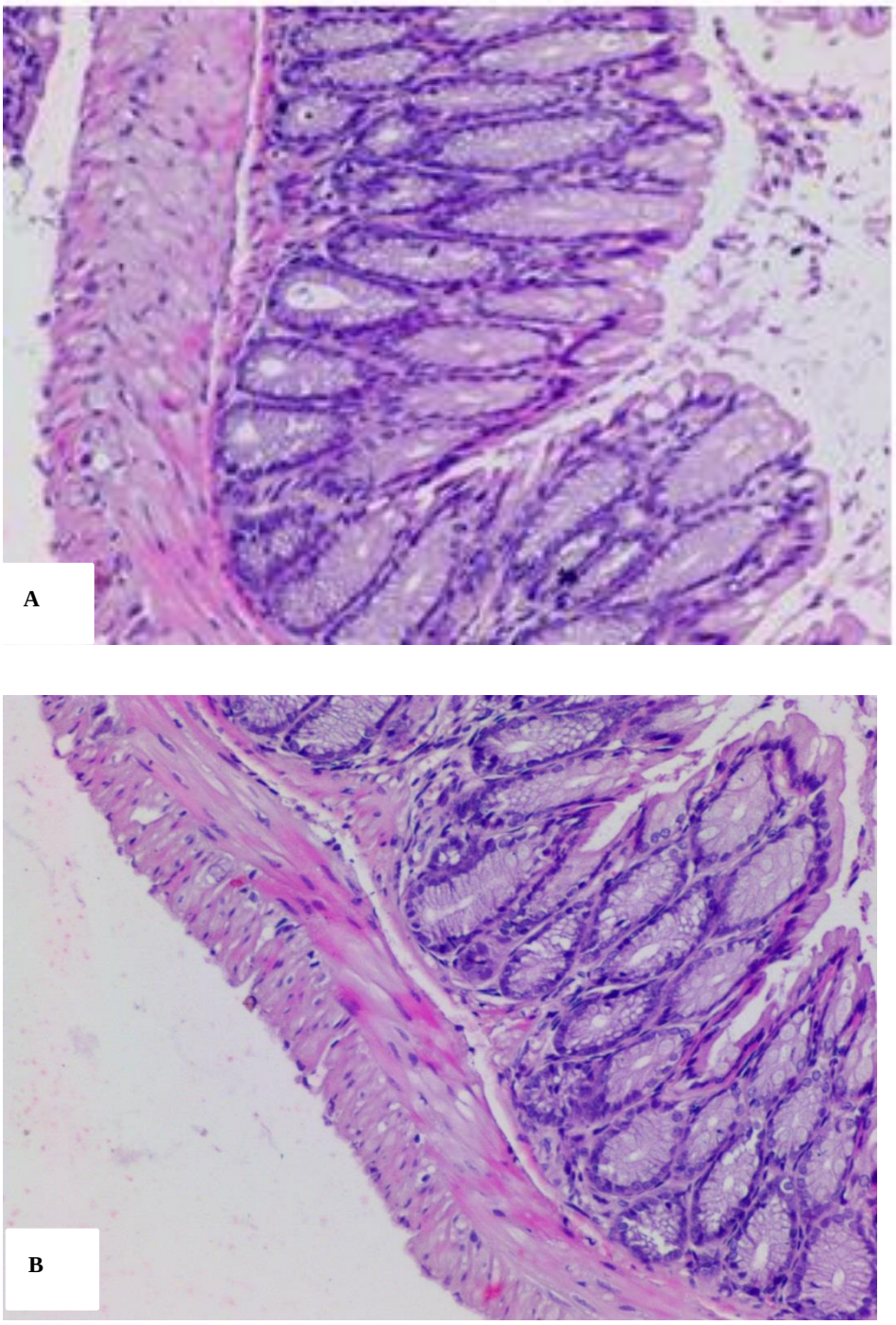


Figure 20 : Colonic tissue from animals treated with the tri-therapy

An almost normal Colonic structure is seen in animals treated with rapamycin probiotics and metformin in non-diabetics and diabetics animals respectively in figures 20A and 20B (x200).

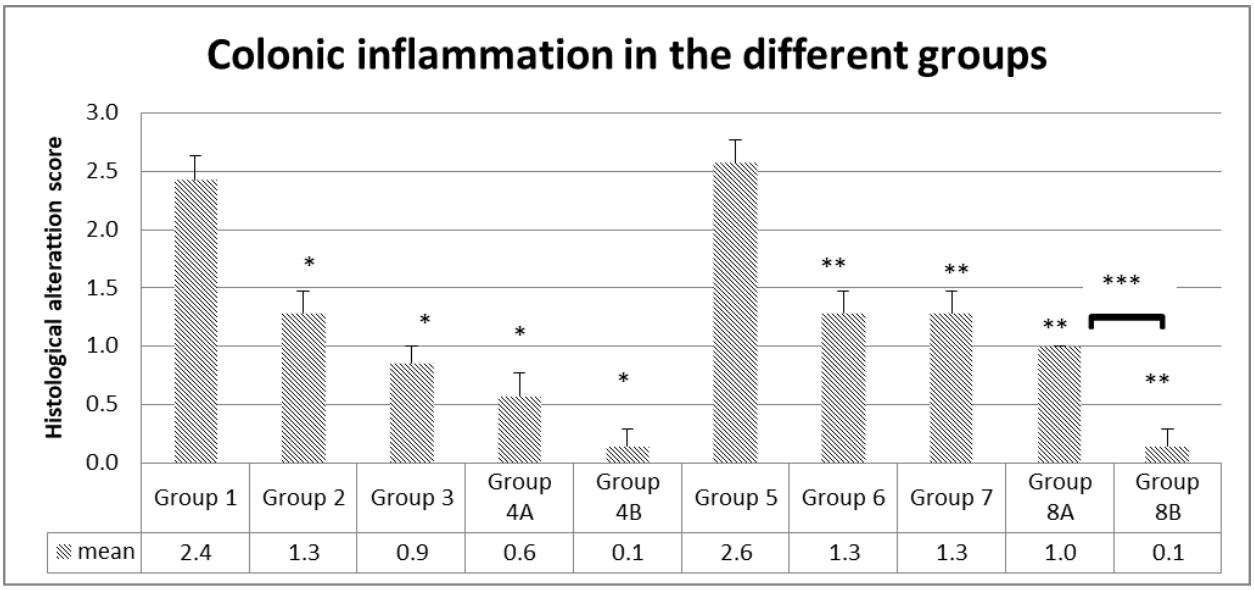


Figure 21: Colonic inflammation average in the different groups

Note the significant drop in inflammation in Groups treated with Metformin, Rapamycin and Probiotics (Group 4B and 8B) where the lowest scores were obtained (0.1). The Values represent mean ± SEM (n = 6). Significance of $p<0.05$ was indicated by (*) when compared to diabetic control, and Non-diabetic control (**); and (***) indicated significance between G8A and 8B.

4. Mast cells number variations

Concerning mast cells, they are normally present in intestinal tissues, they are activated during inflammatory reaction; they degranulate and increase in number. One of the features of inflammatory bowel diseases is mast cell stimulation, secretion and hyperplasia. Hereby, the study of the colonic tissues stained with toluidine blue showed that the high scores encountered in G1 (12.2) and G5 (11.7) decreased and also the mast cell number decreased with the administration of metformin alone and rapamycin alone in a significant way when compared to controls ($p<0.05$); G2 (5.8) and G6 (5.6) for rapamycin, while G3 (3.4) and G7 (4.2) for metformin. The greatest decrease was obtained with the combination of metformin and rapamycin with probiotics; G4B (0.9) and G8B (0.8) with a significant reduction of 92.6% and 93.2% respectively ($p<0.05$).

However, in the diabetic groups G4A and G8A, there were no additive effects of metformin and rapamycin 3.4 and 4.2, respectively (Figures 22 and 23).

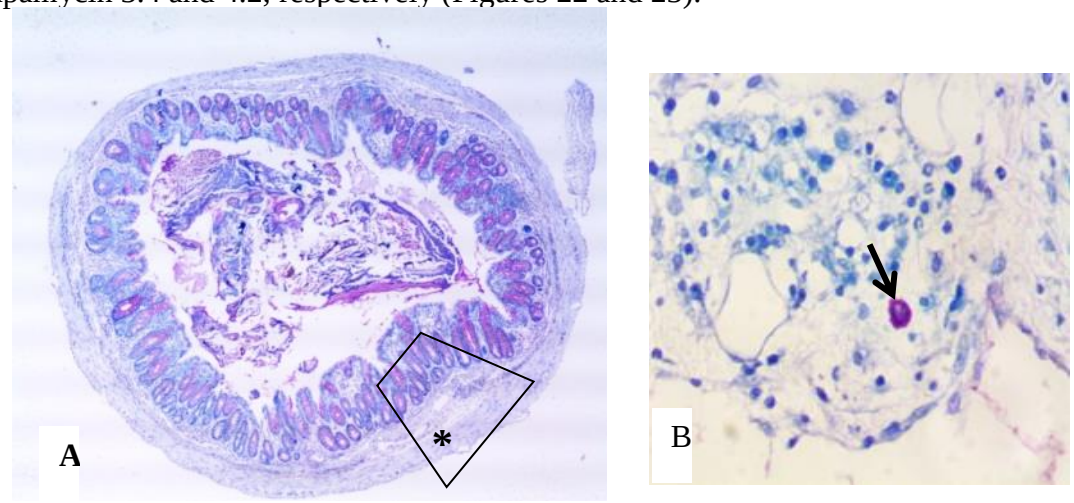


Figure 22: Colonic mast cells

Toluidine Blue stained colonic section (20 B) (x200) showing a typical degranulating mast cell (black arrow) seen in the submucosa of an inflamed colon (20A) (x100).

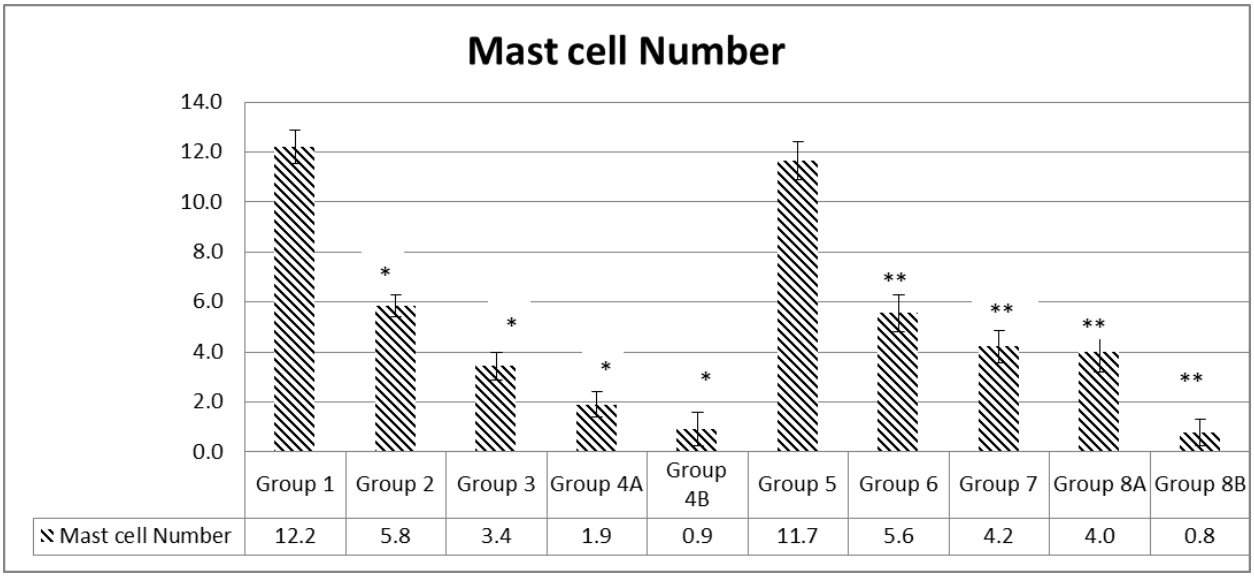


Figure 23 Quantification of Mast cell numbers.

Note that the highest numbers of Mast cells were obtained in Groups 1 and 5. Treatment with Metformin and Rapamycin alone or in combination with probiotics were able to reduce the mast cells number in a significant manner. The lowest values were obtained in Group 4B and 8B when probiotics were administrated to mice in addition to the metformin and rapamycin's combination. The

Values represent mean ± SEM (n = 6). Significance of p<0.05 was indicated by (*) when compared to diabetic control, and Non-diabetic control (**).

5. Reactive Oxygen Species changes

Besides, the modulation in reactive oxygen species (ROS) was significant. In general, cancer cells increase their rate of ROS production compared with normal cells. In this experiment, ROS were assessed in all the colonic samples of the various groups using the DHE staining technique.

In non-diabetics: groups 2, 3, 4(A) and 4(B), the different treatments were able to reduce ROS production in a significant manner when compared to controls in G1 (P = <0.001). In addition, a similar pattern was noted in diabetics: groups 6, 7, 8 (A) and 8 (B) compared to control G5 ROS reduction was significant (p< 0.05). In both G1 and G5, the ROS values were similar and relatively very high (7268 units), regardless of the diabetic or non-diabetic status of the mice.

Actually, in non-diabetics, rapamycin decreased significantly ROS production from 7268 in G1 till 1923in G2, and a similar trend but to a lesser and also significant degree in diabetics (3173units). As for metformin, grossly the effects were similar (G3=1695 and G7=2150), significantly less than G1 and G5, respectively, p<0.05.

On the other hand, there was no additive effect for the metformin and rapamycin combination, the ROS values were significantly less than the non-treated G1 and G5 but relatively more than either metformin alone or rapamycin alone (G4A=3533 and G8A=3147). Furthermore, the presence of the probiotics in the combination therapy made a significant difference in both diabetics (G8B=1903) and non-diabetics (G4A=1918). In brief, all treatments significantly decreased ROS production to various extents; however, the lowest values were with metformin and the combination with probiotics with the absence of additive effect between metformin and rapamycin. All of the differences in the mean values of ROS production among the treatment groups are greater than would be expected by chance; there exist a statistically significant difference (Figures 24 and 25).

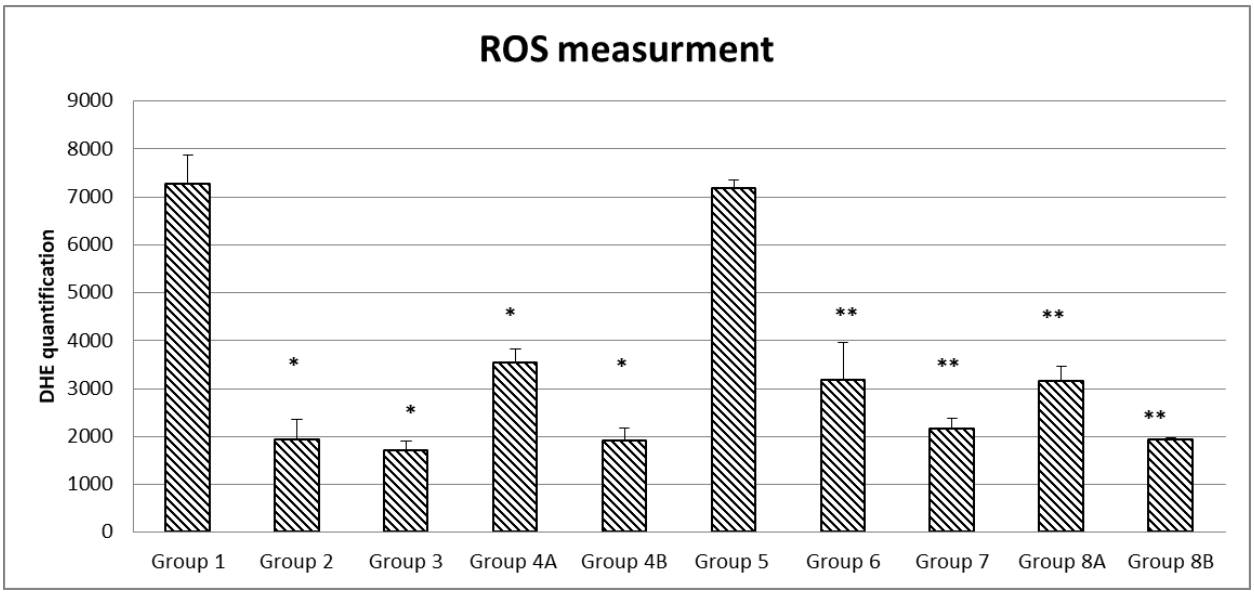


Figure 24: Quantification of ROS formation in the different diabetic and non-diabetic groups.

Note that the highest ROS levels were obtained in the non-treated groups (1 and 5), the different treatments and their combinations were able to reduce ROS levels to a various extent in a significant manner. The Values represent mean ± SEM (n = 6). Significance of $p<0.05$ was indicated by (*) when compared to diabetic control, and Non-diabetic control (**).

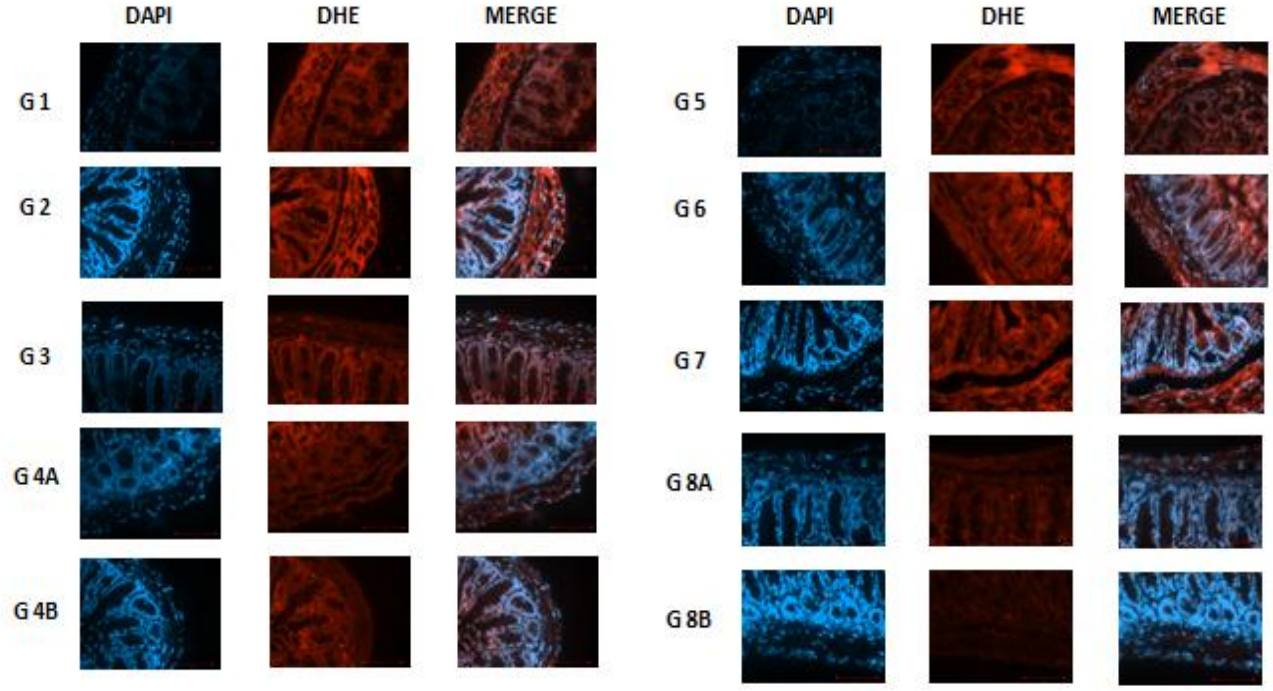


Figure 25: DHE staining in non–diabetic and diabetic animals.

Note the difference in stain intensity when comparing the non-treated Group 1 and 5 to the treated groups. The lowest red fluorescence was obtained in Group 4B and 8B treated with the combination of Metformin, Rapamycin and Probiotics.

6. Molecular analysis of relevant genes and proteins

6.1. Modulation of gene expression of AMPK, mTORC and KI67

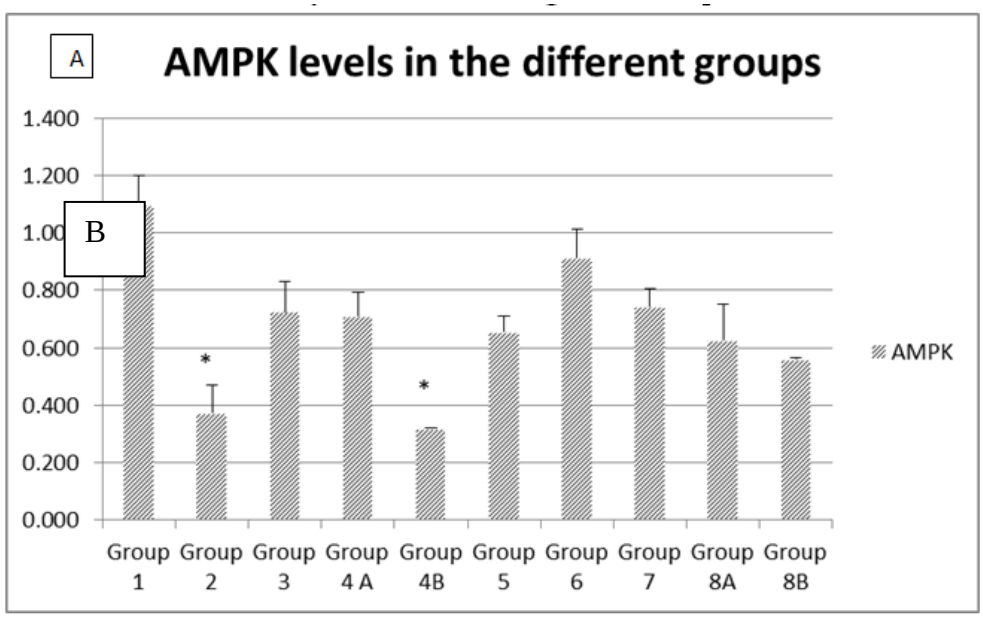
Repeatedly, AMPK gene expression was close in most non-diabetic groups, close to 1.0 in G1, G2 and G3 and decreased by about 31 % in G4A(=0.69) and 39 % in group G4B (0.63), with combination therapy or combination plus probiotics, respectively.

On the other hand, the non-treated diabetic mice in G5 expressed less AMPK by about 31% than the non-diabetics in G1. In addition, the expression in the rest of the diabetics G6, G7 and G8A was not significant. it was less then G1 G2 and G3.in diabetics also, a slight increase in AMPK was observed when metformin and rapamycin were administered alone or in combination. However, when probiotics were added to the combination, a decrease of about 20% was observed in group 8B compared to 8A, similar to non-diabetics with combination G4A and close to 4B. It seems that the triple treatment could show a distinct difference compared to the other groups (Figure 26A).

Concerning mTORC expression, it was suppressed in all groups except in the rapamycin treated diabetics, where the value was highly significant in G6=12.79 compared to very low expression in all other groups. In brief, only rapamycin treatment upregulated mTORC (Figure 26B).

As for KI67 genes, whose level of expression indicates the proliferation of the cells, the data profile was close to the AMPK expression profile. Partial inhibition of proliferation was encountered when combination treatment was used in non-diabetic with (33%) or without (36%) probiotics (Figure27).

Some decrease of proliferation was encountered in all diabetics: 20% in G5, 42% in G6 with rapamycin treatment, 30% with metformin treatment in G7, 25.0 % with rapamycin and metformin G8A and 31% when probiotics were added to the combination. Therefore, the combination, with or without probiotics decreased proliferation by 20-40 % (Figure 27).



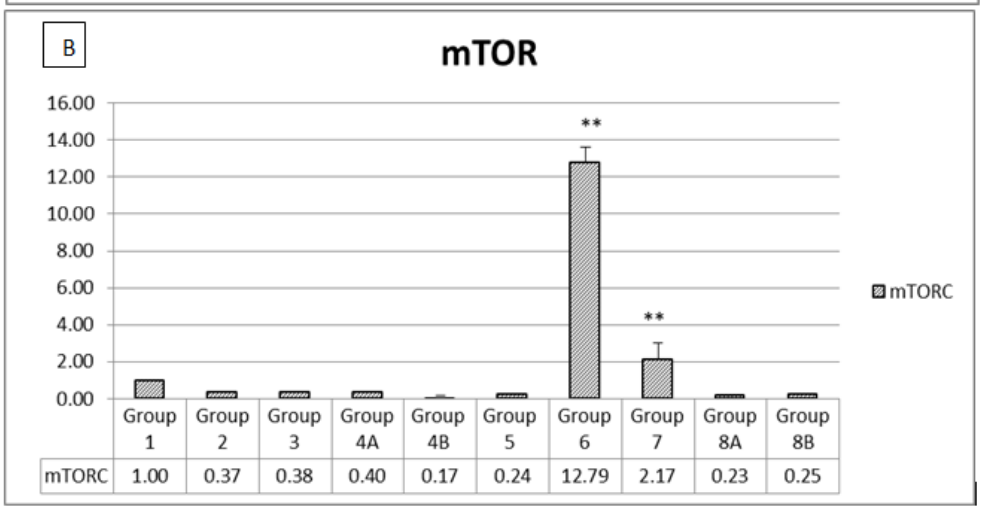


Figure 26: Expression of main genes involved in colorectal carcinogenesis.

The values represent mean ± SEM (n = 6). Significance of $p<0.05$ was indicated by (*) and (**) when compared to diabetic control and Non-diabetic control respectively.

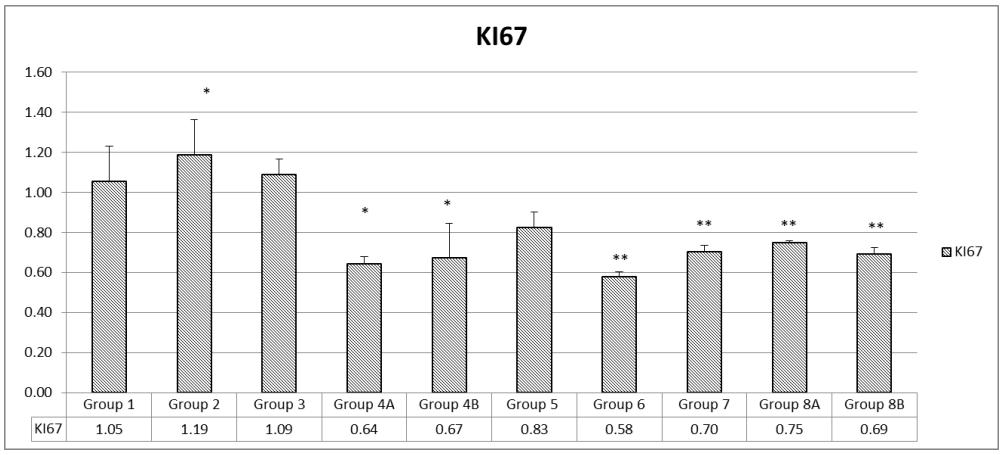


Figure 27: Assessment of Proliferation via KI67

The values represent mean ± SEM (n = 6). Significance of $p<0.05$ was indicated by (*) and (**) when compared to diabetic control and Non-diabetic control respectively.

6.2. Gene expression of pro-inflammatory cytokines: IL-3, IL-6 and TNFα:

As expected the expression of Il-3 genes was relatively the highest in the non-treated mice (G1=1 and G5=0.9). It was inhibited significantly in non-diabetics by rapamycin alone and metformin alone by 89% and 88% respectively. Inhibition by the combination therapy plus probiotics was by 90%. However, again the combination of metformin and rapamycin together inhibited by about 78% in diabetics; again, there were no added effect but rather may be a competitive effect of the 2 drugs. On the other hand, in diabetics, the inhibition was very significant in all groups (G6=90%) G8A=80%, and G8B=90%, However, metformin alone inhibited the expression by only 33% compared to G5 which expressed IL-3 by 91% (Figure 28 A).

Concerning the other interleukin IL-6, its gene expression was suppressed in almost all groups to various extents except in G1, the non-diabetic, non-treated group of mice.

The combination plus probiotics G4B and G8B had relatively the highest suppression, 86% and 83%, respectively. However, the combination without probiotics and rapamycin alone had 84% suppressio7n and 76% in non-diabetics. In brief, there was an additive effect of both drugs in the diabetics but not in the non-diabetics (Figure 28 B). In the non-treated diabetic mice, the expression of IL-6 was low, about 24% with rapamycin, 33% with metformin and 45% with the combination,

and in all the rest of the groups the IL-6 expression was less, G2 =24% G3=33% G4B=45% G5=30% G6=17% G8A and G8B =17%. However, in non-diabetics, met and Rapamycin did not have an additive effect but rather a competitive effect G4A =45% (Figure 28B).

As for TNFα, its gene expression was extremely inhibited >90% in all the non-diabetic groups, and even in the non-treated mice both diabetics and no-diabetics. However, the TNFα gene expression was relatively elevated 4.ww times compared with rapamycin treatment and 8.77 times with metformin treatment compared to G1 (1.0) and G5 (1.01). There was no additive effect; however, in the presence of probiotics G4b and G8B the inhibition was almost complete (Figure 28C).

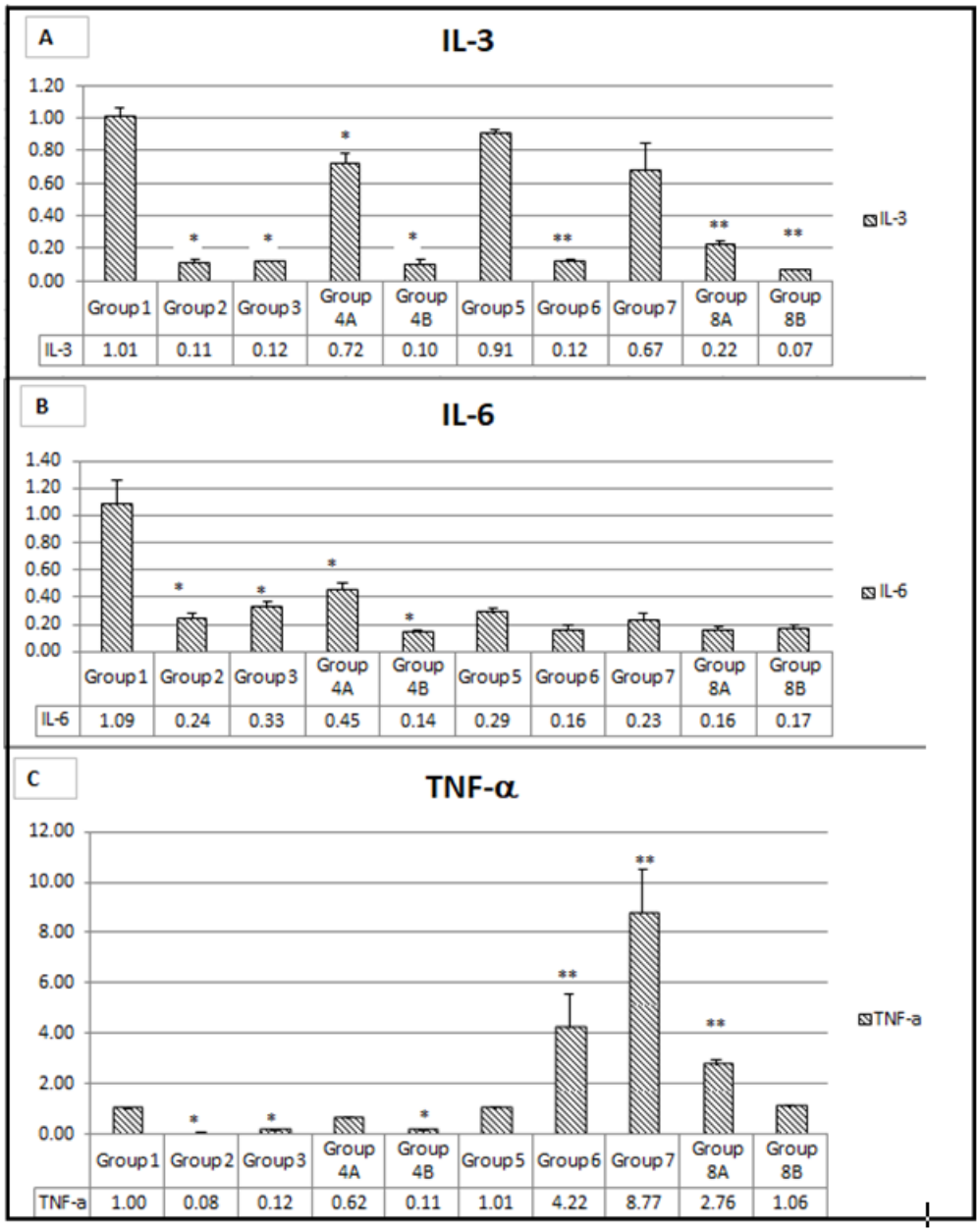


Figure 28: Expression of main genes involved in inflammation

The values represent mean ± SEM (n = 6). Significance of $p<0.05$ was indicated by (*) and (**) when compared to diabetic control and Non-diabetic control respectively.

In brief, the administration of metformin alone or rapamycin alone induced a decrease in the expression of the inflammatory markers, IL-3, IL-6 and TNFα in both diabetics and non-diabetics. The lowest scores were obtained in diabetic and non-diabetic groups taking the triple-therapy (metformin, rapamycin and probiotics) when compared to non-treated controls. However, a slight increase in IL-3, IL-6 and TNFα was noted when combining rapamycin and metformin shedding light on possible alternative signaling pathways (Figure 28, A, B, C).

The three inflammatory markers studied had the similar expression profile to a great extent, implicating that all treatments produced a prominent decrease in the inflammatory response which forms a favorable environment for colorectal carcinogenesis development and progress.

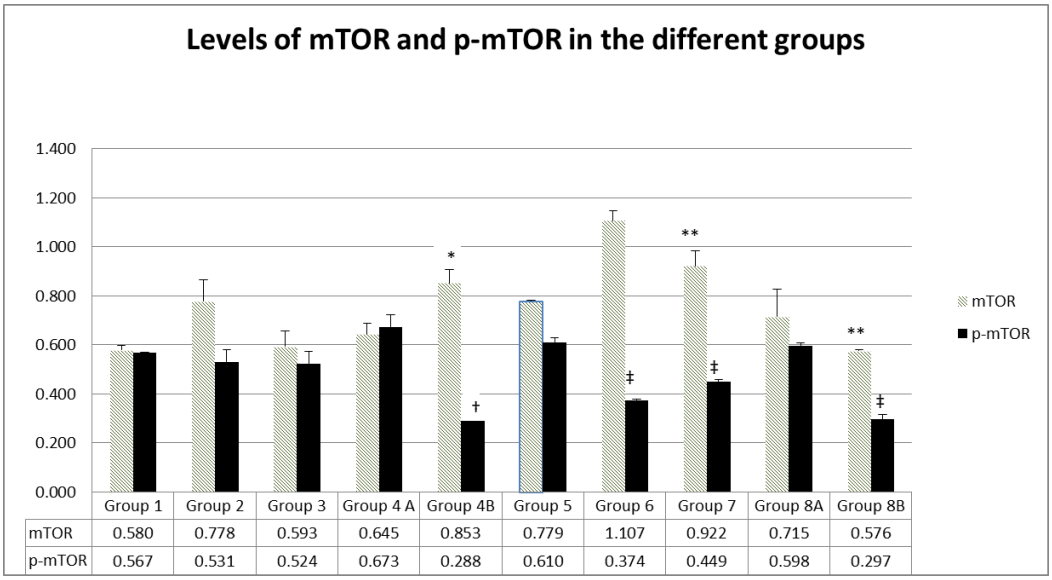
6.3. Variations in the expression of mTOR and p- mTOR proteins in tumors

Data emanating from western blots performed on proteins extracted from tumor sections and assessing the effect of the different treatments on the regulation of mTOR and its phosphorylated form p-mTOR, showed different levels of inhibition in the diabetic and non-diabetic animals (Figure 29).

Data showed that the treatment with Rapamycin increased the levels of mTOR in both diabetics (G6 compared to G5) and non-diabetics (G2 compared to G1). On the other hand, treatment with metformin introduced no change in m-TOR and p-mTOR (G3 versus G1) in non-diabetics but a significant increase in diabetics (G7 compared to G5). Using the combination of Metformin and Rapamycin, did not introduce any significant variations, thus leading us to conclude one more time that the 2 drugs do not have an additive effect on mTOR. However, by adding probiotics to the combination, mTOR expression increased in non-diabetics (G4B=0.85 compared to G1=0.58) and decreased slightly in diabetics, G8B=0.57 compared to G5 0.77).

The p-mTOR decrease was really significant when probiotics were added to metformin and Rapamycin in both non-diabetics and diabetics. In addition, p-mTOR was also significantly inhibited with either rapamycin or metformin treatment in diabetics (G6=0.37, G7=0.449 vs G5=0.61).

In brief, it is important to note that the treatment with rapamycin alone or metformin alone was able to inhibit mTOR activity *via* decreasing its phosphorylation. However, the effect or rapamycin was more significant. The highest inhibition of p-mTOR was obtained when adding probiotics to the combination in diabetic and non-diabetic mice. In addition, there was no additive inhibitory effect of metformin and rapamycin, but the opposite is true, a slight increase in p-mTOR was noted (Figure 29).



	Group 1	Group 2	Group 3	Group 4 A	Group 4B	Group 5	Group 6	Group 7	Group 8A	Group 8B
mTOR	0.580	0.778	0.593	0.645	0.853	0.779	1.107	0.922	0.715	0.576
p-mTOR	0.567	0.531	0.524	0.673	0.288	0.610	0.374	0.449	0.598	0.297

Figure 29: Expression of mTOR and p-mTOR at protein level in the different groups.

The values represent mean ± SEM (n = 6). Significance of $p<0.05$ was indicated by (*) and (†) when compared to diabetic control, (**) and (‡) when compared with Non-diabetic control (**) for mTOR and p-mTOR respectively.

Chapter Four: **DISCUSSION**

Clinical observations and clinical studies indicate that the prevalence of diabetes in newly diagnosed cancer patients ranges from 8 to 18%, suggesting bidirectional association between these 2 diseases (Barone et al., 2010; Richardson & Pollack, 2005; Smith & Gale, 2009). Publications in the past 4 to 5 years have also suggested the link between first line hypoglycemic medications like metformin and the delay in initiation of cancer (Bowker, Majumdar, Veugelers, & Johnson, 2006; Drzewoski, Drozdowska, & Sliwinska, 2011; Schiel, Muller, Braun, Stein, & Kath, 2005). However, the mechanism is still unclear, despite the fact that metformin is capable of activating AMPK involved in tumorigenesis (Evans, Donnelly, Emslie-Smith, Alessi, & Morris, 2005).

On the other hand, rapamycin, originally used as an antifungal agent (Bastidas, Shertz, Lee, Heitman, & Cardenas, 2012),was later approved as a potential anticancer drug (Lamming, 2013), a specific inhibitor of the mammalian target of rapamycin (mTOR) signaling pathway, master regulator of cell growth and metabolism implicated in a number of diseases including diabetes and cancer(Lawrence & Nho, 2018). However, the modest effect of rapamycin-based therapy has prompted investigators, including our laboratory, to consider combination therapy of metformin and rapamycin, especially that metformin administration significantly reduced CRC incidence (Chang et al., 2018)

This in vivo work was also supported by our in vitro data, using either metformin alone, rapamycin alone or a combination of the 2 drugs, on the proliferative activity of HCT116 and HT29 colonic cell lines, and not as much on a leukemic cell line. At physiological and supraphysiological doses the 2 drugs were not toxic. Results showed that the medications were well tolerated by the animals and caused no toxic reactions. Despite the fact that rapamycin and metformin were used in combination with or without probiotics, they did not lead to unfavorable toxicity, while triggering tumor regression, thus suggesting their value for treatment of CRC in diabetes.

As expected, the clinical profile of the controls untreated xenografts was the worst. The individual treatments helped removing some of the symptoms and signs but more so was achieved with the combination therapy, in particular, when probiotics were added to the combination. Such an effect was more remarkable in diabetics vs non-diabetics. Based on clinical observations, the highest

disease activity (DAI) was detected in the non-treated animals to significant extents; however, each drug had some positive effect on the DAI, more so for metformin than rapamycin. On the other hand, there were no significant additive effects between metformin and rapamycin except when supplemented with probiotics, and then all symptoms and signs decreased significantly in all diabetics and non-diabetics.

Metformin significantly reduced or delayed the occurrence of CRC development in the animals; it worked as an independent protective factor against CRC (Chang et al., 2018). Metformin might have exerted its cancer chemo preventive effects by suppressing the transformation and hyper proliferative processes that initiate carcinogenesis (Del Barco et al., 2011). Similarly, Rapamycin has also exercised its potential as an anticancer drug in this rapamycin-sensitive cancer model, but to a very low level, compared to metformin alone or the combination plus probiotics. In brief, the 2 drugs showed remarkable effects in preventing or slowing down the progress of the development of the xenograft. Such an effect was consistent in delaying tumorigenesis particularly when probiotics were added in G4B (for 3/5) after 14 days and G8B (3/5) after 15 days; p-mTOR has been suppressed significantly in both cases.

At the same time, there was a decrease in size of the tumor in almost all treated groups to various extents compared to untreated animals. Again, little but significant effects were encountered with each drug separately with no indication of added effects except when probiotics were in the combination. The probiotics treated animals had decreased size and frequency of all tumors in both diabetics and to a lesser extent in non-diabetics.

By studying the histological alterations of the xenografts, various degrees of necrosis were noted in the xenograft, however, the alterations were more pronounced in the treated diabetics with the combination therapy and especially when probiotics were added: smaller size, less tumor cells, less inflammatory cells, more necrosis and less fluid within the tumor mass compared to larger volume, high cellular density, vascularization and a strong infiltration by inflammatory cells in the controls not treated animals. Each of the 2 drugs had moderate effects which did not add, but were conspicuous in terms of size, lower cell density and less inflammatory cells leading to less production of proinflammatory agents IL-3, IL-6 and NFKB and consequently: more autophagy, more apoptosis and necrosis.

Concerning the colon, most alterations in colonic tissues were encountered in the controls non-diabetic and diabetic animals. The reaction in G1 was typical of inflamed tumorigenic tissue

without any treatment having a high score (2.4 / 3). On the other hand, the alterations were even more severe in G5, the non-treated diabetic control (2.6/3). Treatment with the drug rapamycin alone improved the histology (1.3/3) in G2 and G6. More improvement or less alterations resulted from metformin in G3 (0.9/3) and (1.3/3) in G7 diabetics. Such improvements were more evident when combination therapy was adopted in G4A (0.6/3) and G8A diabetics (1.0/3), whereby most of the alterations disappeared. Such ameliorations were even more prominent when probiotics were added, reaching a histological status very close to normal: absence of inflammatory cells, goblet cells back to normal, and no signs of edema or cryptitis.

In line with such histological changes and their amelioration under the various treatment regimens, the mast cells followed a similar pattern. It seems that the panoply of inflammatory mediators, secreted by these cells decreased as the number of mast cells to various extents in the different groups commensurate of the improvement or limitation of the inflammatory reaction in the various groups. Again, no signs of added effects among the 2 drugs, but the presence of probiotics, one more time, did make a positive impact on the tissues and cells of the colon. Were the drugs working by different mechanisms, were they significantly acting with other substance? And how did the probiotics made the difference? Did they affect ROS production and did they modulate ROS activity?

Actually, the changes in histology were coupled also with ROS modulation as expected.

In general, cancer cells increase their rate of ROS production, compared to normal cells, and increase their susceptibility to ROS-manipulation therapies. The association of ROS with cancer cells could be oncogenic at high levels (Ames, Shigenaga, & Hagen, 1993) thus promoting cancer cell proliferation, survival, angiogenesis and metastasis (Schieber & Chandel, 2014). To maintain redox balance, cancer cells increase their antioxidant capacity by scavenging excess ROS. Results showed that in the absence of the treatments (G1 and G5), ROS production increased as expected in the control xenografted animals. On the other hand, the 2 drugs rapamycin and metformin could decrease significantly ROS levels in both diabetics and non-diabetics without showing any added value to the combination therapy even with probiotics. The highest values of ROS were associated with the non-treated xenografted animals with or without diabetes. The mechanism of ROS reduction by the 2 drugs is probably independent of probiotics. This increase in oxidative stress induced more cancer cell death and smaller tumors (Nogueira et al., 2008). The cancer cells were as sensitive in both metformin and rapamycin treated animals. Actually, ROS was probably

maintained in the various groups at a level that allows for the activation of protumorigenic signaling pathways. Hence, strategies to eliminate ROS or produce ROS may be effective in cancer therapies. Both drugs led to a remarkable decrease in ROS production and consequently a reduction in its pro-cancer effect. ROS also oxidize and inactivate MAPK phosphatases and MAPK/ERK pro-proliferative signaling (Son et al., 2011).They also promote tumor cell survival through the activation of NFKB and NRF2 transcription factors that upregulate the expression of antioxidants to evade ROS –mediated cancer cell death (Morgan & Liu, 2011).

Moreover, data showed that high values of ROS promoted tumor angiogenesis and metastasis as detected in G1 and G5 control non-treated groups. Such cases are usually associated with poor prognosis and activate the AMPK (Ye et al., 2014). Actually, ROS levels increased in these solid tumors and AMPK was activated to probably promote NADPH production. On the other hand, loss or significant decrease of AMPK by metformin or rapamycin, could prevent oncogenic transformation (Laderoute et al., 2006). In brief, ROS has been shown to regulate numerous signaling pathways (e.g. MAPK PI3K/Akt and TNK pathways) and decreasing ROS levels could prevent cancer cell proliferation. Therefore, developing methods to decrease intracellular ROS levels and prevent cancer cell proliferation is an attractive field; are rapamycin and metformin really doing this effect? So far, ROS manipulation strategies have previously focused on antioxidant therapy. A better understanding of the molecular mechanisms of ROS signaling in cancer and the identification of specific ROS targets may provide novel therapeutic avenues for treating cancer. Could probiotics be performing this task?

Emerging research on CRC points out to a complex network of genetic alterations leading to dysregulation of multiple pathways. Moreover, as proposed by CRC subtyping consortium, there are 4 major molecularly distinguishable subtypes of CRC (Dienstmann et al., 2014) and when coupled with T2DM, they tend to have a less favorable prognosis (Suh, Choi, Plauschinat, Kwon, & Baron, 2010). Further, patients with inadequate glycemic control may have an even higher risk of CRC and need to receive polytherapy(Wilkinson & Culpepper, 2011); a potential indication for metformin and rapamycin.

In this context, observational studies have suggested that some anti-hyperglycemic agents like metformin, could decrease or prevent cancer risk (Barnes et al., 2004; Foretz, Guigas, Bertrand, Pollak, & Viollet, 2014) . Metformin could activate AMPK, a central regulator and an important

target for controlling human diseases including T2DM and cancer. AMPK could cause cell cycle arrest in response to metabolic stress through a number of mechanisms (J. Kim, Yang, Kim, Kim, & Ha, 2016). In addition, AMPK might protect sometimes tumor cells against action of cytotoxic agents and hypoxia once tumor is established, or even delay the onset of tumorigenesis in vivo models (Evans et al., 2005). Along this line, studies on the AMPK have shown that mTORC1 and RNA polymerase I transcription factor TIF-1A, both of which are required for rapidly proliferating cells, are under the control of AMPK (Evans et al., 2005).

In this study, AMPK levels in non-treated diabetics (G5) were 31% lower than in non-treated non-diabetics (G1). When metformin was administered alone, a slight decrease in AMPK was observed. Notably, when probiotics were added, these levels decreased remarkably in diabetics and non-diabetics by 40%. This behavior remains unexplained and requires further explanation as to decipher the mechanism which lowered AMPK despite the administration of metformin, an AMPK activator.

On the other hand, the mTOR pathway components are over expressed in CRC (S. C. Johnson, Rabinovitch, & Kaeberlein, 2013). The mTOR combines with raptor(regulatory associated protein of mTOR)to constitute mTOR complex 1(mTORC1) and rictor (rapamycin-insensitive companion of mTOR) to make mTORC2 (Samuels et al., 2004).Consequently, mTOR also emerged as a compelling molecular target for treating several malignancies (Francipane & Lagasse, 2016). There are two different types of mTOR inhibitors, (1) ATP competitive mTOR inhibitors that block the activity of mTORC1/mTORC2, and(2) rapamycin analogs that influence the activity of mTORC1 (Abubaker et al., 2008). Which case applies in this study?

Rapamycin inhibits the mTORC1 activity, suppresses the proliferation of the adenoma cells, inhibits of tumor angiogenesis and decreases the size and number of polyps (D.-D. Kim & Eng, 2012). It also inhibits tumor growth in a dose dependent reduction in HCT116 xenografts (Raymond et al., 2004). All such effects are encountered in this study.

In this study, the mTORC gene expression was minimal or not expressed at all except in the rapamycin treated diabetic animals where it exhibited relatively high values. However, the expression at the protein level was relatively highest in the rapamycin treated diabetics in concordance to the gene expression level. On the other hand, the expression of p-mTOR protein was relatively more suppressed in the combination therapy especially when probiotics were added, both with diabetics and non-diabetics. Among the rest of the groups mTOR and p-mTOR were

moderately expressed. Consequently, the clinical improvements of the health status of the animals or the cancers themselves were do not seem to be related to the p-mTOR levels of suppression.

Data of K167 depicted that the proliferation of the cancer cells continued in these suppressed groups but at a much lower pace, a phenomenon which might explain the decrease in size of the tumors in the suppressed groups. In brief, a reduction in the number of proliferating KI67 positive cancer cells and decrease in mast cells were noted. Such changes were in concordance with the lower levels of gene expression of molecules involved in the mTOR pathway. The uncontrolled mTORC1 mediated signaling could be basically explained by the intricate signaling network of mTOR and possibly the inability of rapamycin to completely block mTORC1 mediated signaling events; the presence of several feedback loops, and the upregulation of compensatory pathways that promote cell survival and growth.

As for the proinflammatory cytokines, IL-6 gene expression was remarkably suppressed in all groups except in the non-diabetic non-treated animals. However, IL-3 had a different profile of suppression; complete suppression by either drugs, metformin or rapamycin in the non-diabetics without an added effect of the drugs, but with a complete suppression in the presence of probiotics. In the diabetics, however, metformin did not show any significant suppression but the combination therapy with or without probiotics did have a significant inhibition of IL-3. However, a different suppression profile was noted with the TNFα ; all non-diabetic groups were suppressed. In diabetics, suppression was also significant in the non-treated and those treated with combination therapy with or without probiotics, however, there was a partial suppression by metformin alone and no suppression by rapamycin. In brief, the drugs did control to various extents the inflammatory process.

The inflammatory cytokines which are supposed to activate mTOR (Zoncu et al., 2011) were suppressed, in particular, when using the combination of rapamycin and metformin, in presence or absence of probiotics. It seems, in this case that the treatment with rapamycin might be further potentiated with the antidiabetic drug metformin (Anisimov, 2013; Martin-Montalvo et al., 2013)and even more so by probiotics. Such results were more evident when the p-mTOR protein was assessed; it depicted a much lower expression than the mTOR especially with metformin, rapamycin, and the 2 drugs in presence of probiotics, both in diabetic and non-diabetic animals. However, the additive effect of both drugs was not apparent; there was actually a relative but not significant rise, with the combination therapy. Such results are congruent with the other data

collected on proliferation, ROS production, mast cells number, decrease in colonic inflammation, improvement of histological alterations, DAI severity and to some extent with the decrease in tumor volume in both diabetics and non-diabetics. In conclusion, the presence of probiotics in the combination of metformin and rapamycin lead through one or more mechanisms, to the suppression of tumor size, delay in their development, significant inhibition of the inflammatory reaction, as well as a decrease in ROS production and cell proliferation, significant decrease in AMPK and inhibition of the phosphorylated mTOR. Further experiments are needed in this area to elucidate the complexity of the pathways involved and eventually the specific targeted molecules as well as the exact role of probiotics and their mechanism of action.

The use of prebiotics, probiotics, and symbiotics can positively affect the microbial balance of the intestinal tract and its ability to function properly. The mechanisms by which probiotics may inhibit colon cancer are not yet fully characterized. However, there is evidence for:

- Alteration of the metabolic activities of intestinal Microflora
- Binding and degrading potential carcinogens
- Short chain fatty acid (SCFA) production
- Production of anti-tumorigenic or anti-mutagenic Compounds
- Elevation of the host's immune response and recognition of probiotics by immune system: toll-like receptors and anti-CRC responses
- Effects on the host's physiology and balancing homeostasis
- Maintenance or enhancement of intestinal barrier function by probiotics
- Inhibitory effect of probiotics on TLR4 and COX-2 Expression
- Folate production and DNA methylation (Chong, 2014; Fotiadis et al., 2008).

Chapter Five: **CONCLUSION AND FUTURE PERSPECTIVES**

The emergence of combination therapy with rapamycin and metformin and/or probiotics may further increase efficacy and bypass possible feedback activation of survival pathways. Significant promise remains for the discovery of new specific signaling inhibitors to reduce mTORC activation, in monotherapy or in polytherapy and decipher the place and role of probiotics in this complex process.

The findings reported in this article suggest that modulation of the gut microbiome with probiotics in combination with the anti-proliferative agents, i.e. rapamycin and the antidiabetic drug metformin, a potential prebiotic agent, could constitute or be a part of new preventive and or therapeutic strategy for CRC management, one of the most common cancers worldwide.

In conclusion, the synergetic action of rapamycin and metformin in association with the probiotics led to reduced expression of early lesions in CRC such as aberrant crypt foci (ACF). Supplementation of the combination by probiotics for two weeks, in mice with xenografts could possibly lead to a decrease in the formation of classical ACF, an increase in apoptosis and reduced rates of inflammation, PCNA and *p*53 positive cells.

It is likely that the established chronic inflammatory process combined with dysbiosis could contribute to an oxidative stress, an increase in reactive oxygen and nitrogen species, as well as proinflammatory cytokines key factors involved in the development of CRC. Such factors were reduced by the various treatments to different extents.

In order to reduce or inhibit carcinogenesis linked to oxidative stress. Consequently, a strategy of chemo prevention involving the administration of exogenous compounds which intervene with the proliferation of cancer cells and with the blocking of their oncogenic transformation, as well as lowering hyperglycemia, could probably constitute a novel strategy. Would metformin and rapamycin coupled with probiotics serve the purpose?

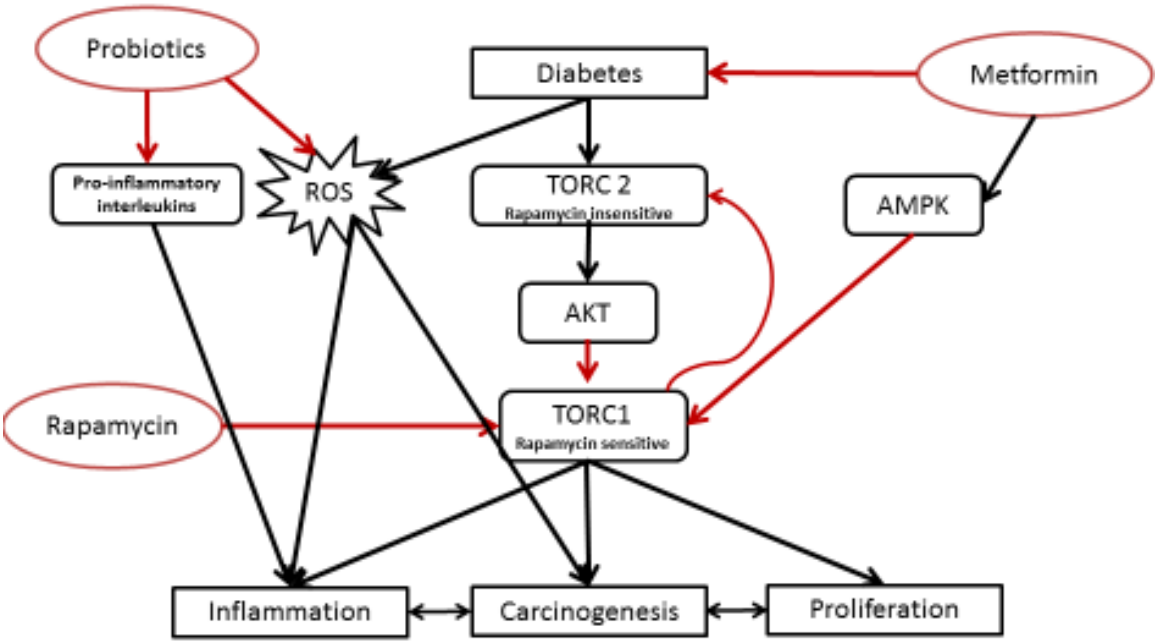


Figure 30: Diagrammatic representation of the proposed mechanism of action of the tri-therapy.

It depicts the interactions and feedback loops within the CRC-diabetes network. Black arrows indicate positive regulations and red arrows indicate inhibition.

In this crosstalk, probiotics act mainly by downregulating pro-inflammatory cytokines and ROS formation. On the other hand, mTOR pathway was inhibited through Rapamycin and Metformin via AMPK activation. As a result of the downregulation of these pathways, an inhibition of the inflammatory and carcinogenic processes was observed in our diabetic CRC model.

Chapter Six: **LIMITATIONS OF THE STUDY**

This study has several limitations. First, a small number of animals per treatment group was used ($n = 6$), especially in the probiotics treated mice, whereby, more controls groups could have been also included. Moreover, this study only assessed the response of male NOD/SCIDs mice, it would have been better if the 2 genders were part of the study.

Another major limitation of this CRC model is the NOD/SCIDs mice; they have a compromised immune system leading to the loss of the complex interactions between tumor and host. Thus, they may not represent the behavior of naturally occurring cancers in humans.

Another restraint is the genetic and epigenetic changes which may occur in the tumor cells during culture and implantation, despite the fact that the cells were in early stages of culture.

Therefore, future studies assessing the effects of rapamycin, metformin and probiotics should be conducted on a larger number of animals from both genders. Clinical studies are also required to demonstrate the beneficial effects of these treatments on patients and to elucidate the safety and correct regimens for the prevention and management of CRC.

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