Université Libanaise

École Doctorale
Sciences et Technologies

THESE de Doctorat en Cotutelle

Pour obtenir le grade de Docteur délivré par

L'Université Libanaise et l'Université de Limoges (France)

L'Ecole Doctorale des Sciences et Technologie

ED 615 Sciences Biologiques et Santé

Cancérologie

Présentée et soutenue publiquement par

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Jeudi 3 Décembre 2020

Role of Metformin and Probiotics in The Crosstalk Between Inflammatory Bowel Disease, Colorectal Cancer and Diabetes

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Dedication

كُنْ مع الله ولا تُبالي

ولا تَنَم غير خالي البالِ

فبين رمشةٍ أنت تَرمِشُها

يُغيّر الله من حالٍ إلى حالِ

To the loving memory of my guardian angel, Weddo

Acknowledgments

First and Foremost, I would like to *thank God*, for all the blessings and Miracles in my life.

I would like to express my sincere gratitude to my Director at the University of Limoges, **Pr. Bertrand Liagre,** the lively, energetic and cheerful Director. You have made this journey much easier and my stay at Limoges memorable. I could have never imagined having a better advisor.

I owe my deepest gratitude to my thesis Director at the Lebanese University, **Pr. Chawki Bou Karim**, for his encouragement, insightful advices and guidance during the completion of this project. Thank you for placing your trust and confidence in my abilities.

Besides my advisors, I would like to thank my thesis committe members**, Pr. Philippe Billiald, Pr**. **David-Yannick Leger**, **Pr. Asaad Eid, and Pr. Mona Diab-Assaf** for generously offering their time, support, and assistance throughout the preparation and review of this thesis.

I would like to thank two beautiful people, **Dr. Rosalyn Jurjus** and **Mrs. Aline Pinon**, I am grateful for your kindness, help and positive vibes.

Special thanks go to my committee chair, **Pr. Abdo Jurjus**. I am grateful for being a part of your lab for the past 6 years, you have pushed me to sharpen my thinking and brought my work to a higher level. Without your guidance and persistent help, this dissertation would not have been possible.

I would also like to thank my friends, for bringing so much fun, and laughter to my life. You are all part of this success story.

I wish to express my love and my gratitude to my Parents, for their payers and sacrifices. Thank you for giving me strength to reach for the stars and chase my dreams. "Ma petite Nanou", you are the better version of me, I love you unconditionally and I will always be there for you.

Finally, I would like to express my gratitude for my Husband, who has been by my side throughout this PhD, living every single minute of it, and without whom, I wouldn't have had the courage to embark on this journey in the first place. You are the best thing that ever happened to me.

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Abbreviations

A

AJs: adherent junctions

AMPs: anti-microbial peptides

ANOVA: analysis of variance

AOM: azoxymethane

APC: adenomatous polyposis coli

C

CAT : catalase

CFU: colony-forming unit

CIMP: CpG island methylator phenotype

CRC: colorectal cancer

D

DAPI: 4', 6-diamidino-2-phenylindole

DCC: deleted in colorectal carcinoma

DHE: dihydroethidium

DNA: deoxyribonucleic acid

DSS: dextran sulfate sodium salt

F

FAP: familial adenomatous polyposis

FDA: food and drug administration

G

GIT: gastrointestinal tract

Gpx: glutathione peroxidase

H

H&E: hematoxylin and eosin

HNPCC: hereditary non-polyposis colorectal cancer

HPF: high power field

I

IBD: inflammatory bowel disease

IECs: intestinal epithelial cells

IL-6: interleukin-6

iSGLT2: sodium-glucose co-transporter 2 inhibitors

K

KRAS: Kirsten rat sarcoma viral oncogene homolog

L

LOH: loss of heterozygosity

LPS: lipopolysaccharides

M

MAPK: mitogen activated protein kinase

MMR: mismatch repair

MSI: microsatellite instability

mTOR: mammalian target of rapamycin

N

NO: nitric oxide

NSAIDs: Non-steroidal anti-inflammatory drugs

P

PBS: phosphate-buffered saline

R

RA-GLP1: glucagon-like peptide 1 receptor agonist

RCS: reactive chlorine species

RONS: reactive oxygen and nitrogen species

ROS: reactive oxygen species

S

SCFA: short-chain fatty acids

SEM: standard error of the mean

SIgA: secretory immunoglobulin A

STZ: streptozotocin

T

T2DM: type 2 diabetes mellitus

TBST: tris-buffered saline and tween

TJs: tight junctions

TNF-α : tumor necrosis factor-alpha

TP53: tumor protein 53

TZD: thiazolidinediones

Introduction

Colorectal cancer (CRC) also referred to as bowel cancer, colon cancer, or rectal cancer is the development of cancer from the colon or rectum. According to the World Health Organization GLOBOCAN database, CRC is the third most commonly diagnosed cancer worldwide with 1.8 million new cases and almost 881,000 deaths in 2018 [1]. Its burden is predicted to increase by 60% in 2030 due to several factors, mainly the aging of the population and the accommodation to the sedentary lifestyle [2]. In Lebanon, CRC has a high prevalence as it accounts for 8.5% of all cancers [3].

The majority of CRC patients are diagnosed with a resectable localized disease. Surgery, followed by adjuvant therapy for high-risk patients, is considered as the optimum curative treatment approach in such cases. However, ultimately, approximately half of all diagnosed CRC patients will develop disseminated advanced disease, which requires medical management and in most cases will be fatal [4]. In parallel, diabetes mellitus affected 451 million people worldwide in 2017 and its prevalence keeps an increasing trend. Actually, this number is expected to rise to 693 million by 2045 [5]. The increasing trend of diabetes makes it imperative that research should focus on its prevention as well as its treatment. Several recent studies focused on the role of inflammation in the onset of diabetes, its poor prognosis and on understanding the mechanisms linking inflammation to diabetes and its related complications. Such interrelationship has stimulated interest in targeting inflammatory pathways as part of the strategy to prevent or control diabetes and its complications, especially in preparing a favorable ground for malignant diseases [6].

Although there has been some debate regarding the effects of diabetes on CRC, meta-analyses consistently show that Diabetes Mellitus (DM) is an independent risk factor for CRC and that diabetic patients with CRC may have worse outcomes than their non-diabetic counterparts [7]. Moreover, these two disease entities share a panoply of common risk factors. The pathophysiological mechanisms of diabetes, including insulin resistance, hyperglycemia and resulting hyperinsulinemia, are all associated with the development and progression of cancer [8]. The co-occurrence of DM and CRC along with inflammation and dysbiosis has been frequently reported by our team [9,10] and by others [11].

In this study, a new therapeutic approach in treating colorectal cancer associated with diabetes was used. Hereby, metformin, an antidiabetic drug frequently and widely used, was tested for its anticancer properties along with probiotics, the microbiota modulating agents.

Metformin is known to inhibit hepatic glucose production and decreases insulin resistance in peripheral tissues, thereby reducing levels of circulating glucose levels and improving insulin sensitivity. Epidemiological studies have shown that patients with type 2 DM who are taking metformin have a lower risk of cancer and better outcomes compared with patients who do not take metformin. Although there has been substantial evidence from *in-vivo* and *in-vitro* studies supporting the potential efficacy of metformin as an anti-cancer agent, there have been no clinical studies investigating the effect of metformin on CRC in presence of probiotics [12].

On the other hand, Probiotics have obtained increasing medical importance because of their beneficial effects upon the host health. In addition to the homeostasis regulation of the intestinal epithelial system and immune responses, probiotics have shown to possess antitumor activity using various mechanisms [13].

In the following chapters, the characteristics of colorectal cancer and diabetes at the clinical, molecular and histological levels will be discussed.

Chapter one will cover the anatomy and physiology of the gastrointestinal tract and the large intestine in particular, with a focus on the intestinal barrier, as well as an extensive definition of the microbiota and its importance in the context of inflammation and carcinogenesis.

Additionally, the key players in the crosstalk between CRC, diabetes and inflammation will be highlighted, mainly oxidative stress and inflammation.

Afterwards, metformin and probiotics' potential in treating and preventing colorectal cancer and ameliorating the diabetic phenotype will be discussed.

The subsequent chapters will outline the work done on the established in-*vivo* model while trying to decipher the mechanisms of action of our treatment combination, metformin and probiotics.

Abstract

Background: the co-occurrence of colorectal cancer (CRC), Inflammatory Bowel diseases (IBD) and diabetes mellitus along with inflammation and dysmicrobism has been frequently reported. Several studies shed light on the anti-oncogenic potential of metformin in colorectal carcinogenesis as well as the beneficial effects of probiotics on inflammatory diseases.

Aims: this study aimed to demonstrate that metformin in association with probiotics act in a synergic effect in breaking the crosstalk, thus inhibiting CRC progression, improving diabetes and reducing inflammation.

Methodology: ninety-six male Balb/c mice, 6-8 weeks old, were divided into 16 control and experimental groups to assess the effect of the different treatments and combinations at the clinical, histological and molecular levels. Pro-inflammatory cytokines IL-6 and TNF-α levels were assessed, as well as reactive oxygen and nitrogen species. Moreover, the proliferation index of colocytes was determined by Ki-67 immunohistochemistry.

Results: metformin and probiotics showed beneficial outcomes on CRC and diabetes, alone and most importantly in combination. Their effects were exerted by reversing the histopathological alterations and by inhibiting the inflammatory process whereby a downregulation of IL-6 and TNF-α as well as oxidative stress were depicted.

Conclusion: the characterization of the effects of probiotics and metformin on CRC and diabetes sheds light on the role of inflammation and microbiota in this crosstalk. Deciphering more the downstream signaling pathways elicited by these compounds will help in developing new effective targeted treatment modalities.

Keywords: Colorectal cancer, Inflammatory Bowel Diseases, Diabetes, Probiotics, Inflammation, Metformin.

Résumé

Contexte: la concomitance du cancer colorectal (CCR), des maladies inflammatoires de l'intestin et du diabète sucré avec l'inflammation et le dismicrobisme a été fréquemment rapportée. Plusieurs études ont mis en lumière le potentiel anti-oncogénique de la metformine sur la carcinogenèse colorectale, ainsi que les effets bénéfiques des probiotiques sur les maladies inflammatoires.

Objectifs: cette étude vise à démontrer que la metformine en association avec des probiotiques agit en synergie en brisant la diaphonie, inhibant ainsi la progression du CCR, améliorant le diabète et réduisant l'inflammation.

Méthodologie: quatre-vingt-seize souris mâles balb/c, âgées de 6 à 8 semaines, ont été divisées en 16 groupes témoins et expérimentaux pour évaluer l'effet des différents traitements et combinaisons aux niveaux clinique, histologique et moléculaire. Les niveaux de cytokines pro-inflammatoires IL-6 et TNF-α ont été évalués, ainsi que les espèces réactives de l'oxygène et de l'azote. De plus, l'indice de prolifération a été déterminé par immunohistochimie Ki-67.

Résultats: la metformine et les probiotiques ont montré des résultats bénéfiques sur le CCR et le diabète, seuls et surtout en association. Les traitements ont réduit le processus inflammatoire en diminuant la production d'IL-6 et de TNF- α ainsi que le stress oxydatif.

Conclusion: la caractérisation des effets des probiotiques et de la metformine sur le CCR et le diabète révèle le rôle de l'inflammation et du microbiote dans cette diaphonie. Décrypter les voies de signalisation en aval induites par ces composés aidera à développer de nouvelles thérapies ciblées efficaces.

Mots-Clés: Cancer Colorectal, Maladies inflammatoires de l'intestin, Diabète, Inflammation, Probiotiques, Metformine.

Chapter 1: Literature review

A- Overview of the gastrointestinal tract

The portion of the alimentary canal extending from the proximal part of the esophagus to the distal part of the anal canal is a hallow tube of varying diameter. This tube has the same structural organization throughout its length. Its wall is formed by four distinctive layers from the lumen outward (Figure 1).

They are as follows:

- Mucosa, consisting of a lining epithelium, an underlying connective tissue called the lamina propria and the muscularis mucosa composed of smooth muscle. These structures differ between the different parts of the alimentary canal to adapt to tits specific functions. The main functions of the mucosa are protection, absorption and secretion.

- Submucosa: consisting of dense irregular connective tissue layer containing blood and lymphatic vessels, a nerve plexus and occasional glands.

- Muscularis externa, consisting in most parts of two thick concentric layers of smooth muscle. Contractions of the muscularis extern mix and propel the contents of the digestive tract.

- Serosa and adventitia: A serous membrane consisting of a simple squamous epithelium, the mesothelium and a small amount of underlying connective tissue. An adventitia consisting only of connective tissue is found where the wall of the tube is directly attached or fixed to adjoining structures (i.e, body wall and certain retroperitoneal organs). It is the outermost layer of the alimentary canal.

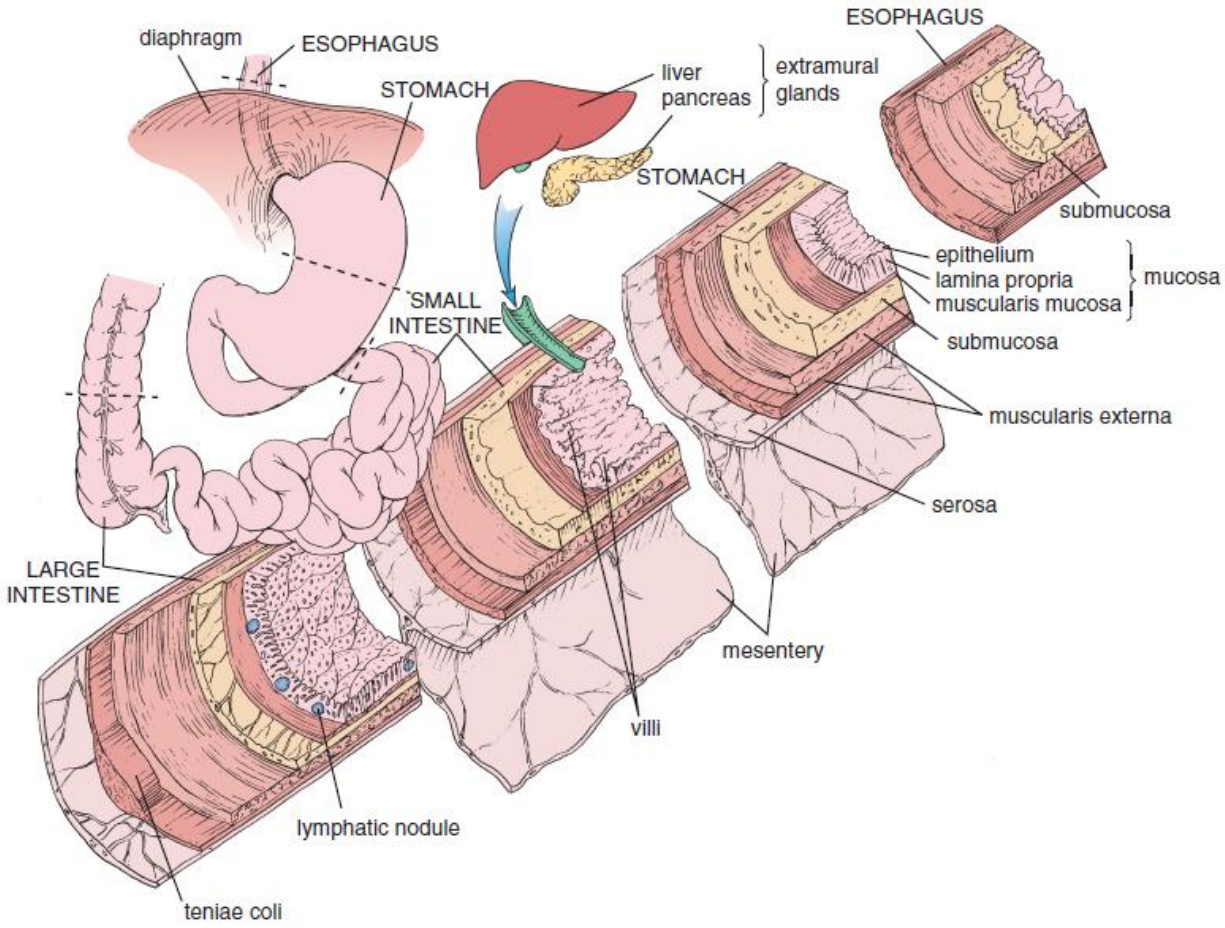

Figure 1: Diagram of general organization of the alimentary canal.

This composite diagram shows the wall structure of the alimentary canal in four representative organs: esophagus, stomach, small intestine, and large intestine [14].

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B- Morphology of the large intestine

The large intestine is a tubular structure about 30 to 40 cm in length at birth, reaching about 150 cm in the adult. Viewed as a whole, the various parts of the large intestine form a horseshoe-shaped arc (Figure 2). It extends from the cecum to the anal canal and consists of different segments: the cecum, the vermiform appendix, colon, rectum, and the anal canal. The colon is further subdivided on the basis of its anatomic location into ascending colon, descending colon and sigmoid colon [14,15].

- **The cecum:** a pouch connected to the ascending colon and the ileum; it prolongs below the ileocecal junction and is surrounded by a mesentery. The cecum has the greatest diameter (7.5 cm approximately), narrowing down to the sigmoid.
- **Appendix:** a narrow, fingerlike extension of the cecum. The appendix contains many lymphoid nodules; it is suspended by a mesentery (the mesoappendix).
- **Ascending colon:** It is located retroperitoneally, and ascends on the right flank to reach the liver, where it turns into the right colic flexure (or hepatic flexure).
- **Transverse colon:** It is attached by the transverse mesocolon, it runs from the right and bends on the left to form the splenic flexure.
- **Descending colon:** It descends retroperitoneally along the left flank to intersect with the sigmoid colon in the left groin area.
- **Sigmoid colon:** It is suspended by the sigmoid mesocolon and runs medially to join the midline rectum in the pelvis. The sigmoid colon has the smallest diameter of about 2.5 cm.
- Rectum and anal canal: They extend from the middle sacrum until the anus [14,15].

The large intestine has a greater luminal diameter than the small intestine, and is characterized by the presence of distinct features at the gross level:

- **Teniae coli:** three longitudinal equally spaced bands of muscularis externa, primarily visible in the cecum and colon. In addition, they are absent in the rectum, anal canal and vermiform appendix. Teniae coli contribute in peristalsis
- **Haustra:** Pouches or sacculations of the colon formed by teniae coli's contractions.
  - Omental appendices: small fatty accumulations covered by visceral peritoneum, suspending on the outer surface of the colon [14,15].

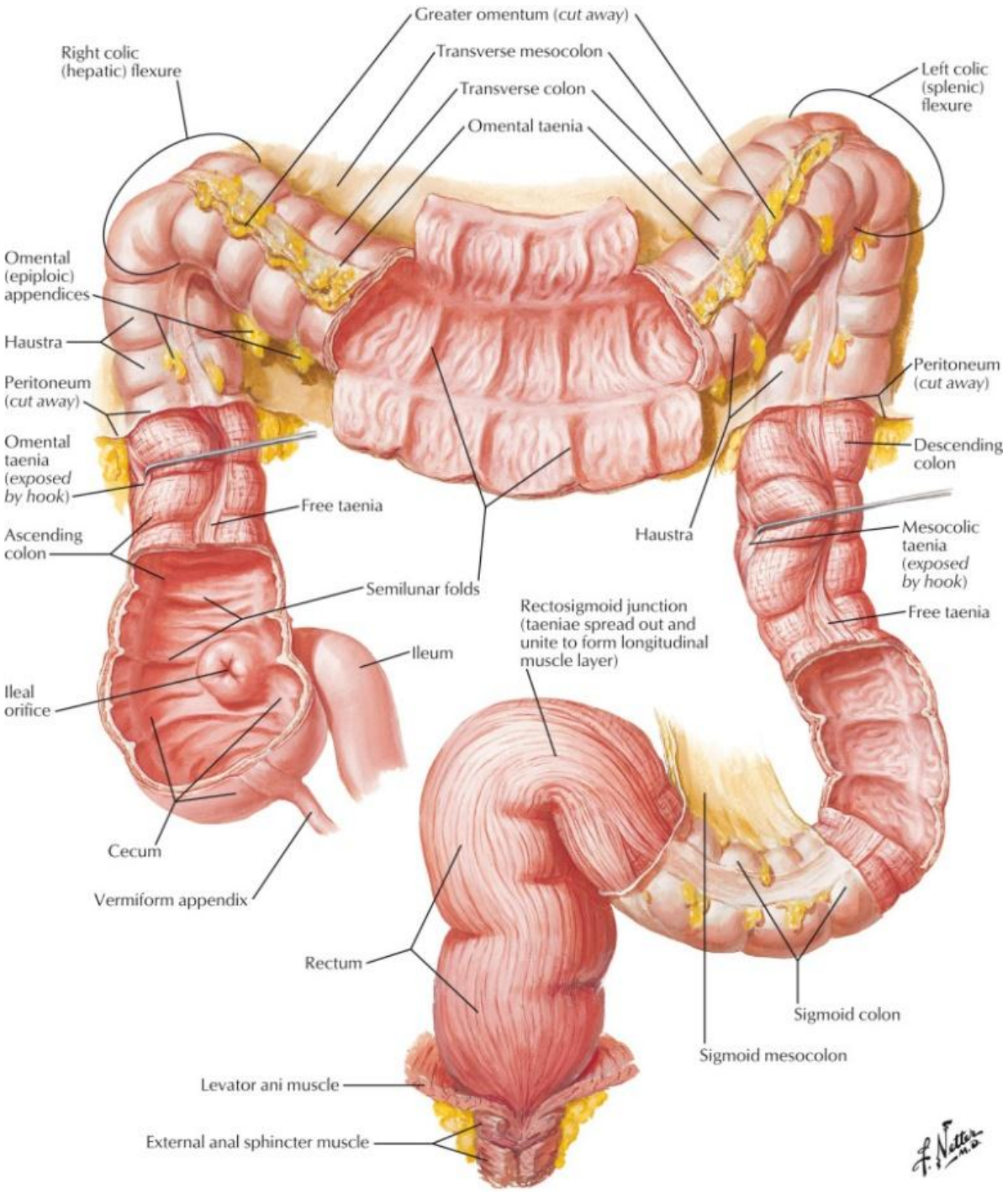


Figure 2: Features of the musculature of the large intestine [16]**.**

The colon, like the entire intestinal tract consists in general of four layers: (1) a mucosa, (2) a submucosa, (3) a double-layered musculosa, and (4) a serosa (Figure 3).

1. Mucosa

The mucosa of the large intestine has a smooth surface; neither plicae circulares nor villi are present. It contains the same cell types of the small intestine except Paneth cells, which are normally absent in humans. It consists of the epithelium and lamina propria.

The epithelium contains numerous straight intestinal glands (Crypts of Lieberkühn) that extend through the full thickness of the mucosae. The gland consists of simple columnar epithelium, as does the intestinal surface from which they invaginate.

The primary function of the columnar absorptive cells is reabsorption of water and electrolytes. This reabsorption is accomplished by the Na+/K+ activated ATPase driven transport system.

Goblet cells are more numerous in the large intestine than the small intestine. They produce mucin that is secreted continuously to lubricate the bowel, facilitating the passage of the increasingly solid content. Goblet cells may mature deep in the intestinal gland. They secrete mucus continuously, even to the point where they reach the luminal surface.

Columnar absorptive cells predominate over goblet cells in most of the colon, the ratio decreases, however, approaching 1:1 near the rectum where the number of goblet cells increases.

- Epithelial cell renewal in the large intestine: All intestinal epithelial cells in the large intestine derive from a single stem cell population; these stem cells are located at the bottom of the intestinal gland. The lower third of the gland constitutes the intestinal stem cell niche, where newly generated cells undergo two to three more divisions as they begin their migration up to the luminal surface, where they are shed about 5 days later.

2. Lamina propria

Although the lamina propria of the large intestine contains the same basic components as the rest of the digestive tract, it demonstrates some additional features such as:

- **Collagen table:** a thick layer of collagen and proteoglycans that participates in the regulation of water and electrolyte transport.
- **Pericryptal fibroblast sheath**: a well-developed fibroblast population of regularly replicating cells. It is suggested that the macrophages of the lamina propria in the large intestine may arise as a terminal differentiation of the pericryptal fibroblasts.
- **GALT:** extensively developed, large lymphatic nodules that distort the systematic spacing of the intestinal glands and extend into the submucosa.

3. Muscularis externa

The outer layer of the muscularis externa of the colon and cecum is marked by the presence of distinct structures, the teniae coli, and haustra coli. The muscularis externa of the large intestine produces two major types of contraction: segmentation and peristalsis.

- **Segmentation:** a local contraction that does not induce the propulsion of bowel load.
- **Peristalsis:** contractions that engender the distal mass movement which occur typically once a day to empty the distal colon.

4. Submucosa and serosa

The submucosa of the large intestine corresponds to the layer of blood vessels, nerves and connective tissue surrounding the mucosa, the submucosa supports the other layers of the large intestine. The outer layer of the large intestine is typically a serosa; however, when the intestine is in direct contact with other structures, the external layer is an adventitia.

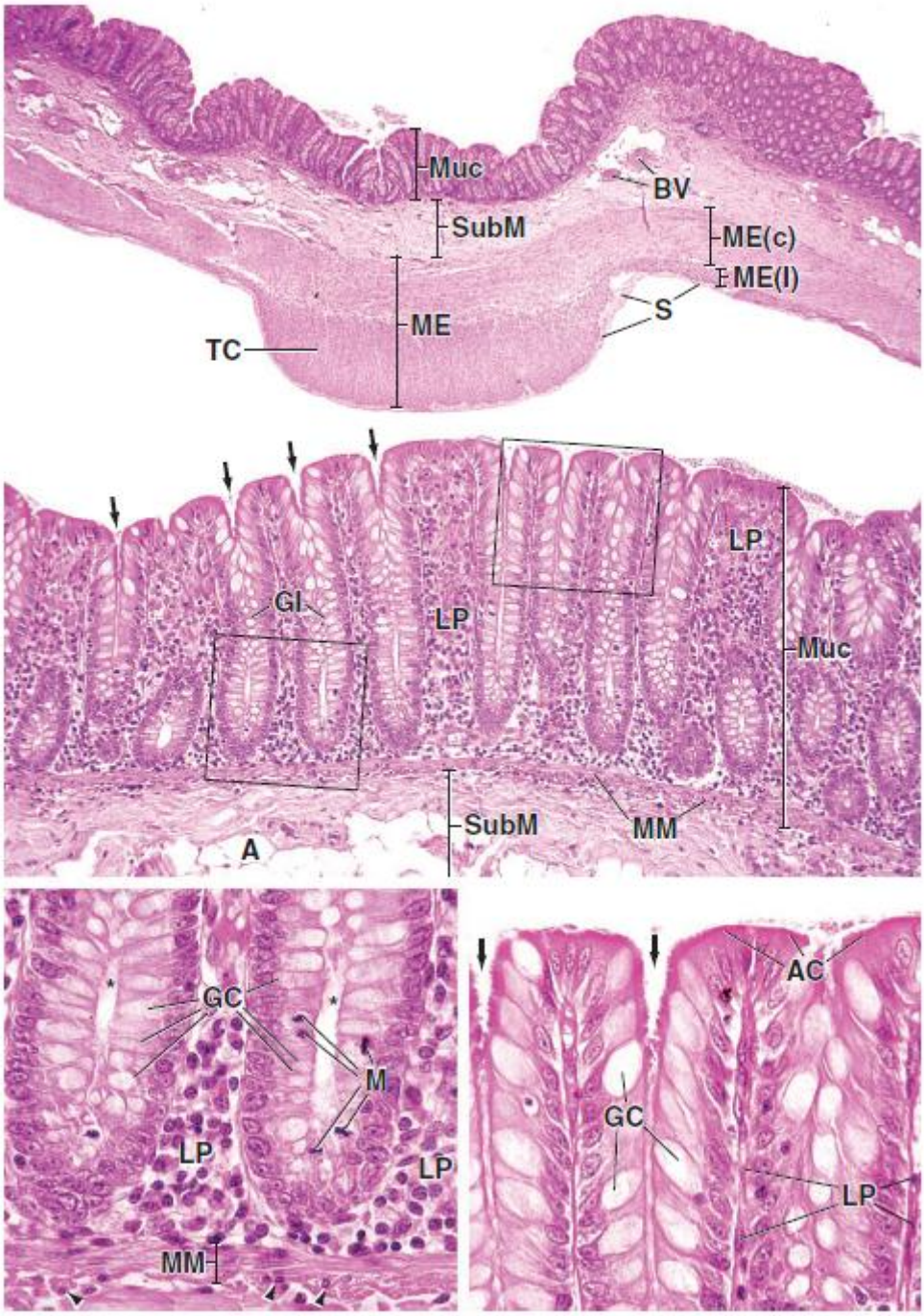


Figure 3 : Histology of the colon (H&E).
A: A cross section through the large intestine is shown at low magnification. Note the four layers that the colon wall: the mucosa *(Muc)*, the submucosa *(SubM)*, the muscularis externa *(ME)*, and the serosa *(S)*.
B: Lamina propria: This figure reveals the muscularis mucosae *(MM)* and the cells in the lamina propria *(LP)*, many of which can be recognized as lymphocytes and plasma cells.
C: Intestinal glands: The cells that line the surface of the colon and the glands are principally absorptive cells *(AC)* and goblet cells *(GC)*. *Arrows* show the opening of the glands.
Note the following abbreviations: A: adipose tissue, BV: blood vessels, GI: intestinal glands
M: mitotic figures, ME(c):circular layer of muscularis externa,ME(l): longitudinal layer of muscularis externa, TC: tenia coli, arrowheads: smooth muscle cells showing rounded nuclei, arrows: opening of intestinal glands [14].

C- The intestinal barrier

The intestinal mucosa is a semi-permeable structure forming a physical and immunological defense barrier (Figure 4). This barrier has a dual role as it allows the selective uptake of substances including essential nutrients and electrolytes. It also restricts the passage of harmful intraluminal entities, including foreign antigens, bacteria, and their toxins.
Three main layers constitute the intestinal barrier: The outer mucus layer, the central single layer and the inner lamina propria [17].

The anatomy of these layers is described in part A. Their specific roles in the activities of the barrier are listed below.

1. The outer mucus layer

The mucosal layer is a chemical barrier overlying the intestinal epithelium; it is considered as the first line of defense that limits the contact between the microbiome and epithelial cells.
In the colon, the mucus is organized as a bilayer composed of an adherent inner layer and a loose detached outer layer; however, the small intestine has only one single detached mucus layer.
The main components of the mucosal layer are mucins. Besides, it comprises the commensal gut microbiota, as well as immune-sensing and regulatory proteins such as antimicrobial peptides (AMPs), and secretory immunoglobulin A (sIgA) [18,19].

- **Mucins**

Mucins are highly glycosylated proteins produced and released by goblet cells, they are the main constituents of the mucus layer. Depletion of mucins leads to mucosal injury, diarrhea and inflammation affect the barrier and confer a predisposition to inflammatory bowel diseases and colorectal cancers [19,20]. In humans there are five oligomerizing secreted mucins (MUC2, MUC5AC, MUC5B, MUC6, and MUC19); with MUC2 as the predominant type in the small and large intestine [21].

- **Antimicrobial peptides (AMPs)**

AMPs are small cationic peptides considered a fundamental component of the innate immunity. The main AMPs in mammals are defensins and cathelicidins. These AMPs exhibit a broad spectrum of antimicrobial activity, their secretion could be continuous or inducible by proinflammatory cytokines or pathogens[22].

- **Secretory IgA (SIgA)**

SIgA is the most abundant class of antibody found in the intestinal lumen of humans and most other mammals. It is known to be the first line of defense in protecting the intestinal epithelium from enteric pathogens and toxins, and maintaining homeostasis [23].

2. The central single cell layer

This is a continuous sheet formed by a monolayer of polarized columnar intestinal epithelial cells (IEC). These cells are tightly attached by apical junctional complexes: the tight junctions (TJs) and adherents junctions(AJs) [18].

Epithelial barrier function is mediated by a series of intercellular junctions that include an apical tight junction (TJ), subjacent adherens junction (AJ), and desmosomes. Given their close structural and functional proximity, the TJ and AJ are collectively referred to as the apical junctional complex (AJC). The backbone of intercellular junctions consists of transmembrane proteins that associate with cytoplasmic plaque proteins anchored to the cytoskeleton [24].

- **Tight junctions**

TJs are the adhesive junctional complexes located at the apical side of the cells, they are multi-protein complexes that function as a selective paracellular barrier facilitating the passage of ions and solutes, while preventing luminal antigens, microorganisms and their toxins [17].

TJs are highly dynamic and their permeability is affected by external and intracellular stimuli and their alteration is implicated in several intestinal and systemic diseases [25].

Under pathophysiological conditions , secreted cytokines such as TNF-α, IFN-γ, IL-1β, IL-4, IL-6, IL-12, IL-13, insulin, and insulin-like growth factor, mediate a dysfunction and

a possible leak of the TJ barrier, resulting in immune activation and tissue inflammation [24,26].

Most importantly, TNF-α-induced alteration of intestinal TJ barrier has been proposed as a chief proinflammatory mechanism in IBD and CRC among others [25].

- **Adherens junctions**

Adherens junctions (AJs) are protein complexes located on the lateral cellular membrane at points of cell to cell contact. They are formed by interactions between transmembrane proteins, intracellular adaptor proteins and the cytoskeleton [17].The main function of adherens junctions is their stabilization of the tight junction which contributes to epithelial barrier function [20].

The major element of the epithelial adherens junction is E-cadherin (or CDH1), a single-spanning transmembrane protein. Disruption of E-cadherin was shown to induce abnormal epithelial differentiation, aggravate inflammatory responses, crypt hyperproliferation and epithelial dysplasia. Moreover, E-cadherin polymorphisms are linked to inflammatory bowel disease and colonic adenocarcinoma [20].

- **Desmosomes**

Desmosomes (DMs) provide mechanical strength to the epithelium. Their transmembrane cadherins include desmoglein and desmocollin proteins. 7 DM cadherins exist including 4 desmogleins and 3 desmocollins. In humans, desmoglein 2 and desmocollin 2 are expressed in human intestinal epithelial cells.

The role of DM proteins in regulating the intestinal epithelial barrier is not fully established yet, however, these junctions play an important role in regulating the mechanical stress generated by the intestinal epithelium [24].

3. The inner lamina propria

This layer, the lamina propria is a supportive layer of conjunctive tissue situated beneath the intestinal epithelium. Innate and adaptive immune cells such as T cells, B cells, dendritic cells and macrophages, reside in the lamina propria [18,27].

These cells play central roles in the immunomodulation and defense mechanisms. Their functions include phagocytosis, elimination of pathogenic substances, cytokine production, as well as the conservation of epithelial barrier function. In addition to their ability to produce prostaglandin E2, they produce also anti-inflammatory cytokines such as IL-10 among others [28].

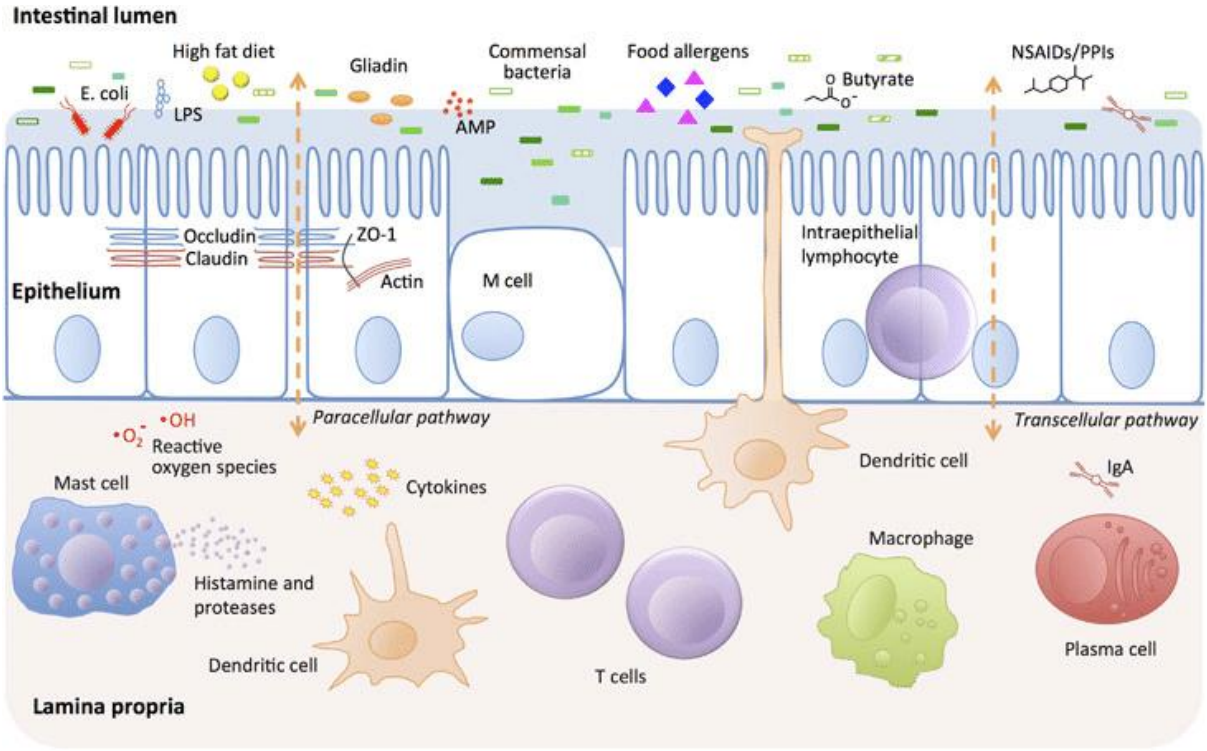


Figure 4: Schematic figure of the intestinal barrier and its components [29].

D- Gut Microbiome

The human body comprises more than 100 trillion microbes, most of which are held in the gut, forming different communities living in a vast range of body niches. This population is called the microbiome and encompasses a wide selection of microorganisms, including archaea, viruses, fungi and anaerobic bacteria, the most studied group since they are the most abundant.

These microbial communities are achieved after birth and are essential for preserving body homeostasis[30].It is estimated that a total of 3.3 million microbial genes form the collective microbial genome, this number is about 150 times greater than the human genome. Thus, the presence of this wide array of genes sheds light on the important impact of intestinal microorganisms on the human body [31].

The dynamic host-microbiome interaction is controlled by a wide range of factors, genetic and environmental, such as age, alcohol, diet, antibiotic use, mode of birth...among others [30]. Intestinal microorganisms play a critical role in human physiology and metabolism. Given its wide spectrum of types and functions, it is not surprising that alterations in normal flora are on the basis of several systemic and intestinal diseases [32]. An alteration in the normal microbiota is defined as dysbiosis, a condition characterized by a disruption in the normal relationship between the host and the intestinal microbiota [33].

1. Metabolic functions of the microbiota

Many reports consider the intestinal microbiota as a metabolic organ as it interacts with the host and upholds human health via various fundamental pathways. These pathways are involved in the metabolism of energy, amino acids, nucleotides, carbohydrates, cofactors and vitamins, as well as the biosynthesis of secondary metabolites. [31].

Importantly, the fermentation of complex carbohydrates by microbiota leads to short-chain fatty acids (SCFAs) production. These SCFAs constitute a vital energy source and essentially they possess immunomodulatory and anti-inflammatory properties [34]. Acetate, propionate, and butyrate are the major SCFAs produced.

- Acetate is the most abundant SCFA, it is a crucial metabolite for bacterial growth , plays a role in cholesterol metabolism and lipogenesis, and in central appetite regulation.
- Propionate is transferred to the liver, where it regulates gluconeogenesis and satiety signaling through interaction with the gut fatty acid receptors.
- Butyrate is the main energy source for colonocytes, it has different functions ranging from induction of cancerous cells apoptosis, to intestinal gluconeogenesis activation, maintaining oxygen balance in the gut and preventing gut microbiota dysbiosis [35].

2. Microbiome perturbations, immune dysfunction, and chronic disease

Several studies shed light on perturbations affecting the composition as well as the functions of gut microbiota in a wide spectrum of diseases. These include Inflammatory bowel diseases (IBD),clostridium difficile Infection, atopic asthma, behavioral disorders like autism spectrum disease (ASD) , celiac disease , colorectal cancer, obesity and type 2 diabetes mellitus (T2DM), cardiovascular disease, as well as autoimmune diseases like rheumatoid arthritis , multiple sclerosis, and type 1 diabetes mellitus [36,37].

3. Dysbiosis and CRC

The microbiome is now considered one of the prime suspects responsible for the onset and evolution of gastrointestinal disorders and specifically colorectal carcinogenesis. Along this line, dysbiosis is now considered to be a key factor in the development of IBD and CRC [38,39]. Although it is not yet clear how dysbiosis could induce colonic carcinogenesis, chronic inflammation appears to be the core mechanism. The first stages of both CRC and IBD diseases involve an alteration to the normal flora, which results in activation of the immune system, thus giving rise to a chronic inflammatory state characterized by an upregulation of pro-inflammatory cytokines and reactive oxygen species, thus creating a tumor-favorable microenvironment [40].

E- Physiology of the large intestine

The large intestine has three major functions: secretory, digestive and absorptive (Figure 5). Within 8-9 hours of ingestion, meals reach the large intestine. About 90% of the ingested water is absorbed by the small intestine, and the rest is absorbed by the large intestine, a process that converts liquid chyme residue into semi-solid stools or feces [41].

The muscular anatomy of the colon is characterized by concentration of the longitudinal muscle into bands named teniae coli. Contraction of the teniae coli and the circular muscle results in haustrations. Segmenting colonic contractions help in water and electrolytes absorption, and contents pass slowly, usually taking days to pass through the colon augmenting the time for water and electrolytes absorption [42,43].

Closure of the anal canal is preserved by a tonic contraction of the internal anal sphincter. Distention of the rectum stimulates the internal anal sphincter to relax and causes the need to defecate. Colonic movements are also controlled by activities of the intrinsic and extrinsic nerves and possibly by hormonal regulations [43].

Feces are formed mostly of bacteria, old epithelial cells from the intestinal mucosa, inorganic waste, undigested food matter and fiber, small amounts fats and proteins. The brown color is due to hemoglobin breakdown products stercobilin and urobilin [42].

Functional problems of defecation, vascular disorders of hemorrhoids, as well as several other gastrointestinal and systemic diseases are related to the anatomy of the large intestine [41].

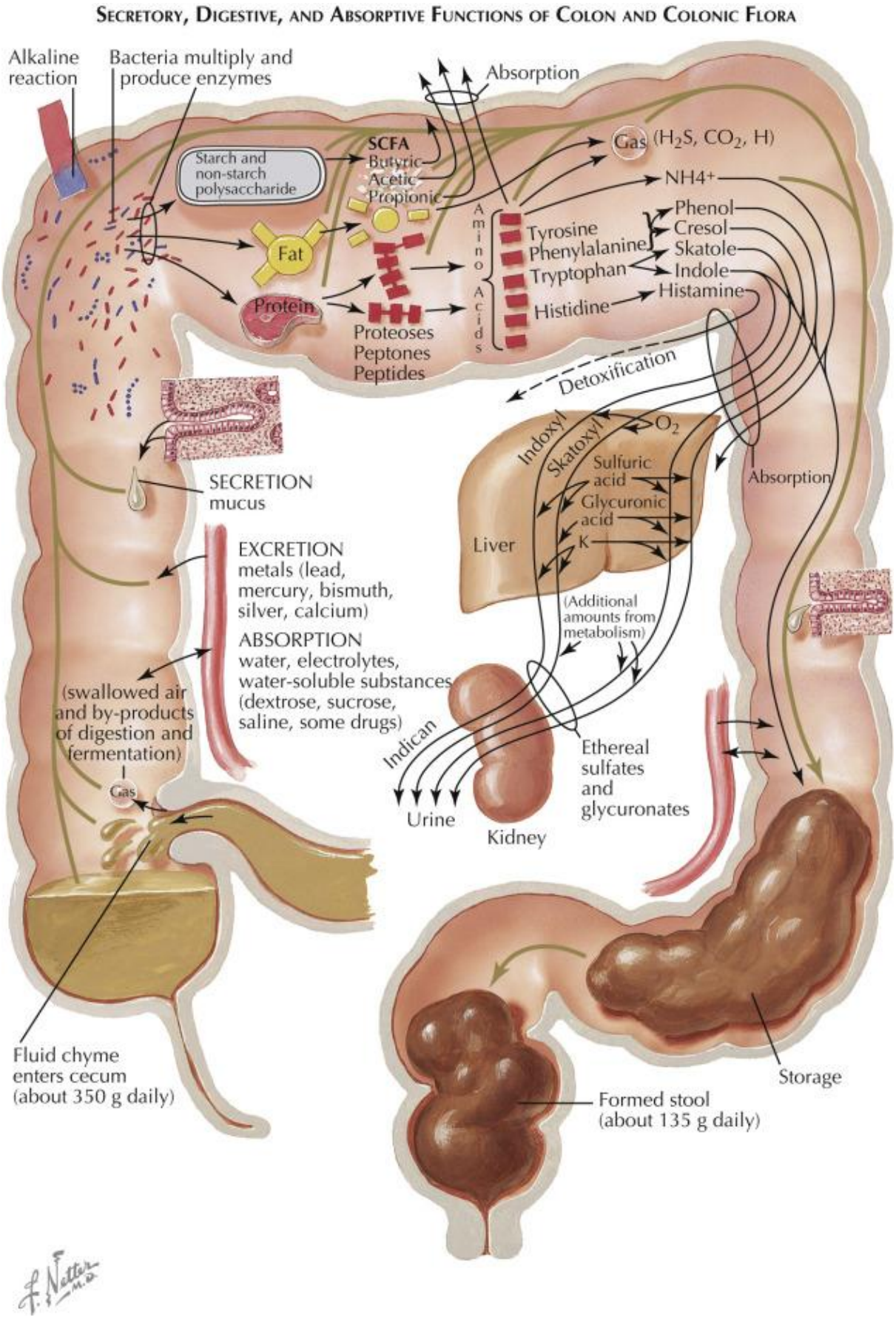


Figure 5: Functions of the large intestine [44].

F- Pathologies of the intestines

A wide spectrum of pathologies affects the intestines, they include:

1. Diarrhea

Diarrhea, described as loose and watery feces is a frequent symptom of gastroenteritis, norovirus or food poisoning, as well as allergies, food intolerances and IBDs.

2. Constipation

Constipation is known as the intermittent and painful evacuation of slow moving hard feces. Complications of constipation include abdominal distension and pain and may lead to gastro intestinal obstruction. Constipation is frequently caused by irregular bowel habits, a diet poor in fibers, and a sedentary lifestyle, however, it could also originate from more serious conditions such as diverticulitis, gastrointestinal tumor mass or paralytic ileus [42].

3. Lactose intolerance

It is defined as the inability to digest dietary lactose leading to abdominal cramps, bloating and diarrhea [42].

4. Celiac disease

Celiac disease is characterized by gluten intolerance which develops in genetically susceptible individuals. The exact etiology of this intolerance in unknown, it is characterized by a release of inflammatory mediators and an impairment of the intestinal mucosal lining [42,45].

5. Bowel obstruction

Obstruction of the large intestine is a serious medical problem requiring urgent attention and intervention. It is commonly caused by colorectal cancer and diverticular disease. Symptoms include abdominal pain, distension and constipation [46].

6. Diverticulitis

Colonic diverticula are pea-sized outpouchings from the colonic lumen caused by a mucosal herniation through the colonic wall at sites of vascular perforation a result of increased pressure; for instance, while straining during excretion [42,47].

7. Malabsorption syndrome

Malabsorption syndrome encompasses a number of disorders in which the small intestine is unable to absorb enough of certain nutrients like proteins, fats, minerals, vitamins and/or carbohydrates as well as fluids, resulting in deficiencies and malnutrition[48].

8. Appendicitis

This condition is defined as an inflammation of the appendix due to an obstruction of the lumen, resulting in ischaemic injury and bacterial infection. Appendicitis is considered a medical emergency, because a probable perforation will lead to peritonitis, possibly leading to death within hours [49].

9. Irritable Bowel Syndrome (IBS)

IBS is a common, chronic gastrointestinal disorder characterized by long-lasting abdominal pain, change in bowel habits, abdominal distension, bloating, and urgency. It is a disorder of gastrointestinal motility, which is influenced by stress and psychosocial dysfunction [46,50].

10. Inflammatory bowel disease (IBD)

Inflammatory bowel diseases (IBD) are a spectrum of immune-related conditions that include ulcerative colitis (UC) and Crohn's disease(CD)empirically defined by clinical, pathological, endoscopic and radiological features [51]. The onset of IBD typically occurs in the second and third decades of life and a majority of affected individuals progress to relapsing and chronic disease. Family aggregation has long been recognized [51].

Several genetic and environmental as well as life style related factors are involved in the onset of IBD and the exact etiology of this multifactorial disease is not well defined yet , However, an active inflammatory status characterized by an upregulation of a wide cytokine network remains the hallmark of this pathology [51,52].

Some differences between UC and CD were described as UC is characterized by a diffuse mucosal inflammation that extends proximally from the rectum to a varying degree. However, in CD, the involvement of the terminal ileum is most common and the earliest mucosal lesions appear over Peyer's patches. Unlike ulcerative colitis, Crohn's disease may be patchy and segmental, and inflammation typically transmural [51].

It is well documented that IBD represents a major healthcare burden of significant global morbidity, with highest prevalence in Europe and North America and increasing incidence in Asia. [52]. Symptoms include abdominal cramps, bloating, gas, urgency, diarrhea and bleeding. The key feature of IBD is dysbiosis, defined as a shift in the composition of the gut microbiota; however, the specific role of dysbiosis in its pathogenesis remains poorly understood. Importantly, IBD is considered an key risk factor for colorectal carcinogenesis [53].

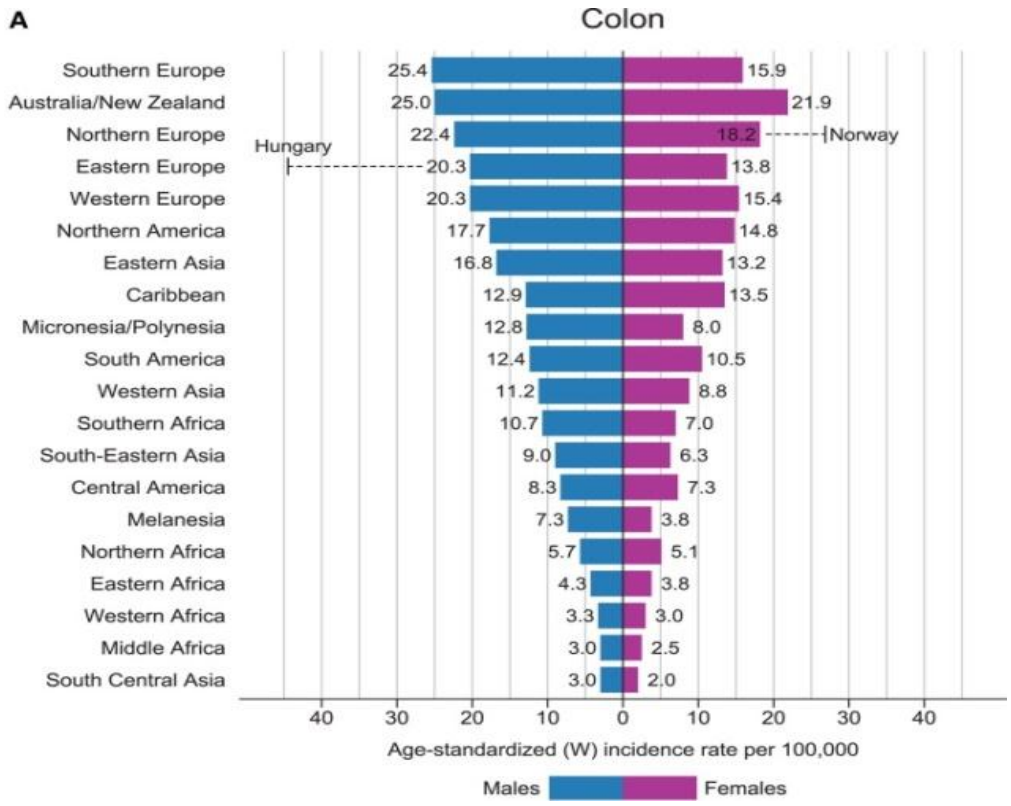
G- Colorectal cancer

1. Epidemiology of CRC

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide (Figure 6) with 1.8 million new cases and almost 881,000 deaths in 2018, according to the World Health Organization GLOBOCAN database [1]. Its burden is predicted to increase by 60% in 2030 [2].

In Lebanon, CRC accounts for 8.5% of all cancers. It is the highest incidence rate in the MENA region, as 1463 cases of CRC were diagnosed in 2018 (Figure 8) in a population of almost 6 million [3].

More than 90% of CRCs are adenocarcinomas, a malignant neoplasm that develops from glandular epithelial cells of the colon and rectum; other rare types include squamous cell carcinoma, adenosquamous carcinoma, spindle cell carcinoma and undifferentiated carcinoma [54] .



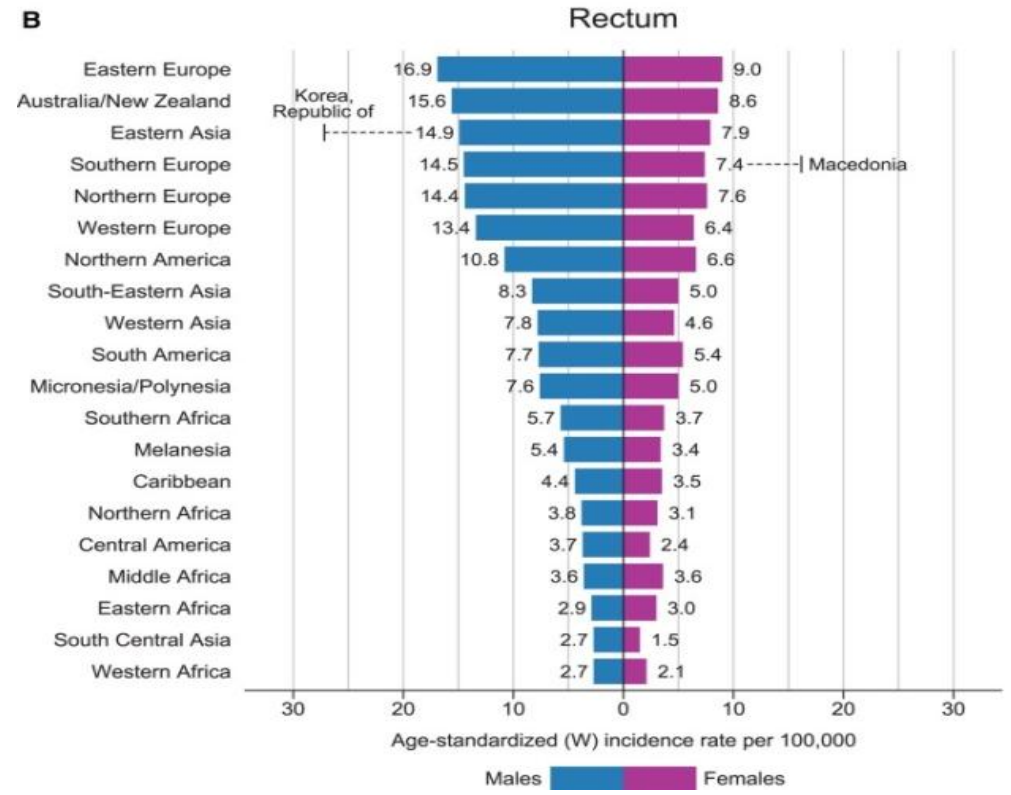


Figure 6: Bar chart of Region-specific incidence age standardized rates by sex for cancers of the (A) colon and (B) Rectum in 2018 [1].

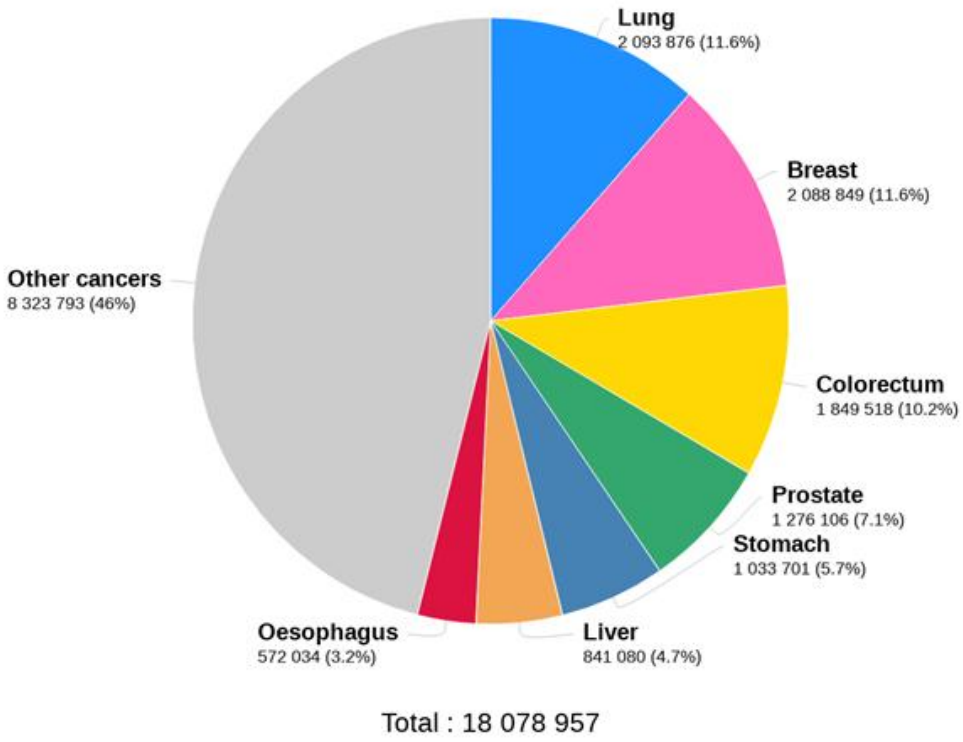


Figure 7: Estimated number of new cancer cases in 2018, worldwide, both sexes, all ages [1].

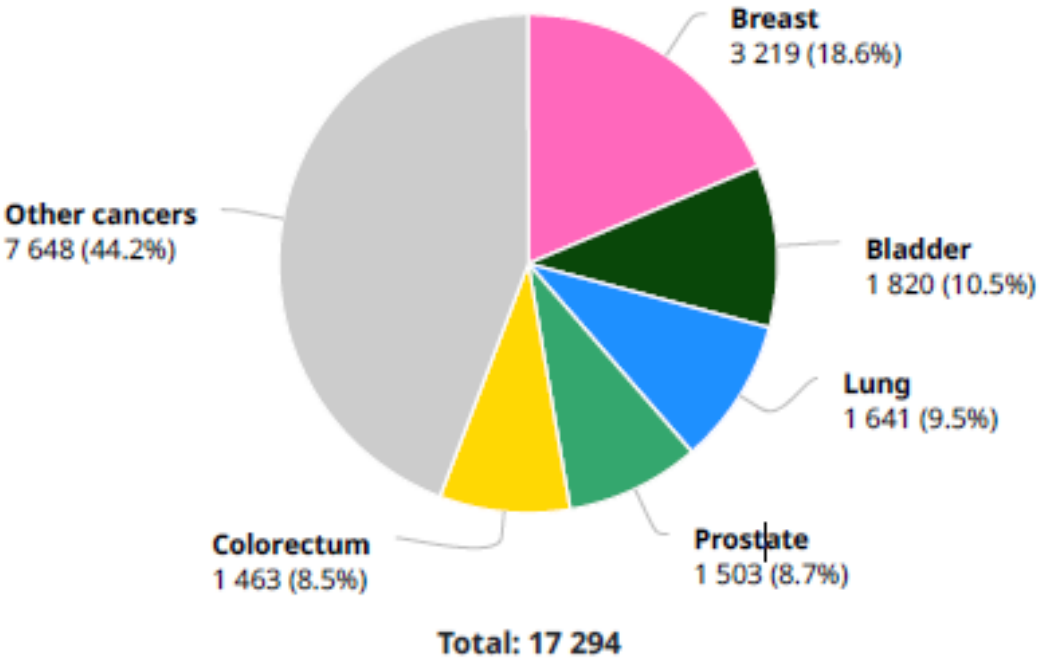


Figure 8: Number of new cancer cases in 2018 in Lebanon, both sexes all ages [1].

2. Classification of CRC

Colorectal cancer is basically due to the onset of mutations in specific genes such as oncogenes, tumor suppressor genes and genes related to DNA repair mechanisms, thus CRC is classified based on the origin of the mutation into 3 categories: sporadic, inherited and familial CRC (Figure 9).

- **Sporadic cancers:**

Seventy percent of CRC cases follow a specific succession of mutations converting an adenoma to a carcinoma. This sequence starts with a mutation of the APC (adenomatous polyposis coli) gene triggering polyp formation, followed by a chain of mutations in *KRAS*, *TP53* and *DCC* (Deleted in colorectal carcinoma) genes, thus leading to the carcinoma state [55].

- **Inherited cancers:**

Inherited CRC account to only 5% of incidences. They are classified into 2 main categories: familial adenomatous polyposis (FAP) characterized by a large number of colorectal adenomas and hereditary non-polyposis colorectal cancer (HNPCC), which is related to mutations in DNA repair mechanisms. The main form HNPCC is Lynch syndrome, it is caused by inherited mutations in one of the alleles coding for DNA repair proteins such as MSH2, MLH1, MLH6, PMS1 and PMS2 [56].

- **Familial colorectal cancer:**

This type accounts for approximately 25% of all cases, it is not included in the inherited type as it occurs in families without evidence for one of the known inherited syndromes. Non-syndromic or familial CRC is a cluster of CRC that is distinguished from the hereditary syndromes. It is an heterogeneous disorder that includes patients with unrecognized hereditary syndromes and an unclear molecular mechanism. Possibly, a combination of environmental and inherited genetic factors play a role in the onset of CRC in these families [57].

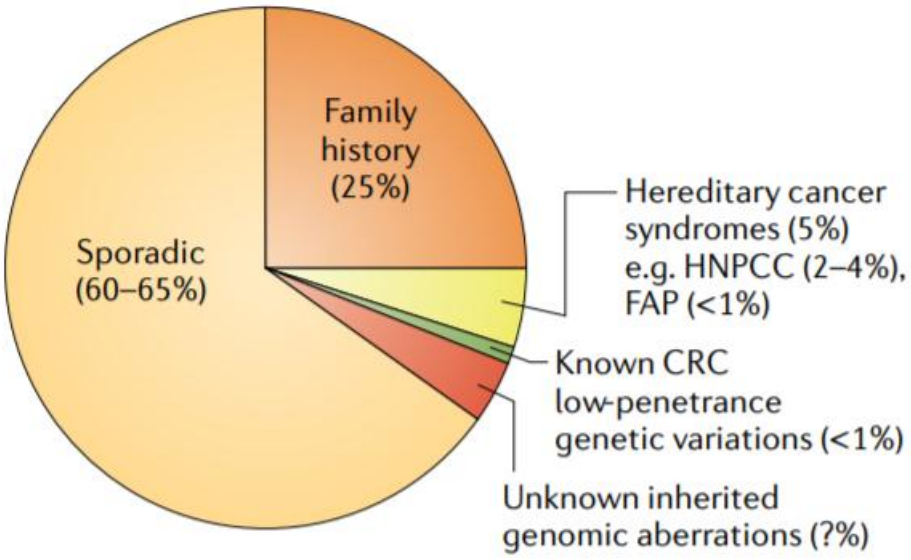


Figure 9: Proportion of colorectal cancer cases associated with sporadic and hereditary factors [58].

3. Risk factors

The etiology of CRC is multifactorial encompassing genetic, environmental and lifestyle related factors including westernized diet, alcohol consumption and obesity… among others. However, chronic inflammation, in particular inflammatory bowel disease (IBD) and dysbiosis in enteric microbiota remain the key players in this process [10,59-61]. Even though CRC is influenced by hereditary components, most CRC cases are sporadic and slowly develop over several years following the adenoma-carcinoma sequence [62].

Although much has yet to be learned about it, several factors are known to increase a person's chance to develop CRC:

- **Age:** the risk of developing CRC is markedly increased after 50 years of age.
- **IBD**: A personal history of inflammatory bowel disease (IBD) increases the chances of having CRC by 2.75 in patients with UC and 2.64% in patients with Crohn's [63].
- **Familial history of CRC:** Having relatives with CRC raises the risk of CRC development.
- **Lifestyle related factors:** This group of factors plays an important role in the predisposition to CRC; it regroups sedentary lifestyle, obesity, bad nutritional habits, red and burned meat consumption, smoking and alcohol consumption…among others [64,65].

4. Dysbiosis and CRC

The microbiome comprised of the collective genome of microbes inhabiting the gut, is now considered one of the prime suspects responsible for the onset and evolution of gastrointestinal disorders and specifically colorectal carcinogenesis. Definitely, dysbiosis is now known to be a key factor in the development of IBD and CRC [38,39,66].

Growing attention has been given to the role of microbial infection in carcinogenesis in recent decades, and microbes are suspected to be involved in approximately 20% of cancers, and especially CRC. Several observations led to the focus on microbiota as a key player in CRC: Variability of the incidence of CRC highly suggested the involvement of certain environmental risk factors, such as high-fat diets, obesity or Western lifestyle. In addition, Knudson's two-hit hypothesis suggested that host factors play an important role in the predisposition to

carcinogenesis. In this scenario, a second environmental hit can lead to uncontrolled cellular proliferation which could be a possible infectious cause [66].

Additionally, experimental studies have focused on the role of dysbiosis on CRC. In 1975, the first observation linking gut microbiota with CRC was reported in germ-free rats that developed less chemically induced colorectal tumor than conventional rats. These results have been reproduced in several in vitro and in-*vivo* CRC models [67].

The contribution of bacteria to CRC could be due to two different scenarios:

- A dysbiotic microbial community with pro-carcinogenic features that are capable of remodeling the microbiome as a whole to drive pro-inflammatory responses and epithelial cell transformation, leading to cancer;
- And the "driver-passenger" theory, wherein intestinal bacteria, termed "bacteria drivers", initiate CRC by inducing epithelial DNA damage and tumorigenesis, in turn promoting the proliferation of passenger bacteria that have a growth advantage in the tumoral microenvironment [66].

Although it is not yet clear how dysbiosis could induce colonic carcinogenesis, chronic inflammation appears to be the core mechanism. The first stages of both CRC and IBD diseases involve an alteration to the normal flora, which results in activation of the immune system, thus giving rise to a chronic inflammatory state characterized by an upregulation of pro-inflammatory cytokines and reactive oxygen species among others, creating a tumor-favorable microenvironment [40].

5. Staging and prognosis of CRC

Pathologic staging for CRC is done by the tumor-node-metastasis (TNM) system as defined by the American Joint Committee on Cancer.
In TNM staging system, T describes the size of the tumor and any spread of cancer into nearby tissue, N describes spread of cancer to proximate lymph nodes; and M is for metastasis.

In colorectal carcinogenesis, TNM is based on depth of invasion of the bowel wall, extent of regional lymph node involvement, and presence of distant sites of disease. The classification for CRC is described below [68].

Primary Tumor (T)

- TX Primary tumor cannot be assessed
- T0 No evidence of primary tumor
- Tis Carcinoma in situ: intraepithelial or invasion of lamina propria
- T1 Tumor invades submucosa
- T2 Tumor invades muscularis propria
- T3 Tumor invades through the muscularis propria into pericolorectal tissues
- T4a Tumor penetrates to the surface of the visceral peritoneum
- T4b Tumor directly invades or is adherent to other organs or structures

Regional Lymph Nodes (N)

- NX Regional lymph nodes cannot be assessed
- N0 No regional lymph node metastasis
- N1 Metastasis in 1–3 regional lymph nodes
    - N1a Metastasis in one regional lymph node
    - N1b Metastasis in 2–3 regional lymph nodes
    - N1c Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
- N2 Metastasis in 4 or more regional lymph nodes
    - N2a Metastasis in 4–6 regional lymph nodes
    - N2b Metastasis in 7 or more regional lymph nodes

Distant Metastasis (M)

- M0 No distant metastasis
- M1 Distant metastasis

- M1a Metastasis confined to one organ or site (for example, liver, lung, ovary, nonregional node)
- M1b Metastases in more than one organ/site or the peritoneum

TNM staging is the most common staging system (Figure 10); it predicts the disease prognosis.

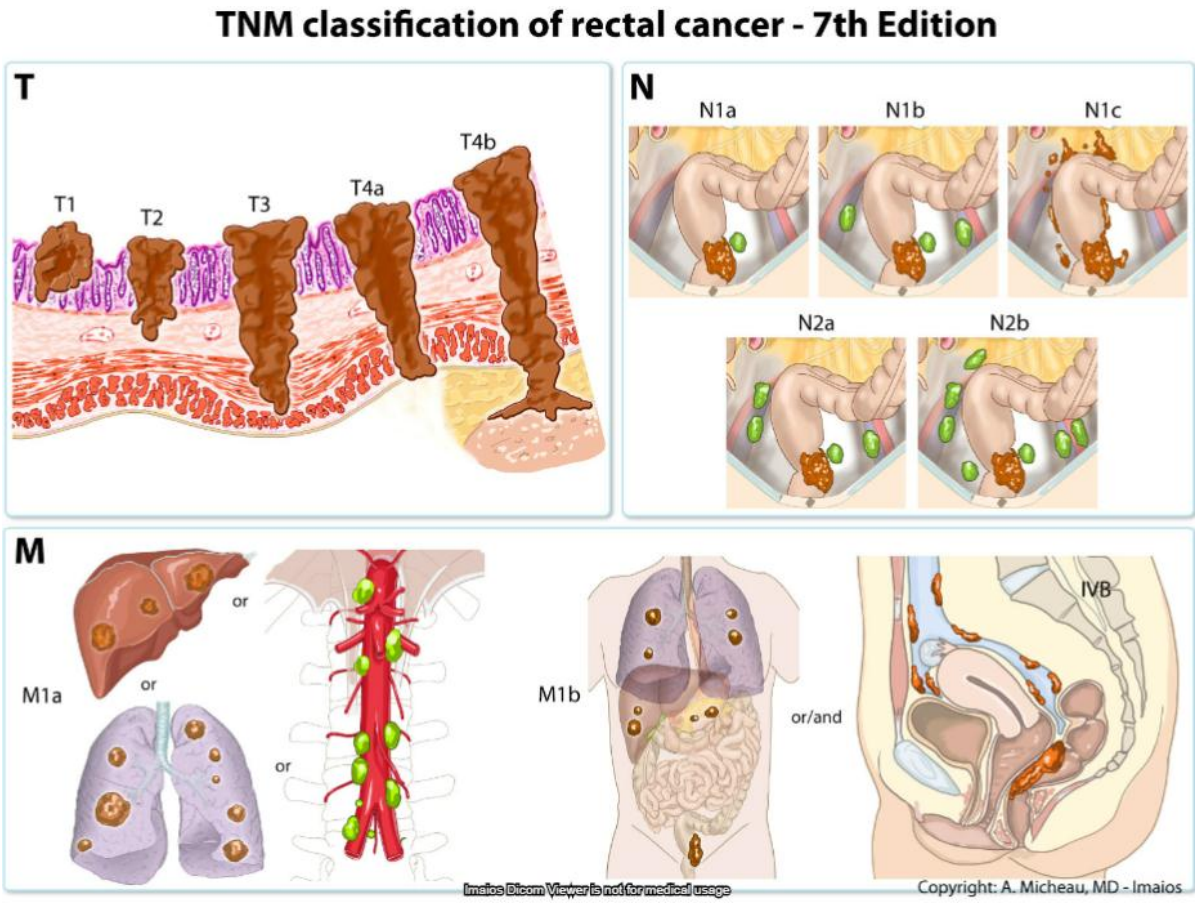


Figure 10: TNM classification of colorectal cancer [68].

6. Molecular pathways of CRC

The mechanism of CRC development consists of four major stages: initiation, promotion, progression and metastasis, passing through a stepwise accumulation of multiple genetic and epigenetic aberrations, subsequently leading to invasive and metastatic tumors (Figure 11).

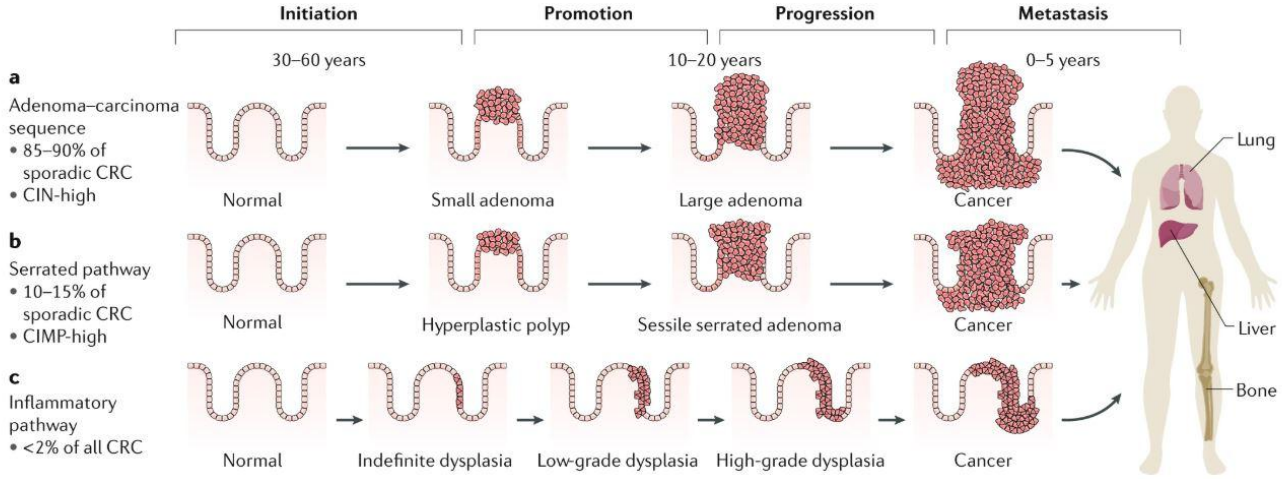


Figure 11: Pathways of colorectal carcinogenesis [58].

Currently, three pathogenic mechanisms have been identified in the passage from normal colon to colorectal cancer: Chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP).

These sequences implicate the progression of normal colon epithelial cells to aberrant crypt foci, followed by early and advanced polyps with subsequent progression to early cancer and then advanced cancer.

- **The Chromosomal instability (CIN) pathway**

The Chromosomal instability (CIN) is the classical pathway, it accounts for 80%–85% of all CRC cases. It is characterized by disparities in the number of chromosomes, thus leading to loss of heterozygosity (LOH) [69].

Several mechanisms are implicated in this process such as alterations in chromosome segregation, telomere dysfunction and DNA damage response, affecting crucial genes such as *APC*, *KRAS*, *PI3K* and *TP53* … among others. Importantly, *APC* mutations lead to a nuclear

translocation of β-catenin thus enhancing tumorigenesis and invasion. On the other hand, the *KRAS* and *PI3K* mutations activate MAP kinase pathway which leads to cell proliferation. *TP53* mutations also affects the p53 *gene* and leads to uncontrolled cell cycle progression [30].

- **The Microsatellite instability (MSI) pathway**

The MSI pathway is caused by a loss of DNA repair mechanisms leading to mutations in oncogenes or tumor suppressor genes such as *MLH1, MSH2, MSH6, PMS1* and *PMS2* . Loss of expression of mismatch repair genes (MMR) can be caused by spontaneous events (promoter hypermethylation) or germinal mutations such as those found in Lynch syndrome. In general, MSI tumors present a better prognosis than sporadic tumors [30].

- **CpG island methylator phenotype (CIMP)**

The CpG island methylator phenotype is due to epigenetic instability, a common feature in colorectal carcinogenesis. The main characteristic of CIMP tumors is the hypermethylation of oncogene promoters MINT clones, p16, THBS, and MLH1, which leads to genetic silencing and a loss of protein expression [30,70].

CIMP has a distinct phenotype , it is characterized by key clinical, pathologic, and molecular features, including female sex, old age, high MSI, *BRAF* mutations, and right-sided tumor location [70].

7. Screening for CRC

Screening is considered a secondary prevention as it can reduce CRC incidence and mortality by enabling detection and, therefore, treatment of precancerous lesions before their malignant transformation. Such a screening can be performed by:

- **Fecal Occult Blood (FOBT)**

This is a commonly adopted technique, the fecal occult blood test detects small amount of blood in stool samples, supporting CRC diagnosis. The two main types of FOBT are the

immunochemical FOBT (or fecal immunochemical test -FIT) and the guaiac-based FOBT (Figure 12).

The guaiac-based test (g-FOBT) is the most commonly used. This test is based on the oxidation of the paper-embedded guaiac by hydrogen peroxide (developer) in presence of the heme component of hemoglobin thus generating a blue-colored product (Figure 12-A).

The i-FOBT was approved by the United States Food and Drug Administration in 2001 has been added to recent American Cancer Society (ACS) and GI Consortium guidelines for colorectal cancer screening. This test is based on an antibody-antigen detection of human hemoglobin (see Fig. 12-B) [71].

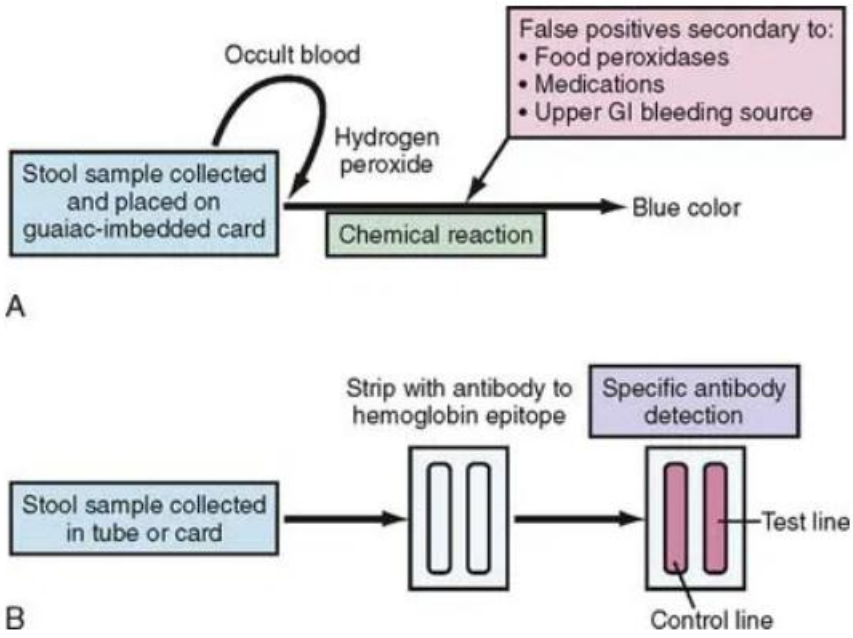


Figure 12: Schematic representation of the g-FOBT (**A**) and i-FOBT (**B**) blood detection reactions.
g-FOBT, guaiac-based fecal occult blood test; i-FOBT, immunochemical fecal occult blood test [71].

- **Colonoscopy**

Colonoscopy is known as the gold-standard screening method. It is a highly sensitive but costly test. The efficacy of colonoscopy in CRC screening is explored by several studies shedding light on the decreased CRC risk and mortality in patients undergoing colonoscopy compared to their non-screened counterparts.

Colonoscopy is a safe technique. Complications, including colonic perforations and intestinal bleeding, are relatively rare.

Regardless of one's lifestyle habits, screening and detection of polyps with subsequent polypectomy plays a crucial role in preventing CRC development in asymptomatic patients and is recommended to begin in average-risk patients who are 50 years old [44]. However, for individuals with predisposing conditions, earlier and more frequent testing is recommended [44,58] .

8. CRC Treatments

The choice of first-line treatment for CRC patients currently involves a multimodal approach based on tumor and patient related characteristics such as the number and localization of metastases, tumor progression, and prognosis among others [72,73].

Besides surgical resection, several regimens of these chemotherapy drugs are used sometimes in combination:

a. Intravenous Fluorouracil:

Fluorouracil is considered the cornerstone of systemic treatment for colorectal cancer. It is a fluorinated pyrimidine that acts primarily through inhibition of thymidylate synthase and is commonly administered with leucovorin, a reduced folate that is thought to stabilize fluorouracil's interaction with this enzyme.

b. Irinotecan:

It is a semisynthetic derivative of the natural alkaloid camptothecin that acts by inhibiting topoisomerase I, an enzyme that catalyzes breakage and rejoining of DNA strands during DNA replication .

c. Oxaliplatin:

It is a diaminocyclohexane platinum compound that forms DNA adducts, leading to impaired DNA replication and cellular apoptosis.

d. Bevacizumab:

It is a monoclonal antibody working as an Angiogenesis Inhibitor in a strategy to control malignant proliferation and spread through the inhibition of neoangiogenesis.

e. Epidermal Growth Factor Receptor (EGFR) Inhibitors:

Such inhibition is mainly performed by cetuximab and panitumuma: these antibodies are directed against the extracellular domain of EGFR. EGFR plays a crucial role in tumorigenesis as it enhances cellular growth and proliferation [72,73].

In addition to traditional chemotherapy, adjunct treatments are emerging with the aim of inhibiting inflammation , increasing chemotherapy efficacy and minimizing its resistance and side effects as well as the risk of developing secondary tumors. These include the use of agarose tumor macrobeads, anti-inflammatory drugs, metformin, probiotics, and gold-based drugs…among others [30].

H- Diabetes

Diabetes mellitus is a group of metabolic disorders characterized by chronic hyperglycemia and dysregulated metabolism of carbohydrates, lipids and proteins induced by insulin insufficiency [74]. According to the International Diabetes Federation, diabetes affected 451 million people worldwide in 2017; this number is expected to rise to 693 million by 2045. This rising trend has been promoted by a shift into urban lifestyle, the spread of western style diet and lack of physical activity [5].

1. Types of diabetes mellitus

According to the American Diabetes Association 2019, diabetes is classified into four main types:

Type I diabetes: It is Insulin dependent diabetes, it is immune-mediated and characterized by a destruction of the pancreatic beta-cells and complete insulin deficiency.

Type II diabetes: This type is non-Insulin dependent, and it is characterized by insulin resistance.

Gestational diabetes: It is described as an intolerance to glucose with onset in pregnancy.

Specific types of diabetes: They are due to other causes, such as monogenic diabetes syndromes (neonatal diabetes and maturity-onset diabetes of the young), diseases of the exocrine pancreas (such as cystic fibrosis and pancreatitis), and drug- or chemical-induced diabetes (such as with glucocorticoid use, in the treatment of HIV/AIDS, or after organ transplantation) .

2. Complications of diabetes

Diabetic patients are more prone to life threatening complications when compared to non-diabetic counterparts, these complications could be categorized into two main types:

- **Acute metabolic complications*:*** These complications are short term and comprise hypoglycemia, diabetic ketoacidosis and hyperosmolar non-ketotic coma.

- **Late systemic complications*:*** These are long term chronic complications, further classified into two other sub-categories: macrovascular and microvascular.

    - Macrovascular complications comprising coronary heart disease, peripheral vascular disease and stroke.
    - Microvascular complications including neuropathy, retinopathy and nephropathy
    - Diabetic foot is considered a micro- and macrovascular complication. Other complications exist such as depression and erectile dysfunction [75].

Notably, diabetic complications constitute the major cause of hospital admissions, disability, and mortality in diabetic patients [76].

3. Risk factors

Several risk factors predispose an individual to diabetes mellitus, such as age, inactive lifestyle, high blood pressure, poor glycemic control, smoking, obesity, bad lipid profile and cardiovascular diseases….among others [76].

4. Treatment of diabetes

While lifestyle modifications and metformin are considered the cornerstone of the initial management of diabetes mellitus, an increasing array of second and third-line pharmacological agents is being used.

Different families of oral and injectable drugs are available for the treatment of diabetes including sulfonylureas, meglitinides, insulin, alpha-glucosidase inhibitors, Thiazolidinediones as well as Glucagon-like peptide-1 (RA-GLP1) receptor agonists, Dipeptidyl peptidase-4 inhibitors, and Sodium glucose cotransporter 2 (SGLT2) inhibitor. In addition, insulin analogues that better simulate endogenous insulin secretion have been developed.

However, it is worth noting that metformin remains the first choice of treatment for most patients. Other alternative treatments should be individualized, taking into consideration patient characteristics such as the level of hyperglycemia and the presence of co-morbidities, and the treatment properties such as its durability of lowering blood glucose, risk of hypoglycemia, effect on diabetic complications , effect on body weight, as well as its side effects and contraindications [77].

I- Oxidative stress

The term "oxidative stress" was first used in 1970, "cells were subjected to oxidative stress" to describe the addition of H_2O_2 to erythrocytes. Since 1985, the term was used to denote oxidative damage to cells and organs [78].

1. Free radicals

Free radicals are unstable, highly reactive molecules containing one or more unpaired electron(s) [75]. Free radicals can be classified into three main types [78]:

- Reactive oxygen species (ROS)
- Reactive Nitrogen species (RNS)
- Reactive Chlorine species (RCS)

Among the most important ROS are the hydroxyl radical (OH), the superoxide radical anion (O_2^-), nitric oxide (NO), and peroxyl radicals (ROO), as well as non-radical species such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), and peroxynitrite ($ONOO^-$) as depicted in Figure 13 [79].

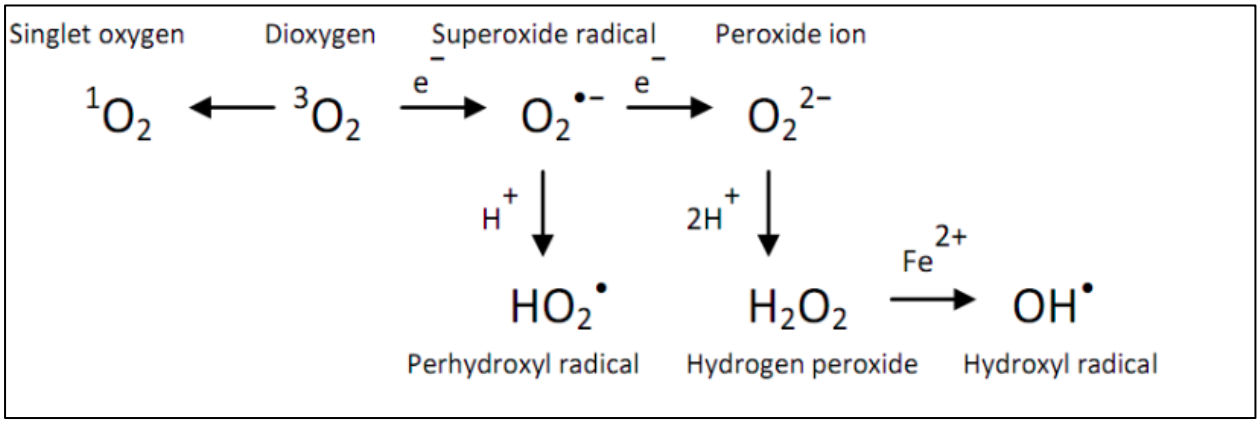


Figure 13: Commonly encountered reactive oxygen species [80].

Reactive oxygen and nitrogen species (RONS) are produced by all aerobic cells and play chief roles in several systemic functions and disease. RONS generation could be endogenous or exogenous.

- Endogenous sources of RONS include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, myeloperoxidase (MPO), lipoxygenase, and angiotensin II.
- Exogenous sources of RONS encompass air and water pollution, tobacco, alcohol, heavy or transition metals, drugs (e.g., cyclosporine, tacrolimus, gentamycin, and bleomycin), industrial solvents, cooked food (e.g., smoked meat, waste oil, and fat) and radiation, which are metabolized into free radicals inside the body.

Whether they are endogenous or exogenous, RONS cause oxidative modification to carbohydrates, lipids, proteins, and cellular DNA [81].

2. Antioxidants

Natural antioxidants are considered as defense mechanisms against reactive species, these substances could be classified into different categories according to their properties:

- Endogenous antioxidants : glutathione, lipoic acid, coenzyme Q, ferritin, uric acid, bilirubin, l-carnitine, melatonin, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPXs), thioredoxins (TRX) and peroxiredoxins (PRX), among others[82].
- Natural antioxidants: have a dietary origin, such as ascorbic acid (Vitamin C), tocopherol (Vitamin E), β-carotene (Vitamin A), lipoic acid, uric acid, glutathione and polyphenol metabolites
- Synthetic antioxidants: N-acetyl cysteine (NAC), pyruvate, selenium, butylated hydroxytoluene, butylated hydroxyanisole, and propyl gallate… among others [83].

3. Oxidative stress in colorectal cancer

In the last decade, association between oxidative stress and CRC has been broadly studied. In the gastrointestinal tract, ROS generation is obtained via different enzymatic reactions (Table 1). Additionally, in colorectal carcinogenesis, markers of oxidative stress were found to be upregulated. This is depicted by a multitude of studies on human colorectal tumors whereby increased levels of ROS, nitric oxide (NO), 8-oxodG in DNA, lipid peroxides, glutathione peroxidase (GPx), catalase (CAT) and decreased methylation of cytosine in DNA, in addition to lipid modification and increased leukocyte activation, were found, linking oxidative stress to inflammatory cells and inflammation and consequently colorectal cancer [84].

One other possible way by which oxidative stress exerts its tumorigenic effect is through cell cycle modification, alteration of p53 expression, thus influencing proliferation of cancerous cells [85].

Several studies also explored ROS inhibition as a treatment modality in CRC. This inhibition was found to ameliorate CRC phenotype especially in colitis associated colorectal cancer [85], thus linking again oxidative stress to colorectal carcinogenesis.

Table 1: Enzymatic reactions that participate in ROS/NOS generation in the GI tract [85].

Enzyme	Reaction	Site of action
Complex I and III/ubiquinone of the mitochondrial electron transport chain	Complex I (NADH dehydrogenase): O_2 + NADH → $O_2^{\bullet-}$ + NAD^+ Complex III (cytochrome bc_1): $O_2 \rightarrow O_2^{\bullet-}$	Mitochondria
Xanthine oxidase	Xanthine + O_2 + NADPH → $O_2^{\bullet-}$ + H_2O_2 + $NADP^+$ + uric acid	Plasma and cytoplasm of epithelial cells
NADPH oxidase	$2O_2$ + NADPH → $2O_2^{\bullet}$ + $NADP^+$ + H^+	Cell membrane
Haber-Weiss reaction	$H_2O_2 + O_2^{\bullet-} \rightarrow O_2 + OH + OH^{\bullet}$	Plasma and cell's cytoplasm
Fenton reaction	$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH + OH^{\bullet}$	Plasma and cell's cytoplasm
Catalase (CAT)	$2H_2O_2 \rightarrow O_2 + H_2O$	The cytoplasm and peroxisomes of epithelium and lamina propria; leukocytes.
Glutathione peroxidase (GPx)	H_2O_2 + 2GSH → GSSG + $2H_2O$	GPx1- peroxisomes of colon lymphatic tissue and the lamina propria, submucosa, muscularis and serosa;

		GPx2- peroxisomes of the luminal epithelium; GPx3- secreted by the intestinal epithelial cells; GPx4- peroxisomes of colonic and ileal tissues.
Endothelial nitric oxide synthase (eNOS)	l-arginine + O_2 → l-citrulline + $NO^{\bullet}$	Cell membrane of the endothelial cells
Inducible nitric oxide synthase (iNOS)	$NO^{\bullet} + O_2^{\bullet-} \rightarrow ONOO^-$	Cytoplasm of inflammatory and epithelial cells
Superoxide dismutase (SOD)	$2H^+ + 2O_2^{\bullet-} \rightarrow O_2 + H_2O_2$	SOD1- cytoplasm and small amount in nucleus; SOD2- mitochondria; SOD3- plasma.
Glutathione reductase (GRd)	GSSG + NADPH → GSH + $NADP^+$	Like GPx

4. Oxidative stress and diabetes

It is well established that oxidative stress is considered as the main cause behind diabetes complications especially cardiovascular events, however, the exact mechanism by which oxidative stress facilitates the development of diabetic complications is not fully understood. In diabetes, an increased intracellular glucose leads to an increased RONS production, which surpasses the neutralizing antioxidant capability of the cell. Thus, the activation of different molecular pathways involved in hyperglycemia-induced oxidative tissue could lead to the production of growth factors and pro-inflammatory cytokines, linking again diabetes to oxidative stress and inflammation [81].

J- <u>Inflammation and CRC</u>

Chronic inflammation is characterized by a continuously active inflammatory reaction and tissue destruction. Many of the immune cells contributing to the pathology of chronic inflammation (macrophages, neutrophils and eosinophils) are involved in the carcinogenic process, directly or through the production of inflammatory cytokines and other secretory factors [86,87].

Cytokines that promote colorectal and colitis-associated tumor development include TNF-α, IL-6, and IL-1 among others. On the other hand a variety of cytokines showed protective effects on CRC such as IL-10 and TGF-β that inhibit colorectal tumorigenesis [88]. Most tumor-promoting cytokines are produced by lamina propria macrophages and dendritic cells (DC) during early stages of CRC development or by T cells during late-stage tumor progression [89].

It is well established that chronic inflammation promotes carcinogenesis via several pathways, mainly by inducing gene mutations, inhibiting apoptosis, or stimulating angiogenesis and cell proliferation. Moreover, this inflammatory microenvironment promotes the accumulation of additional mutations and epigenetic changes, whereby, activated inflammatory cells produce reactive oxygen species (ROS) and reactive nitrogen intermediates that can induce DNA damage and mutation [90].

Importantly, two key genes in the inflammatory process, cyclooxygenase-2 (COX-2) and nuclear factor kappa B (NF-κB), provide a mechanistic link between inflammation and cancer and are targets for chemoprevention, particularly, in CRC [91,92]. Most, but not all, tumor-promoting cytokines trigger receptors on intestinal epithelial cells that activate oncogenic transcription factors and other oncogenic signaling pathways, such as extracellular signal-regulated kinase or Akt/mammalian target of rapamycin (mTOR) [93,94].

1. Nuclear factor-κB

Nuclear factor-κB (NF-κB) transcription factors play a crucial role in many physiological and pathological processes (Figure 14). Given their important role in mediating inflammatory signals, a lot of attention has been given to NF-κB and its upstream activator, IκB kinase (IKK), and their involvement in inflammation and carcinogenesis [95] NF-κB regulates the expression of genes, many of which play important roles in the regulation of inflammation and apoptosis and have been associated with tumor progression, whereby activated NF-κB was found in 40% of CRC tissues and 67% of CRC cell lines [96]. The upstream IκB kinases are also activated in several types of cancer. Additionally, defective IKKα was found in several solid tumors such as breast, colon, ovarian, pancreatic, bladder, prostate carcinomas and melanoma [97].

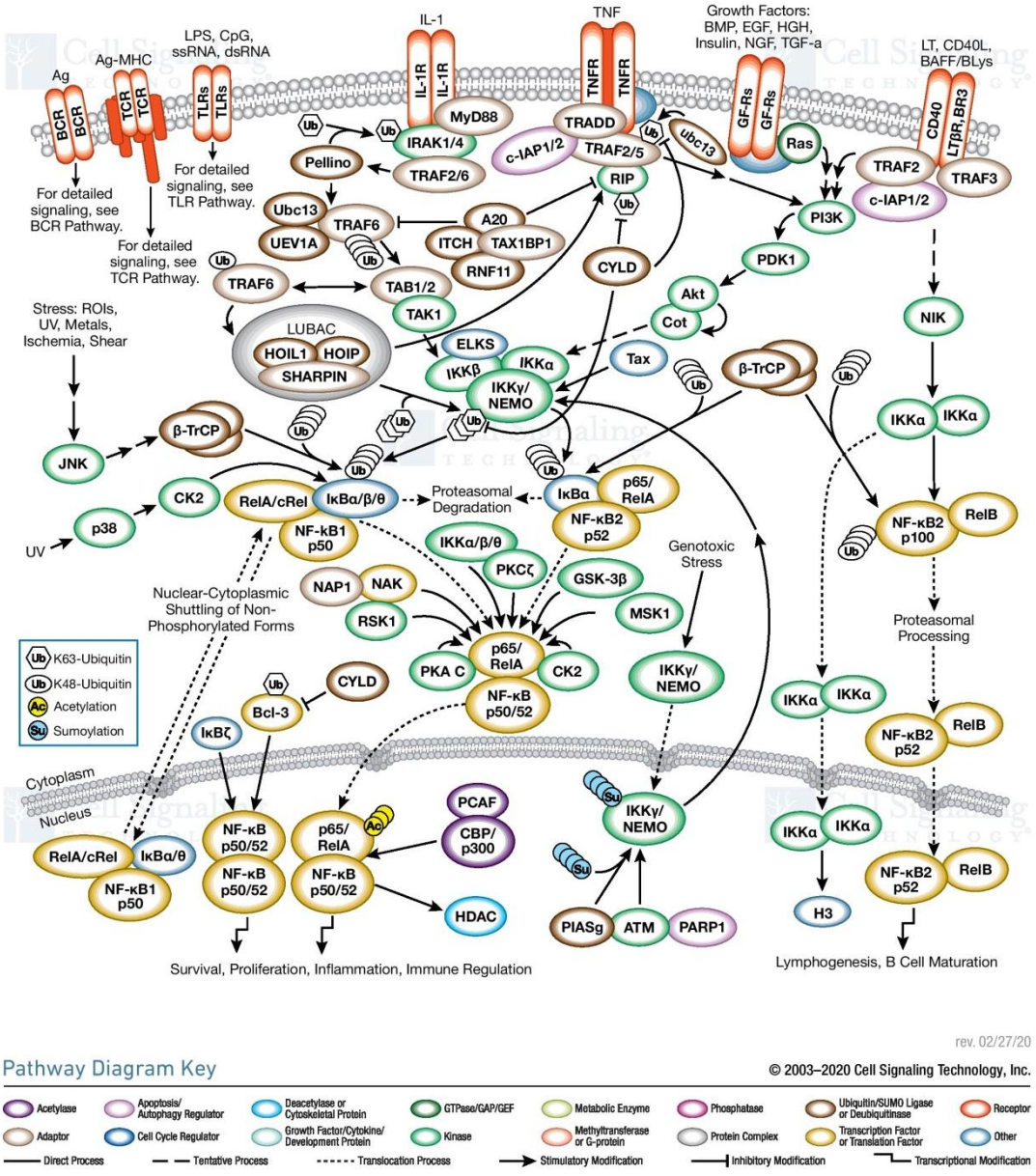


Figure 14: The NF-κB signaling pathway [98].

NF-κB pathway Description

Nuclear factor-κB (NF-κB) proteins include NF-κB2 p52/p100, NF-κB1 p50/p105, c-Rel, RelA/p65, and RelB. These proteins function as transcription factors that regulate the expression of genes influencing a broad range of biological processes including innate and adaptive immunity, inflammation, stress responses, B-cell development…among others.

The two characterized molecular pathways of NF-κB are the classical (or canonical) pathway, and the alternative (or noncanonical) pathway.

In the classical pathway, NF-κB/Rel proteins are bound and inhibited by IκB proteins. These IκB are phosphorylated thus activated by proinflammatory cytokines,

Lipopolysaccharide (LPS), growth factors, and antigen receptors. The activation of IκB is followed by the activation of NF-κB/Rel complexes that translocate to the nucleus where they induce target gene expression.

On the other hand, in the noncanonical NF-κB pathway, the activation of the kinase NIK, leads to the activation of IKKα complexes, which leads to the phosphorylation of C-terminal residues in NF-κB2 p100. This leads to the translocation of NF-κB complexes to the nucleus thus inducing target gene expression [98].

2. Cyclooxygenase-2

Many human cancers show elevated prostaglandin (PG) levels owing to upregulation of cyclooxygenase-2 (COX-2). The chief downstream mediator of COX-2 is PGE_2 that promotes cellular proliferation and angiogenesis, inhibits apoptosis, enhances invasiveness, and modulates immunosuppression. COX-2 is found to be significantly overexpressed in a variety of tumors, including CRC. It is regularly expressed at low levels in colonic mucosa, however, its activity increases dramatically following mutation of the APC gene, thus linking COX-2 to the pathogenesis and progression of colorectal carcinogenesis [99,100].

Selective inhibitors of COX-2 (coxibs) have established a noticeable efficacy in the treatment of pain and inflammation comparable to that of non-selective Non-steroidal anti-inflammatory drugs (NSAIDs) with better gastrointestinal safety. Subsequently, additional pharmacologic activities have emerged outside of coxibs' analgesic activity, such as their ability to induce apoptosis and anti-neoplasic effects [100].

3. Interleukin-6

Interleukin-6 (IL-6) was found to stimulate proliferation of premalignant enterocytes. Additionally, IL-6 is a potent stimulator of colon cancer cell proliferation and tumor growth. It has an important role in colitis as it mediates a pathogenic immune response, hence its inactivation completely blocks colitis in several animal models and in patients [88].

Most of the effects of IL-6 in cancer cells are mediated by STAT3, a transcription factor that is activated by many growth factors and cytokines, including IL-11; IL-22; TGF-α, TGF-β and EGF…among others.

IL-6 also promotes Th17 cell differentiation, which can promote and sustain IBD, and regulates the survival of other proinflammatory T cells, such as Th1 cells, while inhibiting the function of Treg cells [88].

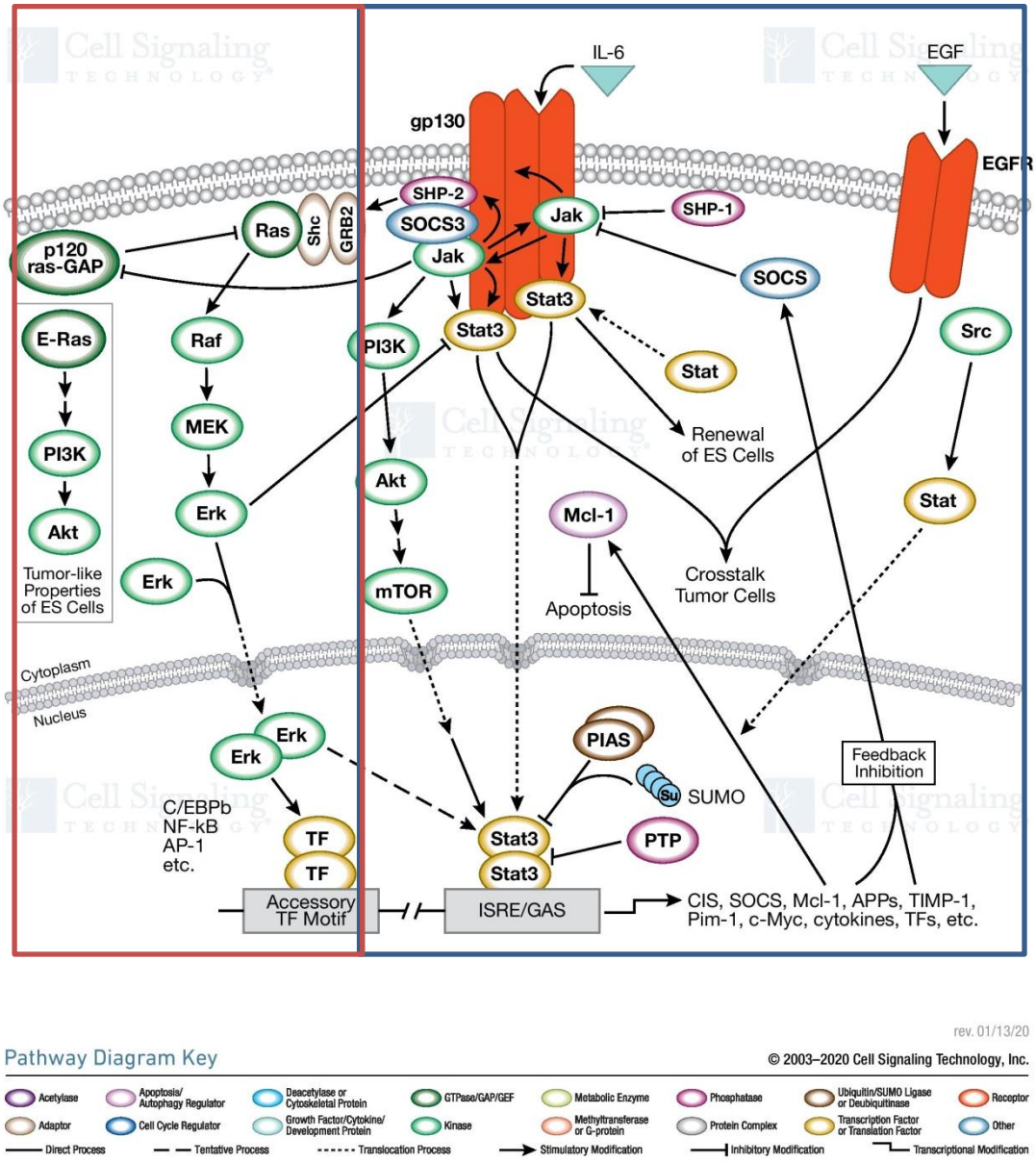


Figure 15: IL-6 signaling cascade [101].

IL-6 cascade description

IL-6 signaling is induced by the complex formed with IL-6 and a specific IL-6R leading to a series of signals through two main pathways: the JAK/STAT pathway and the Ras/MAPK (mitogen-activated protein kinase) pathway (Figure 15).

The JAK/STAT pathway (Blue rectangle): it is a member of a tyrosine protein-activated kinases family. The main transcription factors involved are STAT3 and STAT1. They can be

released after tyrosine phosphorylation, thus forming a homologous or heterodimer, transferred to the nucleus, thereby activating specific DNA binding sequences to promote gene expression. The Ras/MAPK pathway (Red rectangle): Activation of the membrane-bound guanosine triphosphatase or Ras protein usually induces activation of downstream signaling proteins such as MAPK cascade phosphorylation. The main substrates for phosphorylation of MAPK are c-Myc, c-Jun, c-Fos [101].

4. TNF-α

Tumor Necrosis Factor alpha (TNF-α) is a multifunctional proinflammatory cytokine, playing an important role in various physiological and pathological processes, including cellular proliferation, differentiation and apoptosis…among others. TNF-α is produced during the initial inflammatory response. It induces and maintains inflammation through the production of cytokines, chemokines, endothelial adhesion molecules, and the recruitment of activated leukocytes to the site of infection or injury. A large number of cells are able to produce TNF-α, mainly monocytes and macrophages but also lymphocytes, neutrophils, NK cells …among others [102].

TNF-α acts through two distinct receptors: TNFR1 p55 (TNF Receptor-1) which is found in all human tissues and TNFR2 p75 (TNF Receptor-2) which is mainly expressed in immune cells. Collectively, TNF-α is considered as a promoter of inflammation, angiogenesis, and tumor promoting factor, specifically, TNF-α expression is shown to be upregulated in CRC [88].

However, the binding of TNF-R1 and TNF-R2 results in distinct downstream pathways, which favor both cell survival and apoptosis. Importantly, it has been shown that the activation of JNK, Erk1/2, p38 MAPK, and NF-κB pathways promotes cell survival. On the other hand, the activation of caspase-8 induces apoptosis [103-106].

5. TGF-β

Despite a primary tumor suppressor role, there is increasing evidence suggesting that the transforming growth factor-beta (TGF-β) can promote tumor growth, invasion, epithelial-

mesenchymal transition (EMT) and metastasis in advanced stages of CRC. In addition, TGF-β has been shown to attenuate an anti-tumor immune response through the induction of regulatory T cells in spontaneous and inflammation-associated cancer [107].
Importantly, TGF-β is a multifunctional growth factor that possesses dual roles in tumor progression, as it acts as both anti-tumorigenic and pro-tumorigenic agent depending on the stage and characteristics of the tumor [107,108].

6. Interleukin-10

Interleukin-10 (IL-10) is also considered as a cytokine with a double role; interestingly, discrepancies are found between animal and human observations. For example, human IL-10 serum levels increase over time during CRC progression and correlate with poor CRC prognosis, shedding light on the tumor promoting role of this cytokine [109].
In contrast, several studies showed that IL-10-deficient mice develop colitis and colitis-associated cancer within two to three weeks after birth, shedding light on the importance of this cytokine in colorectal inflammation and carcinogenesis and posing IL-10 as a tumor protective agent. Additionally, the experimental IL-10 knockout mouse model results in a disease similar to human IBD and, therefore, has been proven useful as an experimental model for developing new and effective therapies for IBD and its associated carcinogenesis [100,108].

K- Inflammation and diabetes mellitus

Inflammation plays a major role in the onset and progression of diabetes, importantly diabetes is now considered as an inflammatory disease. Moreover, increased levels of circulating inflammatory markers were found in serum of diabetic patients. It is an etiological factor which promotes the onset of insulin resistance leading to DM. It also contributes to the predicted diabetes complications.
One important factor linking diabetes and inflammation is obesity, it is closely associated with the development of insulin resistance in inflammatory state and peripheral tissues.

Furthermore, the activation of the immune system is also a crucial factor that when associated to chronic inflammation, contributes extensively to the pathogenesis of DM. All these mechanisms and others are illustrated in Figure 16. The occurrence of diabetes correlates with multiple health conditions originating due to inflammatory mechanisms such as IBD [10,76].

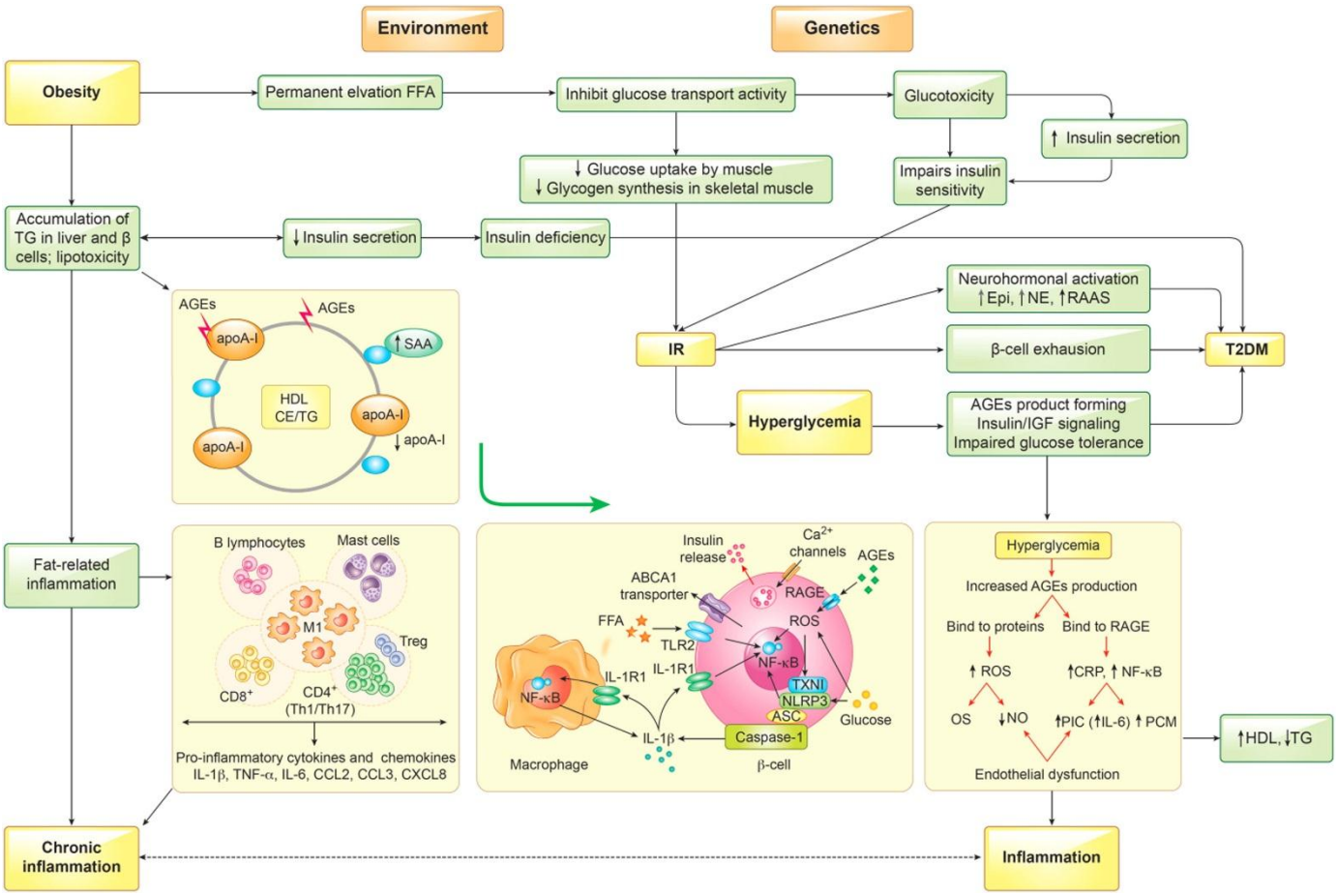


Figure 16: The relationship between inflammation and diabetes [110].

L- Metformin

Metformin is one of the most extensively prescribed metabolic modulators. It was first synthesized in the 1920s and has been used worldwide for treating diabetes mellitus, metabolic syndrome and polycystic ovary syndrome among others. Metformin is a safe, inexpensive medicine suitable for daily use. It is also suitable to patients who need chemopreventive agents

as a long term therapy. Multiple studies have elucidated the mechanism of action for metformin. Metformin is known to activate AMP-activated protein kinase (AMPK), which inhibits the mTOR pathway that plays an important role in cellular translational processes and progression [111-113]. These properties prompted interest in metformin as a potential anti-cancer agent. Subsequently, a number of observational studies and meta-analysis have associated lower cancer incidence with metformin use as well as a lower risk of nonspecific cancer-related mortality [9,114] . Several studies have also demonstrated that metformin has an anti-proliferative effect associated with cell cycle arrest and apoptosis by down regulation of anti-apoptotic proteins as well as AMPK [115,116], however, its mechanism of action is not fully elucidated, especially in the context of CRC.

M-Probiotics

Having demonstrated the role of microbiota in colorectal cancer onset and progression, it is reasonable to suppose that it could be switched to a "non-carcinogenic" microbiome, thereby preventing the tumorigenic process [30].

Dietary interventions and food supplements, such as selected probiotics, have emerged as a valid alternative to manipulate microbiota and manage several diseases such as diabetes, inflammatory diseases and colon cancer among others [117,118].

The World Health Organization /Food and Agriculture Organization (2001) defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [119].

Multiple reports showed the relevance of probiotic oral supplementation and the scarcity of adverse effects. However, contradictory results still exist in literature about the anticarcinogenic activities of probiotics [117].

Probiotics were also shown to have beneficial effects on diabetes mellitus, with numerous studies reporting their ability to reduce insulin resistance,HbA1C levels and improve glucose tolerance [120]. In brief, probiotics possess a wide spectrum of mechanisms through which they exert their protective effects [119,121,122]:

- Shift in gut microbial composition
- Preservation of epithelial barrier function
- Defense against harmful gut flora
- Competitive elimination of pathogens
- Enhancement of host immunity against pathogens by producing anti-microbial peptides that result in the suppression of specific microorganisms.
- Anti-carcinogenic effects
- Anti-inflammatory actions
- Improvement of lactose intolerance symptoms
- Amelioration of glucose metabolism
- Decrease in insulin resistance
- Lipid profile amelioration.

In the last few years, a growing interest in studying and using probiotic microorganisms has been observed, not only for the treatment of gastrointestinal diseases but also for the overall improvement of human health [30].

Indeed, several studies and reviews highlighting both the systemic activity of probiotics and their beneficial role in ameliorating diabetes and allergic diseases such as ectopic eczema management have been published [10,123].

Consequently, it is clear that the manipulation of intestinal microbiota composition by means of probiotics may be a promising approach to ensure the correct maintenance and improvement of human health, in general and colorectal cancer in particular.

Chapter 2: Aims of the study

The concept of this research emerged after a thorough analysis of the literature, focusing on the links between colorectal cancer and diabetes and the crucial role of microbiota and dysbiosis in these links. This study was established to further investigate the interplay between colorectal cancer and diabetes in a colitis-associated colorectal cancer mouse model in the presence of probiotics and metformin *in-vivo*, using the Azoxymethane/Dextran sodium sulfate (AOM/DSS) colitis-associated CRC model that recapitulates key aspects of human colorectal cancer progression.

Moreover, an emphasis on the role of diabetes in this process was explored by inducing diabetes in the same mouse model using Streptozocin (STZ). On this basis, the specific aims include:

Aim 1: Study the effects of Probiotics on diabetes in a mouse model of STZ-induced diabetes

Aim 2: Evaluate the effects of metformin alone or in combination with probiotics on Colitis-associated colorectal cancer mouse model.

Aim 3: Investigate the mechanisms by which these two compounds (probiotics and metformin) exert their effects.

Chapter 3: Materials and Methods

1. Animals

In this study, a total of 96, 6-weeks old, male Balb/c mice were grouped according to their body weights and housed in medium sized polysulfone cages at the animal care facility of the American university of Beirut. The animals were kept at a constant temperature (21°C ± 2°C) with an alternating 12-hour light/dark cycle. Animal chow (provided from Teklad-Envigo) and water were provided ad libitum.

Personnel working with the animals were trained and certified by the Animal Care facility of the American University of Beirut. Moreover, the study was approved by the Institutional Animal Care and Use Committee of the American University of Beirut, Lebanon (IACUC#16-04-370).

All animal experiments and procedures followed strictly the institutional and international ethical guidelines of the care and use of laboratory animals.

2. Experimental design

The Balb/c mice were divided into 4 main categories: diabetics and non-diabetics , CRC and non-CRC as illustrated in table 2. Animals were then randomly arranged according to the different treatment combinations:

- Non-treated controls
- Treated with metformin (M) alone,
- Treated with probiotics (P) alone
- Treated with the combination of the 2 drugs (MP)

As detailed in Table 2, there were 16 animal subgroups.

Mice were individually labeled by tail coloring to facilitate the tracking of each mouse and the average group weight was adjusted to eliminate any significant weight variance among the different animal groups.

Table 2: Experimental design.

Non-diabetic animals	
With CRC induction	**Without CRC induction**
CRC (CRC)	Normal controls (NC)
CRC + metformin (CRC+M)	Metformin (M)
CRC + probiotics (CRC+P)	Probiotics (P)
CRC + metformin and probiotics (CRC+MP)	Metformin and probiotics (MP)
Diabetic animals	
With CRC induction	**Without CRC induction**
Diabetic CRC (DCRC)	Diabetic (D)
Diabetic CRC + metformin (DCRC+M)	Diabetic + metformin (D+M)
Diabetic CRC + probiotics (DCRC+P)	Diabetic + probiotics (D+P)
Diabetic CRC + metformin and probiotics (DCRC+MP)	Diabetic + metformin and probiotics (D+MP)

3. Induction of CRC

It is well established that the Azoxymethane (AOM)/Dextran sulfate sodium (DSS) model is able to induce CRC in mice and this model is widely used in experimental colitis-associated colorectal cancer studies, in our laboratory and elsewhere [124].

- **DSS preparation:**

DSS (purchased from Sigma-Aldrich, Thermo Fisher Scientific, Inc), is the pro-inflammatory agent. DSS powder was weighed and dissolved in autoclaved water and administered to the animals, at optimized concentrations, in their drinking water. A DSS cycle consists of one week DSS and the two following weeks of regular drinking water.

Since the colitogenic effect of DSS is influenced by environmental factors , the used animal batches and strain as well as other factors, pilot studies were performed to define the optimal concentration and needed number of DSS cycles [125].
AOM (Sigma-Aldrich, Thermo Fisher Scientific, Inc) is the carcinogenic agent, it was injected intraperitoneally at a Maximum Tolerated Dose (MTD) of 10 mg/kg body weight [124].

4. Induction of Diabetes

Streptozotocin (STZ) (Sigma Aldrich, Fisher Scientific, Inc) at a single 150 mg/kg dose has been used to induce diabetes mellitus. Preceding its administration, STZ was directly suspended in citrate buffer (pH 4.4-4.5) and injected intraperitoneally [126,127].

5. Administration of probiotics and metformin

The commercial probiotic (P) used regroups a combination of seven strains of lactic acid-producing bacteria: Lactobacillus rhamnosus, Saccharomyces boulardii, Bifidobacterium breve, Bifidobacterium lactis, Lactobacillus acidophilus, Lactobacillus reuteri and Lactobacillus plantarum. A daily dose of 10^8 CFU per animal was administered in their drinking water.

Metformin (Glucophage) treatment was administered via drinking water at a dosage of 150 mg/kg body weight. .

6. Clinical course assessment

During the experimental period, clinical parameters were recorded, animals were monitored for body weight, stool aspect and rectal bleeding. The clinical disease activity index (DAI) emanated from these parameters with a score ranging from zero to four. DAI is calculated based on the formula : DAI = (Stool consistency + Fecal bleeding + Weight loss)/3 considering the following values : stool consistency (0, normal; 2, loose; 4, diarrhea), gross bleeding (0, absence; 2, blood stained; 4, presence) and weight loss (0, none; 1, 1%-5%; 2, 5%-10%; 3, 10%-20%; 4, >20%) [128].

7. Determination of blood glucose levels

Tail vein blood was used to measure blood glucose levels by am Accu-Chek® Performa blood glucose meter system. The reading spectrum for the Accu-Chek is 10-500 mg/dl values exceeding 500 mg/dl were registered as "HI".

Glucose measurements were done before diabetes induction and weekly after STZ injection. Diabetes was diagnosed with BGL > 250 mg/dl. Moreover, all blood glucose measurements

were taken at the same time, in the fed state early in the morning to eliminate inconsistency in blood glucose values caused by feeding patterns of the mice [129].

8. Measurement of fecal occult blood

In order to collect feces, each mouse was placed for a couple of minutes in an empty cage with no bedding, using a clean forceps, feces were removed and occult blood was measured using Guaiac fecal occult blood test kit, as per the manufacturer instructions [130].

9. Blood and serum collection

Terminal bleeding was performed on the experimental time point by cardiocentesis in accordance with the approved institutional animal care protocols. The blood was then centrifuged at 2500 rpm for 10 min and the serum was separated and stored at -20°C for further analysis.

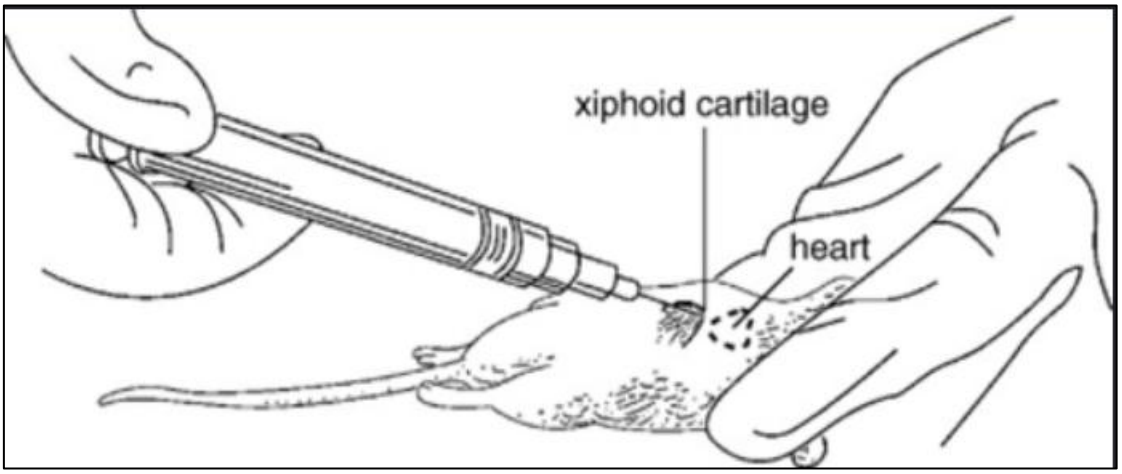


Figure 17: Blood collection by cardiac puncture (cardiocentesis).

10. Dissection

At the experiment endpoint, animals were sacrificed by cervical dislocation and dissected in order to remove their colon. Isolated colon was quickly flushed on ice with cold phosphate-buffered saline (PBS) in order to clean the colon and remove its content.

A portion of this clean colon was fixed in 10% buffered formalin for histological processing and the other bigger portion was snap frozen and kept in a liquid nitrogen tank for further molecular studies.

11. Histological studies

After being fixed in the liquid fixing agent (10% Formalin) for more than 48 hours, colon biopsies went through a series of processing steps: dehydration with graded ethanol, clearing using xylol, wax infiltration and paraffin embedded. Blocks were then cut into 5 μm sections, placed on a glass slide and stained for general morphology with hematoxylin and eosin (H&E) [131].

The different sections were examined under light microscopy and photographed at different magnifications using an Olympus CX41 microscope. A scale from Hussein et al. as illustrated in table 3, was adapted and used to do the histologic scoring of the H&E stained sections [132].

Table 3: Histological 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

12. Determination of intestinal cellular proliferation by immunohistochemistry using Ki-67 stain

Paraffin-embedded sections were used to check for ki-67 labelling, slides were deparaffinization using a series of xylol and graded alcohol (Table 4). Then, antigen retrieval was done by immersion in a citrate buffer at pH=6. After a TBST wash, the primary antibody (Ki-67 Encorbio) was incubated overnight at a dilution of 1/1000 at 4°C .The next day the sections were washed and incubated with goat anti-rabbit IgG highly cross-adsorbed Secondary Antibody, Alexa Fluor 594 (Thermo Fisher Scientific, Inc Scientific) at a dilution of 1/1000 for 2 hours at room temperature. The nuclei were counterstained with DAPI and the slides coversliped and stored at 4°C until analysis.

Ki-67 was evaluated by examining the crypts, which were photographed and the number of Ki-67 positive cells per HPF (40x objective) were counted [133,134].

Table 4: Deparafinization protocol adapted from Abcam IHC.

Solution	Incubation time
Xylene	3 min
Xylene	3 min
1:1 Xylene: 100% Ethanol	3 min
100% Ethanol	3 min
100% Ethanol	3 min
95% Ethanol	3 min
70% Ethanol	3 min
50% Ethanol	3 min

13. Reactive oxygen species measurement by dihydroethidium (DHE)

Frozen slides were prepared from tissues, snap frozen in liquid nitrogen (-80°C), and a solvent resistant pen was used to demarcate colonic tissue. DHE solution (Thermo Fisher Scientific, Inc) was dispensed over the tissue at a 1/1000 dilution. The slides were then placed in an incubator at 37°C for 30 min, the DHE residues removed, and slides counterstained with a mounting medium containing DAPI. Slides were coversliped and stored at 4°C at dark until

microscopic evaluation and photography. DHE intensity was quantified using Zen 2.3 software [9].

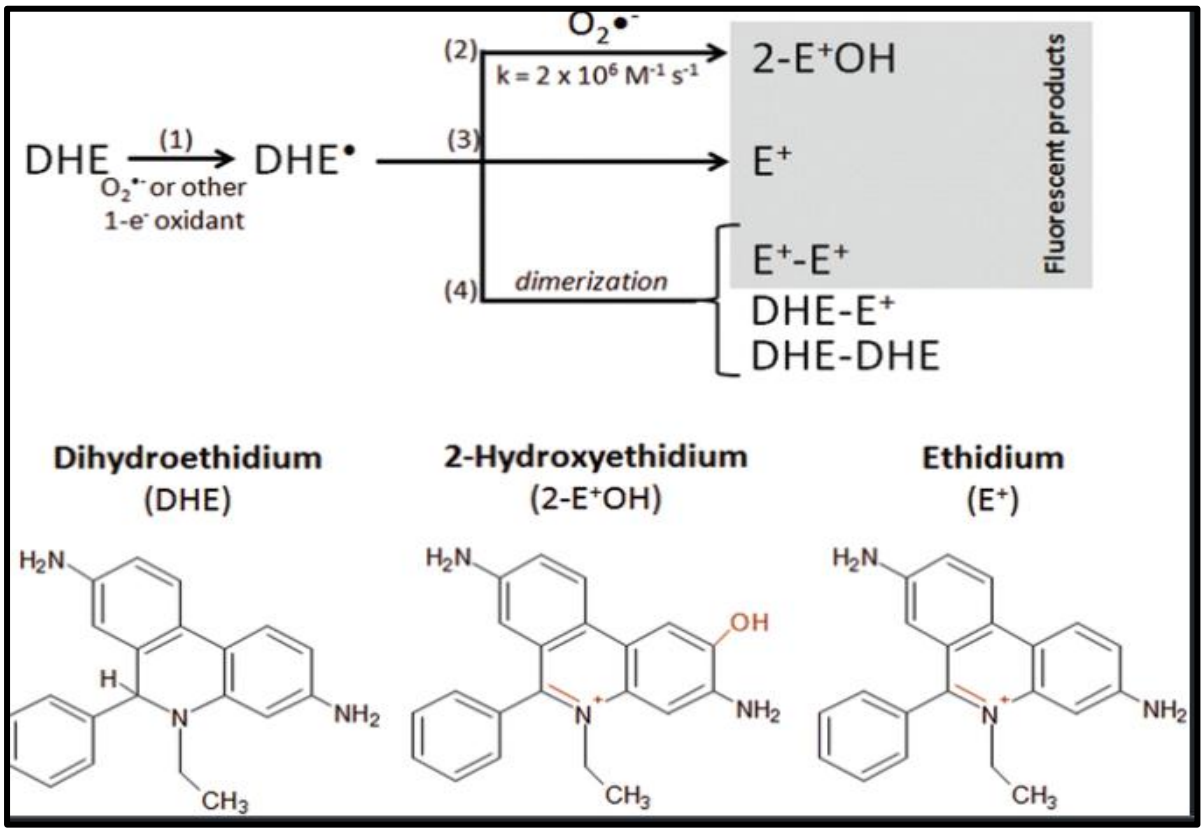


Figure 18: The principle of DHE protocol for ROS detection and quantification [135].

14. Determination of nitrite levels

The classic colorimetric Griess reaction was used to measure serum nitrite levels (Figure 19). Greiss reagent is composed by two light sensitive reagents: A and B.

Reagent A: 1% sulfanilamide (0.3g) in 30% acetic acid (9 ml acetic acid +21 ml H_2O)

Reagent B : 0.1% N-naphthylethylenediamine dihydrochloride (0.1g) in 60% acetic acid (30 ml acetic acid + 20 ml H_2O).

The solution used for the standard curve is sodium nitrite ($NaNO_2$) with $C_0 = 10mM$ (the curve should include at least 7 points = 7 concentrations by serial dilution).

Preparation of 10 mM $NaNO_2$ solution: 690 mg of $NaNO_2$ (powder) dissolved in 1 L dH_2O and for the serial dilutions, the used formula is: $C_i\ \mu M \times V_i = C_f\ \mu M \times V_f\ \mu L$.

At first, Griess reagent was prepared by mixing an equal volume of reagents A and B.

A 96 well microtiter plates were used, whereby 100 µl of Griess reagent was added to 100 µl of serum sample. The plate was incubated in dark for 10 min at room temperature and absorbance was measured at 550 nm. A sodium nitrite standard curve was established using a serial dilution of a $NaNO_2$ solution. This curve was used to calculate the nitrite concentration (µM) [136,137].

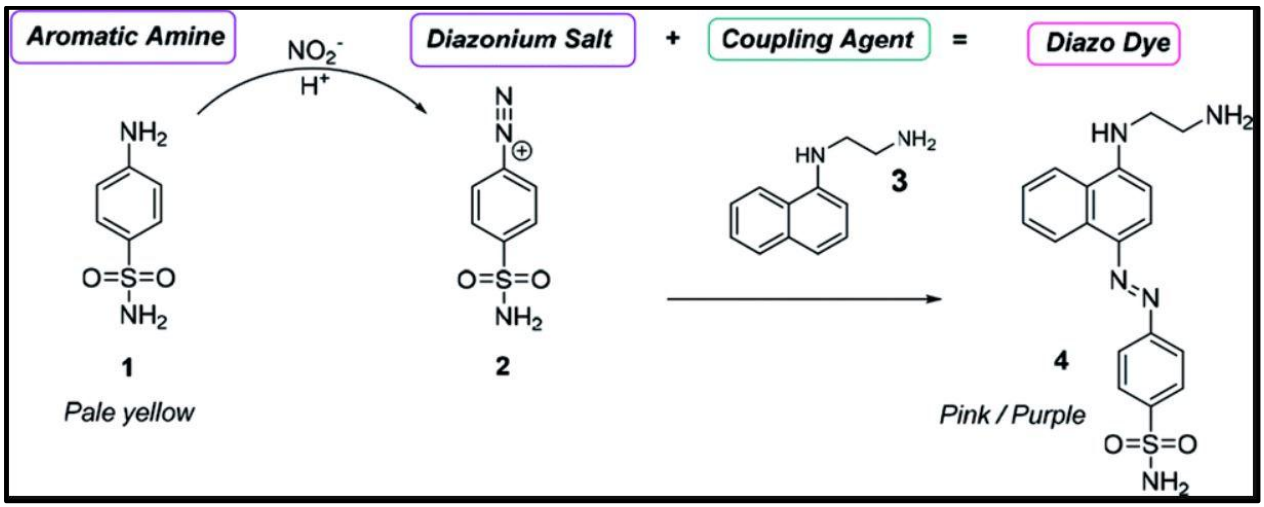


Figure 19: Griess reaction principle [138].

15. Assessment of cytokine levels

The levels of IL-6 and TNF-α were assessed in both plasma and colon tissue using ELISA assay (Figure 20) performed according to the manufacturer's instructions (Thermo Fisher Scientific, Inc). The optical density was measured using a microtiter plate reader on 450 nm (Multiskan Ascent 96/384 plate reader, Thermo Fisher Scientific, Inc). The final results were expressed in pg/ml with the limit of detection of IL-6 and TNF-α as 4-500 pg/ml and 8-1000 pg/ml, respectively.

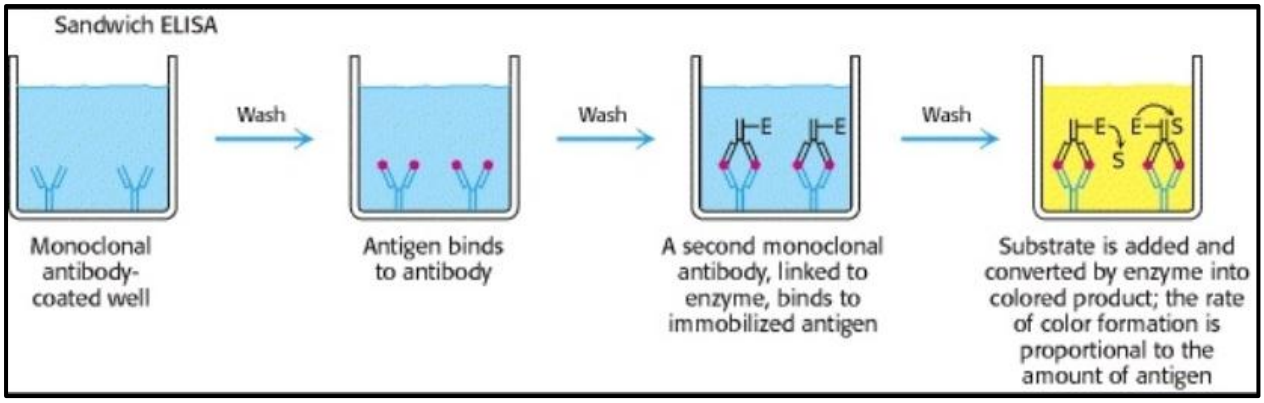


Figure 20: Principle of sandwich Enzyme-linked Immunosorbent assay ELISA [139].

16. Statistical analysis

Statistical analyses were done using GraphPad Prism 8.0.1 software and data were expressed as a mean±SEM. Significant differences were assessed using the one-way ANOVA followed by Tukey-Kramer multiple comparisons test. A value of $P<0.05$ was considered significant [140].

Chapter 4: Results

1. Effect of metformin and probiotics on glycemia

All the emanating clinical, histological, immunohistochemical and molecular data were analyzed with an emphasis on the effect of the combination therapy, M and P, in treating CRC in non-diabetic and diabetic animals.

First of all, diabetes induction in all intended animals was successful. Blood glucose level was measured on a weekly basis as a direct indicator of diabetic status.

As expected, one week after an IP injection of 150 mg/kg STZ, glycemia levels increased beyond 250 mg/dl. These elevated levels were effectively maintained in all diabetic untreated mice (group D and DCRC) during the experimental period (Figure 21b).

All animals in the non-diabetic groups (NC, CRC, CRC+M, CRC+P, CRC+MP, M, P and MP), had glycemia levels less than 250 mg/dl at all time points and the difference between these groups was not statistically significant (ns) as shown in Figure 21a.

Nevertheless, animals in the CRC group that were not exposed to diabetes induction (group CRC), showed elevated glycemia peaks specifically in weeks 3, 5, 8 and 11 in which DSS was administered. Importantly, at week 8, these fluctuations in blood sugar levels were statistically significant when comparing normal animals in group NC to CRC group. These peaks were significantly lowered in animals treated with the MP mixture (CRC+MP versus CRC, $^{\#}p<0.05$) as shown in Figure 21a.

Diabetic (DCRC and D) groups showed the highest blood glucose levels at all time points with no statistical difference (ns) between the two groups. This reveals that colorectal carcinogenesis did not affect the animals' glycemia levels. In diabetic mice, the treatment with P and M alone or in combination significantly reduced glucose levels throughout the experimental period. When compared to the untreated diabetic mice, with CRC induction (DCRC group), M and P single drug administration in DCRC+M and DCRC+P groups reduced significantly blood glucose values (Figure 21b, $*p<0.05$). Remarkably, the M and P mixture showed a significant effect, superior than either drug alone, as showed when comparing DCRC+M versus DCRC+MP groups ($^{\dagger}p<0.05$) and DCRC+P versus DCRC+MP groups ($^{\dagger}p<0.05$). These observations indicated that P helped M in alleviating the hyperglycemic phenotype induced by STZ (Figure 21b).

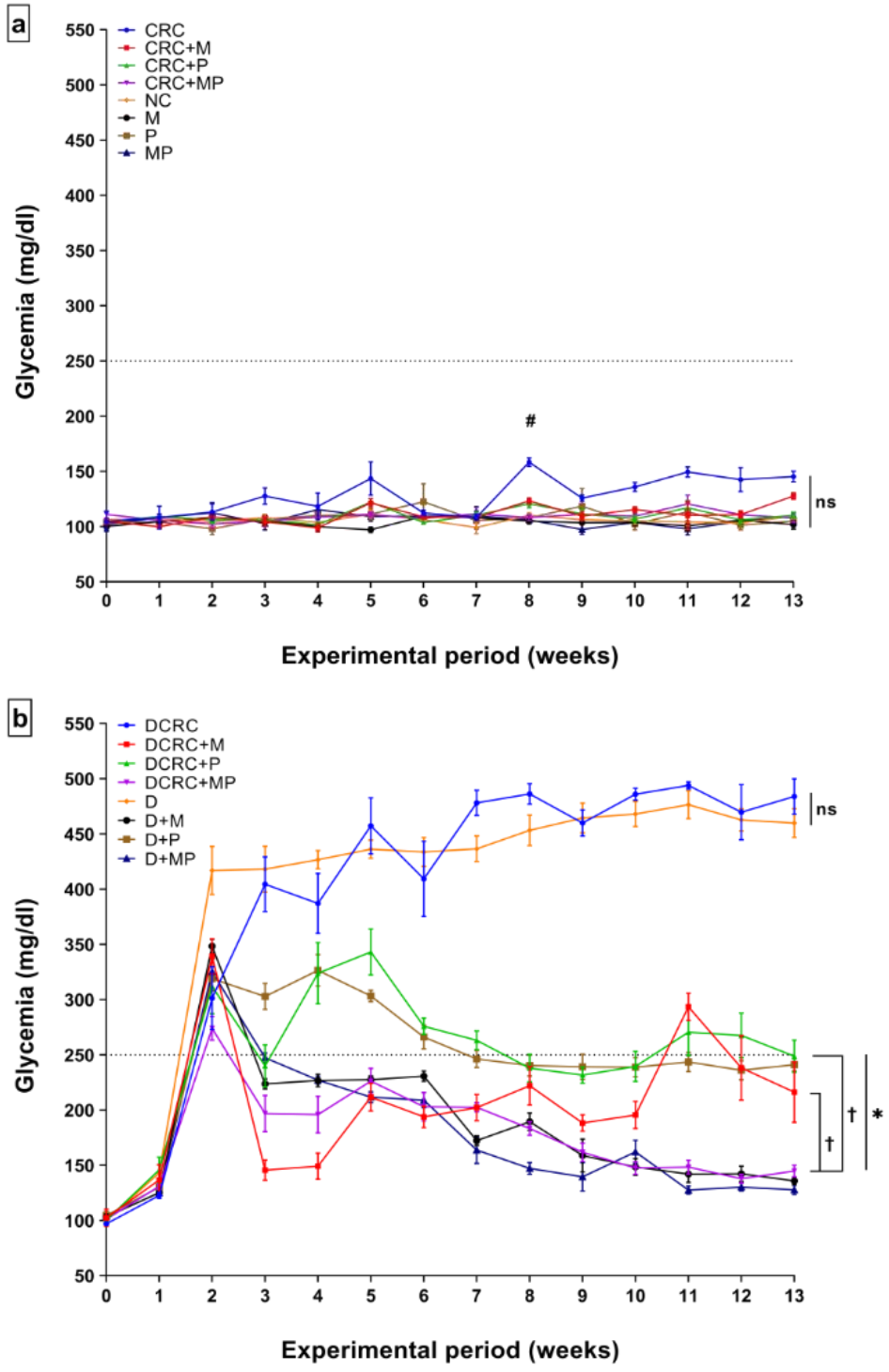


Figure 21: Effects of probiotics and metformin on glycemia levels in non-diabetic (a) and diabetic (b) mice.

2. Effect of metformin and probiotics on CRC

The optimal procedure adopted for CRC induction was successful in all mice. The concentration of Dextran sulfate sodium (DSS) and its number of cycles were defined by pilot studies in which using different concentrations of DSS ranging from 1 till 3% were tested. Treatment of male Balb/c mice with 1.5% DSS in their drinking water for 4 cycles, in addition to an injection of 10 mg/kg Azoxymethane (AOM), resulted in clinical signs and symptoms, gross and histological changes characteristic of CRC.

Animals that underwent this optimized DSS/AOM protocol showed signs of sickness starting the first cycle and these signs were intensified by each consecutive DSS cycle.

Weight loss (Figure 22), loose stools, diarrhea and rectal bleeding were the main detected changes, as calculated by the DAI. Additionally, these animals presented various signs of sickness such as bad posture, hunched back, decreased grooming, reduced mobility and responsiveness (Figure 23).

A weight gain was seen in non-diabetic and diabetic animals without colorectal cancer (i.e. in groups NC, M, P, MP, D, D+M, D+P and D+MP). Conversely, groups with colorectal cancer presented a weight loss; whereby the lowest weight averages were noted in CRC and DCRC groups with no statistically significant difference between these 2 groups. Treatment with metformin or probiotics alone and most importantly in combination had a positive effect in preventing the weight loss in the non-diabetic and diabetic CRC animals with $^{\#}p<0.05$ and $^{*}p<0.05$ respectively (Figure 22).

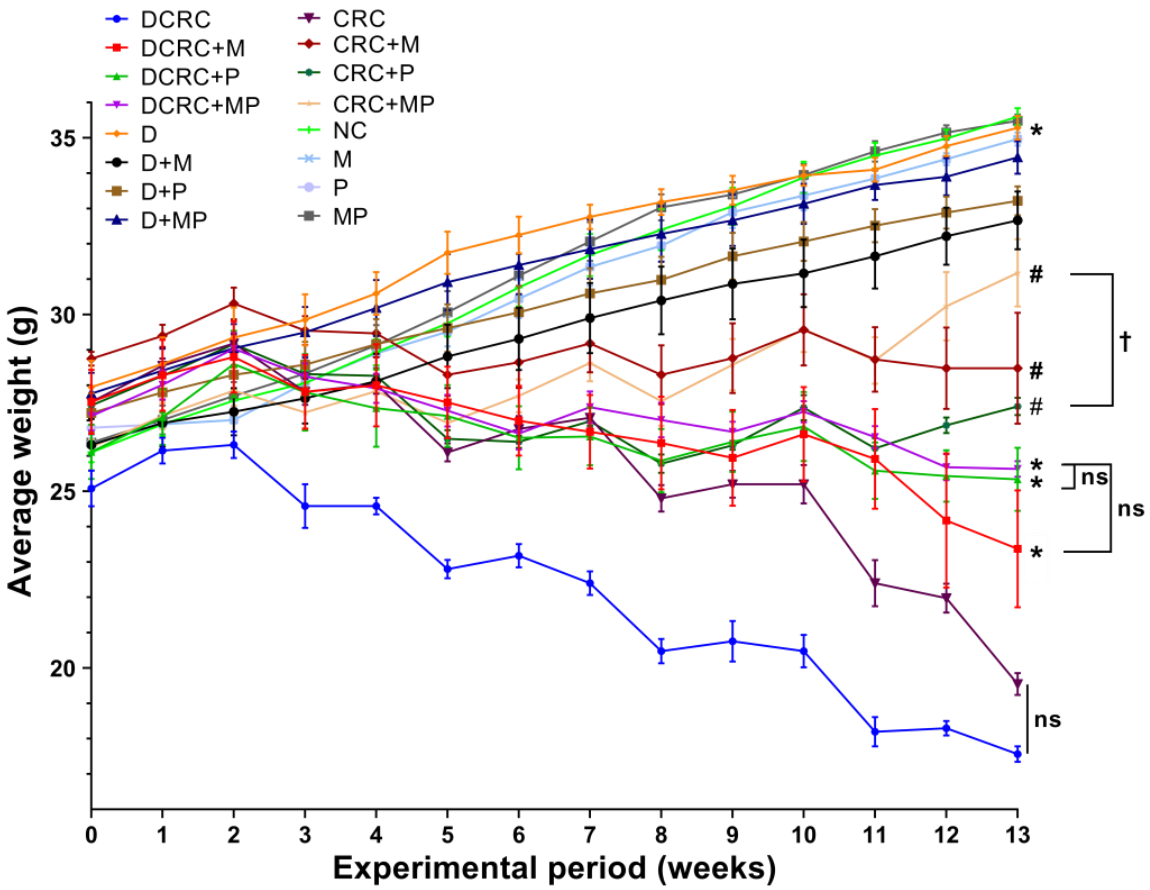


Figure 22: Weight changes in the different animal groups during the experimental period**.**

Figure 23: Hunched back animals with unhealthy clinical profile (left side), a mouse with diarrhea and rectal bleeding (right side).

When looking at fecal occult blood, negative values were recorded in all animals belonging to the groups that were not exposed to CRC induction (NC, D, M, D+M, P, D+P, MP and D+MP groups). On the other hand, animals that had the CRC induction, displayed rectal bleeding and had positive fecal blood to varied extents. 17% of CRC and DCRC animals had positive occult blood on the first DSS administration (at week 3), they reached 100% positive rates by the end of the experiment (Table 5).

Metformin and probiotics treatment alone or most importantly in combination reduced the frequency of blood in the stools and hindered their appearance until week 5.

CRC+MP and DCRC+MP groups showed the best scores whereby blood in stools was detected in only 67% of the animals at week 5 and the positive rates were 50% and 33%, respectively , at week 13. In addition, when paralleling animals treated with single drug in non-diabetic and diabetic CRC, i.e. groups (CRC+M, CRC+P, DCRC+M and DCRC+P) to the MP combination groups (CRC+MP and DCRC+MP), lower proportions of positive occult blood in the combination treatment, were encountered at all time points (Table 5). Treatments elicited a decrease of the blood in the stools, thus indicating a recovery in the mucosa.

Table 5: Effects of metformin and probiotics on fecal occult blood (FOB). Note the color scale extending from strong to faint red whereby the shade of the red color indicates the value of the cell.

Group	Experimental period (weeks)													
	0	1	2	3	4	5	6	7	8	9	10	11	12	13
NC	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
D	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
CRC	0%	0%	0%	17%	33%	100%	100%	50%	100%	100%	100%	100%	100%	100%
DCRC	0%	0%	0%	17%	17%	100%	100%	80%	100%	100%	100%	100%	100%	100%
CRC+M	0%	0%	0%	0%	0%	100%	83%	33%	100%	83%	67%	100%	100%	100%
DCRC+M	0%	0%	0%	0%	0%	100%	33%	17%	100%	50%	33%	100%	100%	100%
CRC+P	0%	0%	0%	0%	0%	83%	33%	33%	100%	100%	67%	100%	100%	100%
DCRC+P	0%	0%	0%	0%	0%	100%	50%	33%	100%	33%	33%	100%	100%	60%
CRC+MP	0%	0%	0%	0%	0%	67%	17%	0%	67%	50%	33%	67%	67%	50%
DCRC+MP	0%	0%	0%	0%	0%	67%	17%	17%	100%	17%	17%	100%	100%	33%
M	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
D+M	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
P	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
D+P	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
MP	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
D+MP	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

As for the DAI, the highest disease activity indices were obtained in animals belonging to the CRC and DCRC groups (Figure 24). Treatment of CRC animals with M alone or P alone improved their clinical profile and decreased DAI, but not significantly.

Importantly, the combined MP treatment induced a decrease in DAI levels in non-diabetic CRC and diabetic CRC ($^{\#}p<0.05$ and $*p<0.05$, respectively), as these groups (CRC+MP and DCRC+MP) had the lowest DAI along with an amelioration in their clinical status.

The addition of probiotics to the metformin treatment was beneficial as this combination decreased DAI in non-diabetics as seen when comparing animals treated with metformin alone and animals treated with the MP combination in CRC and DCRC groups (Figure 24).

normal controls and in all of the other groups that were not subject to AOM/DSS CRC induction (i.e. M, P, MP, D, D+M, D+P and D+MP groups) had normal DAI close to zero.. Additionally, it is worth noting that when comparing diabetic animals to their non-diabetic counterparts, diabetics were shwon to present more signs of discomfort and sickness without significant alteration of the DAI score (Figure 24).

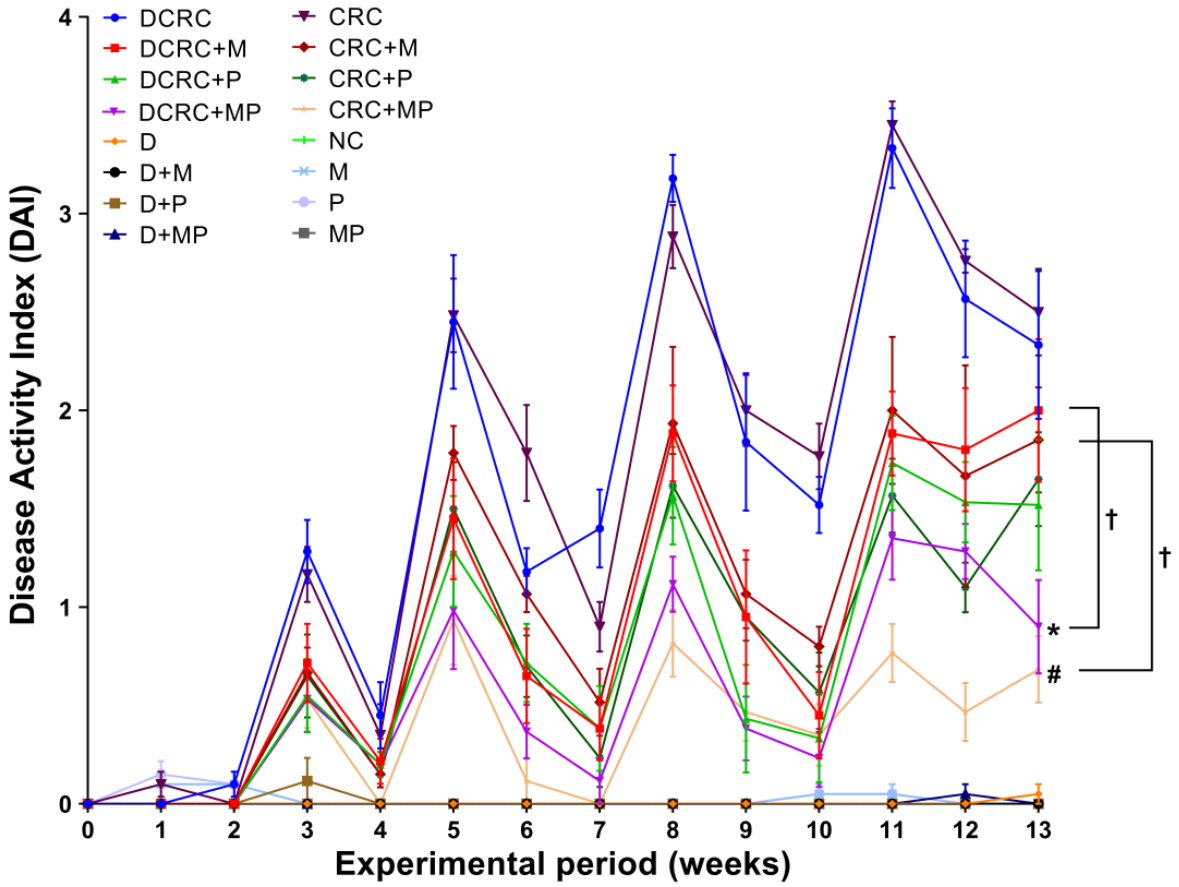


Figure 24: Disease activity index variations in the different groups.

3. Effect of metformin and probiotics on the survival rates

A fluctuation in survival rates was detected between the different groups based on their experimental condition and administered treatment. The lowest survival rate was obtained in in diabetic CRC (DCRC) animals whereby 50% of the animals were dead by the experimental endpoint (week 13). Animals receiving the M and P treatments had survival rates of 67% and 83% in groups DCRC+M and DCRC+P, respectively. Importantly DCRC+MP group receiving the combined therapy had 100% survival rates (Figure 25).

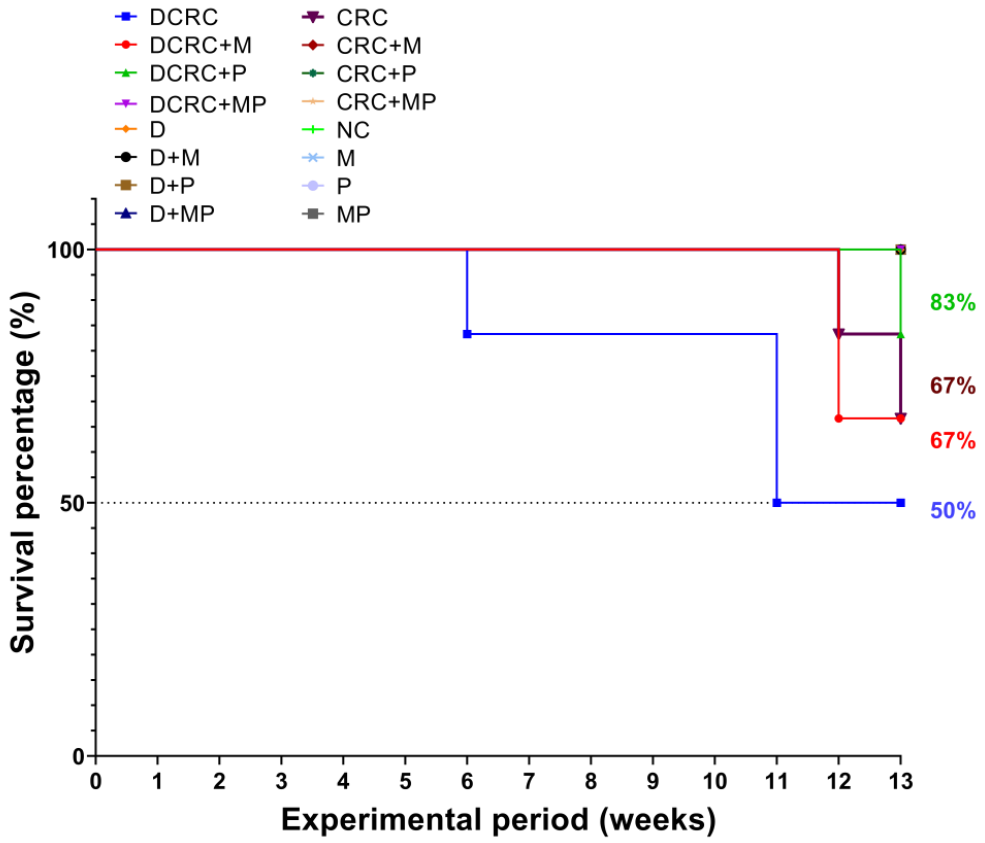


Figure 25: Kaplan-Meier survival curves of animals in the different groups.

CRC non-diabetic animals had a survival rate of 67%, the survival was ameliorated when animals were treated with M and P alone or in combination in CRC+M, CRC+P and CRC+MP groups, as their survival rates reached 100% similarly to normal animals in groups NC, M, P and MP (Figure 25).

4. Metformin and probiotics effect on colon length

It is well known that a shortening of the colon reflects the extent of colorectal inflammation. In this study, measurement of the colon showed differences between the various groups: the shortest colons were seen in mice exposed to AOM/DSS (CRC and DCRC groups) with 6.75±0.78 cm and 6.67±0.17 cm respectively, (Figures 26 and 27).

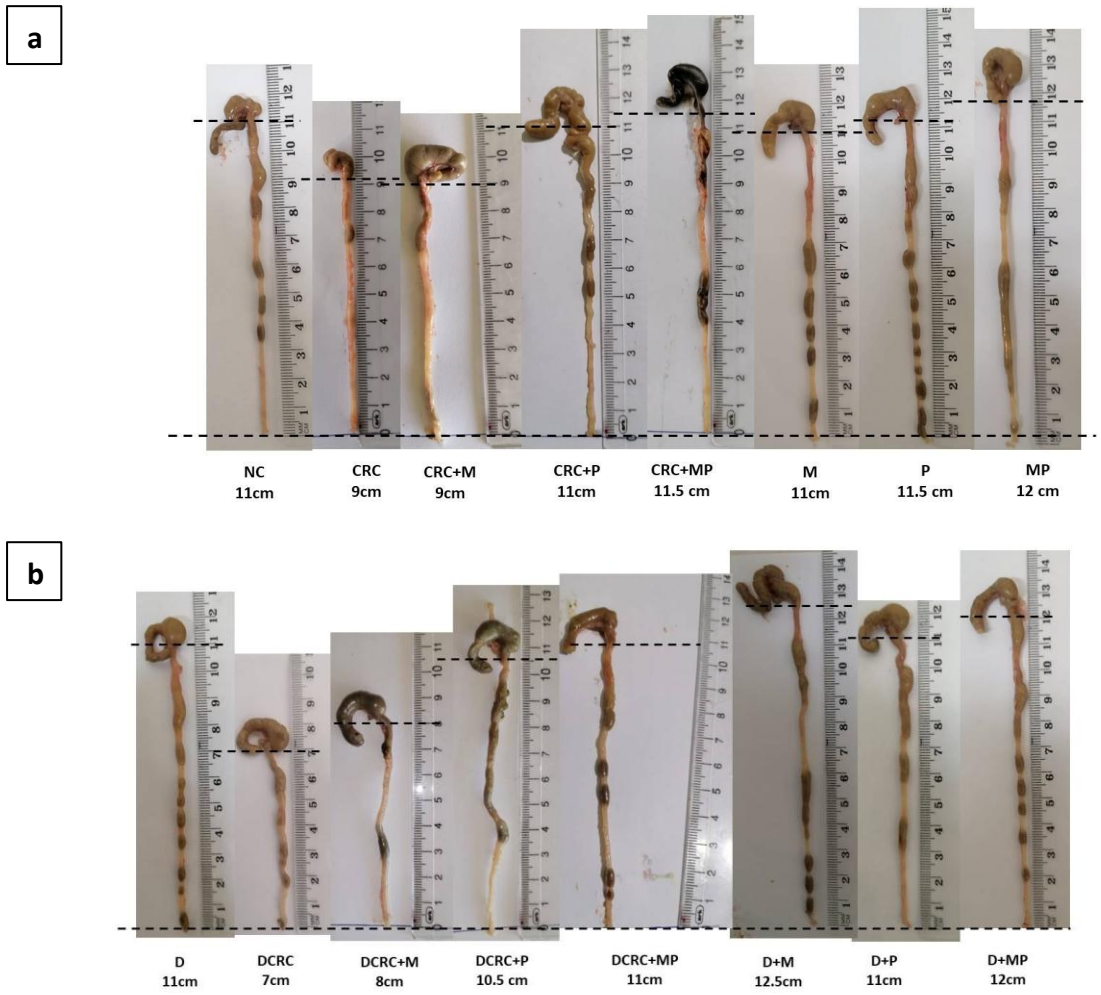
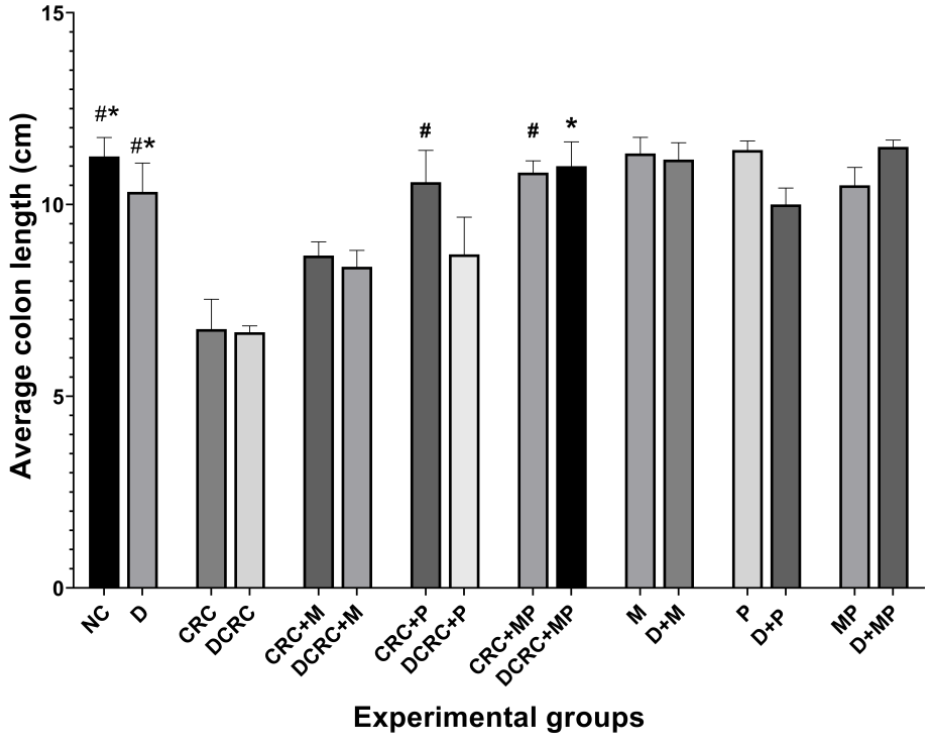


Figure 26: Gross macroscopic images of the colon from non-diabetic groups (a) and diabetic groups (b).

In non-diabetics, animals that were subject to CRC induction (CRC group), showed significantly shorter colons than the normal controls (NC) ($^{\#}p<0.05$). Metformin administration in (CRC+M) group augmented the colon length, but this was not statistically significant. However, administration of P alone in (CRC+P) group or in combination with metformin (CRC+MP) significantly increased colon length to reach 10.58±0.83 cm and 10.83±0.31 cm, respectively, with $^{\#}p<0.05$ (Figure 27), close to normal control (NC) group.

Significantly shorter colon were obtained when comparing DCRC group and D group with $*p<0.05$. Treatment with single drug, M or P ameliorated the colon length but not significantly in DCRC+M and DCRC+P groups. However, when the MP combination is administered, the colon length was significantly ameliorated, even that it reached the normal values (11±0.63 cm in DCRC+MP group) ($*p<0.05$) (Figure 27).

Diabetes alone did not affect colon length, as no statistically significant difference was obtained when comparing non-diabetic and diabetic animals in all subgroups.



Non-diabetic groups	NC	CRC	CRC+M	CRC+P	CRC+MP	M	P	MP
Mean±SEM	11.25±0.50	6.75±0.78	8.67±0.36	10.58±0.83	10.83±0.31	11.33±0.42	11.42±0.24	10.5±0.47

Diabetic groups	D	DCRC	DCRC+M	DCRC+P	DCRC+MP	D+M	D+P	D+MP
Mean±SEM	10.33±0.75	6.67±0.17	8.37±0.43	8.70±0.97	11.0±0.63	11.17±0.44	10.0±0.43	11.5±0.18

Figure 27: Colon length variation.

Average colon length (cm) was recorded on the day of sacrifice at week 13, bar graphs represents the mean±SEM for each group (n=6). Significant differences between the groups were determined by one-way ANOVA followed by Tukey's Multiple Comparison Test. Non-diabetic groups were compared to their experimental CRC control, significance was expressed by $^{\#}p<0.05$. On the other hand, diabetic groups were compared to their experimental diabetic CRC control, significance was expressed by $*p<0.05$

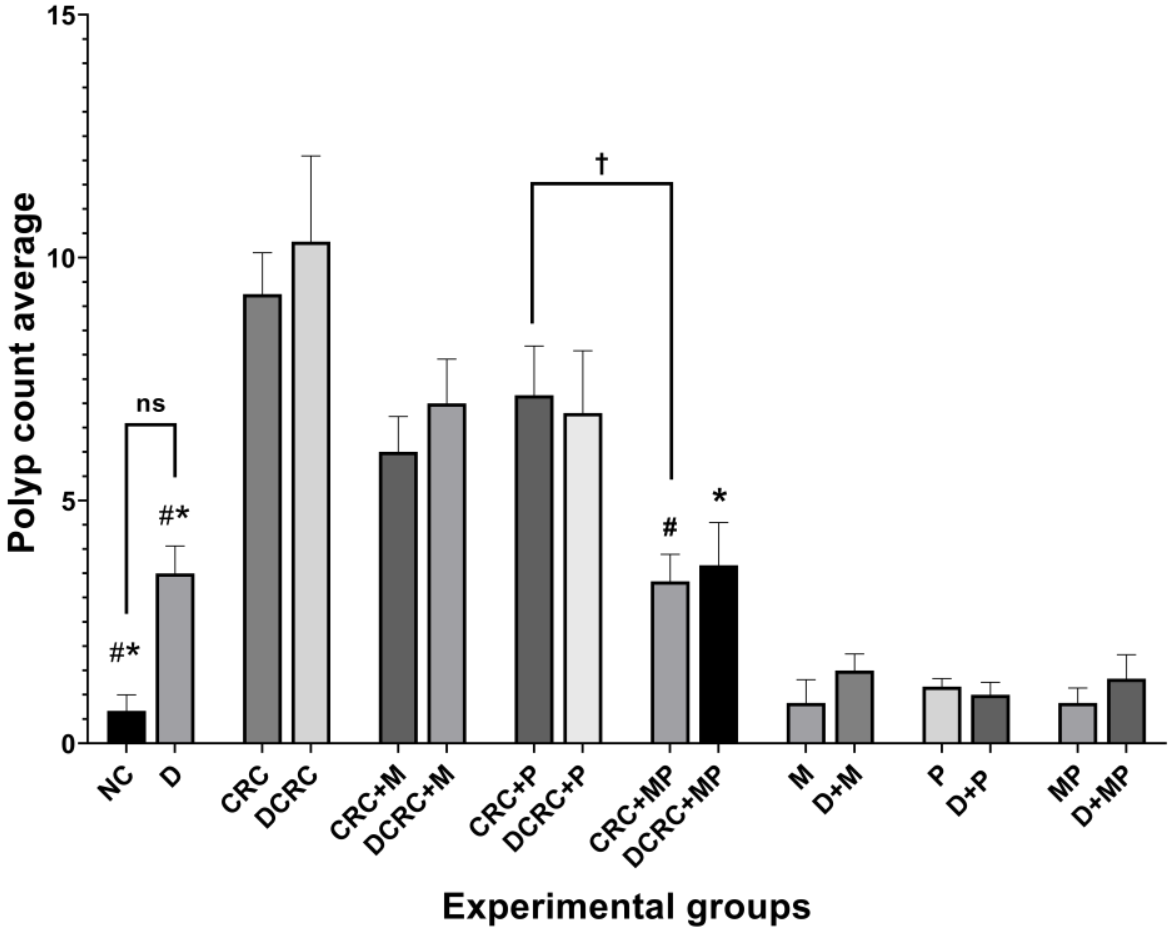
5. Effect of metformin and probiotics on polyp formation

Polyp formation was evaluated by quantifying the number of formed bumps within the colon from the ileocecal junction until the distal end of rectum.

Animals in CRC and DCRC groups had an average number of 9.25±0.85 and 10.33±1.76 polyps, respectively. These numbers were significantly higher than the very low and negligible number obtained in the normal controls (0.67±0.33) $^{\#}p<0.05$ and $*p<0.05$, respectively.

Treatment with either drug alone in non-diabetic and diabetic CRC animals reduced the number of polyps; however, this reduction was not statistically significant. Conversely, animals receiving the MP combination therapy showed significantly less polyps with 3.33±0.56 polyps in CRC+MP group and 3.67±0.88 in DCRC+MP group ($^{\#}p<0.05$, $*p<0.05$), respectively (Figure 28).

In D group, diabetic mice had an average of 3.5±0.56 polyp, this number was higher than their non-diabetic counterparts (0.67±0.33 polyps in NC group) with no statistical significance (Figure 28).



Non-diabetic groups	NC	CRC	CRC+M	CRC+P	CRC+MP	M	P	MP
Mean±SEM	0.67±0.33	9.25±0.85	6±0.73	7.17±1.01	3.33±0.56	0.83±0.48	1.17±0.17	0.83±0.31
Diabetic groups	**D**	**DCRC**	**DCRC+M**	**DCRC+P**	**DCRC+MP**	**D+M**	**D+P**	**D+MP**
Mean±SEM	3.5±0.56	10.33±1.76	7.±0.91	6.8±1.25	3.67±0.88	1.5±0.34	1.0±0.26	1.33±0.49

Figure 28: Polyp count variation in the different groups.

Polyps were counted on the day of sacrifice, bar graphs represents the mean±SEM for each group (n=6). Significant differences between the groups were determined by one-way ANOVA followed by Tukey's Multiple Comparison Test. Non-diabetic groups were compared to their experimental CRC control , significance was expressed by #$p<0.05$. On the other hand, diabetic groups were compared to their experimental diabetic CRC control, significance was expressed by *$p<0.05$. Moreover, when comparing only 2 groups, connecting lines were used to indicate the compared groups with †$p<0.05$, (ns) stands for non-significant.

6. Histological alterations of the colon due to metformin and probiotics treatment

No alterations were depicted when examining colon sections of the normal mice in groups NC, and mice receiving metformin and probiotic alone or in combination in groups M, P and MP. These groups showed low histological scores, they had normal colon histology characterized by the straight unbranched crypts reaching the muscularis mucosa, the intact columnar epithelium, with enterocytes lining the crypts, presence of numerous goblet cells , a thin smooth muscularis mucosae , as well as normal presence of inflammatory cells aggregates (Panel 26A-a).

In contrast, in CRC and DCRC groups, animals that were exposed to the AOM/DSS protocol showed significant histological alteration upon histological inspection of their respective colon tissues.

These animals in these groups had the highest histological alterations with 19.0±1.35 and 20.0±0.58, respectively (Figure 29C). Several changes were noted indicating the different levels of inflammation and dysplasia occurring in the colon. Mainly, an epithelial ulceration, an inflammation of the crypts, formation of crypt abscesses, as well as a dysregulation in the normal crypt architecture were noted. In addition, huge inflammatory cells infiltration in the mucosa and submucosa were present, along with a discontinued muscularis mucosa and goblet cells depletion ….as represented in Panels 26A-e and 26B-m for CRC and DCRC, respectively.

The comparison of the histological scores in the different non-diabetic and diabetic groups shed light on the beneficial effect of the combination therapy on the colonic tissue.

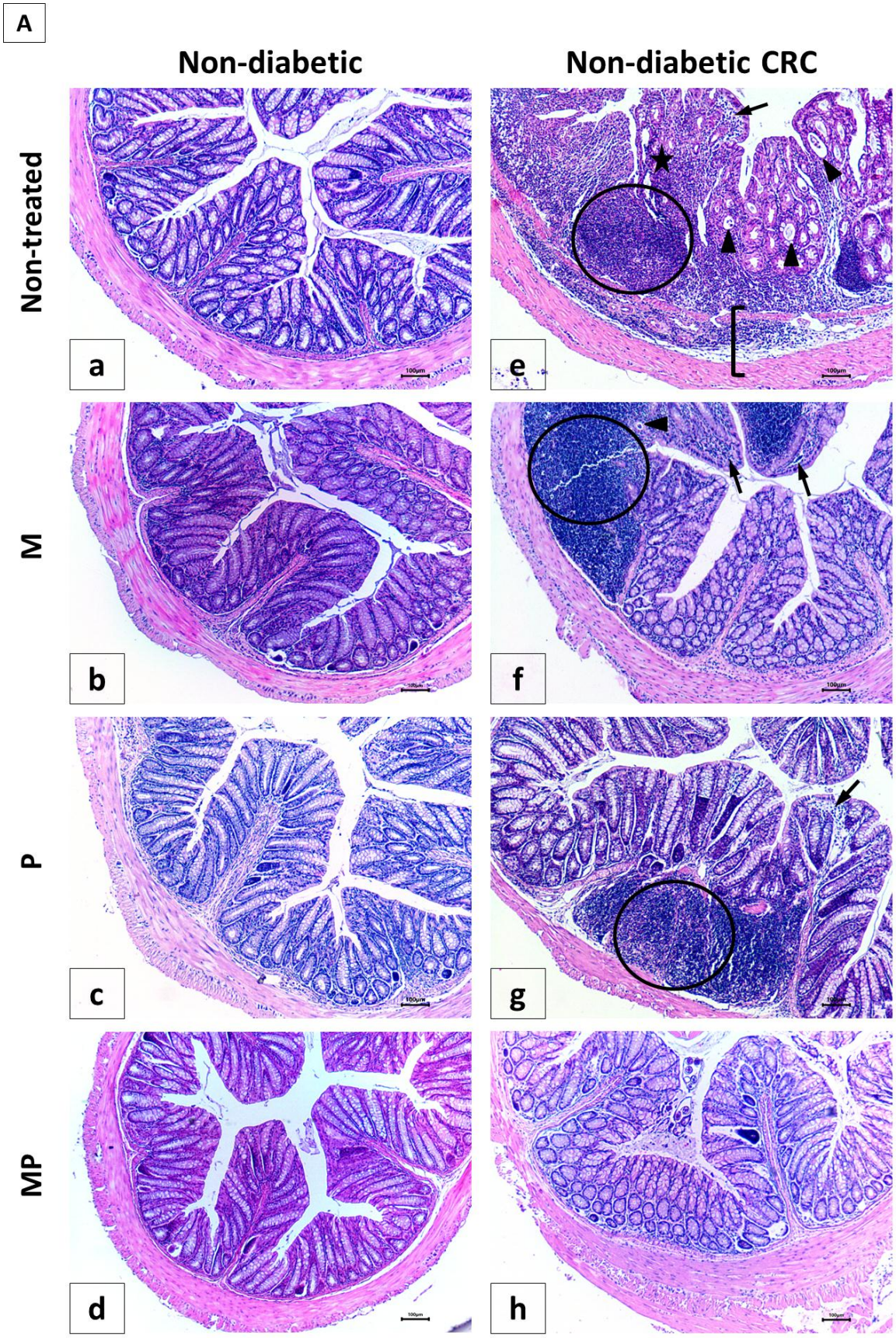
An improvement in colon crypts was noted in groups CRC+M and CRC+P, as seen in panels 28A-f and g, and this single drug treatment reduced the histological score to reach 14±1.0 and 11.83±0.31, respectively, with $^{\#}p<0.05$ when compared to untreated CRC (Figure 29C). The administration of MP combination, had an improved effect better than either drug alone and presented the lowest histological score (8.67±1.17 in CRC+MP group with $^{\#}p<0.05$) (Figure 29).

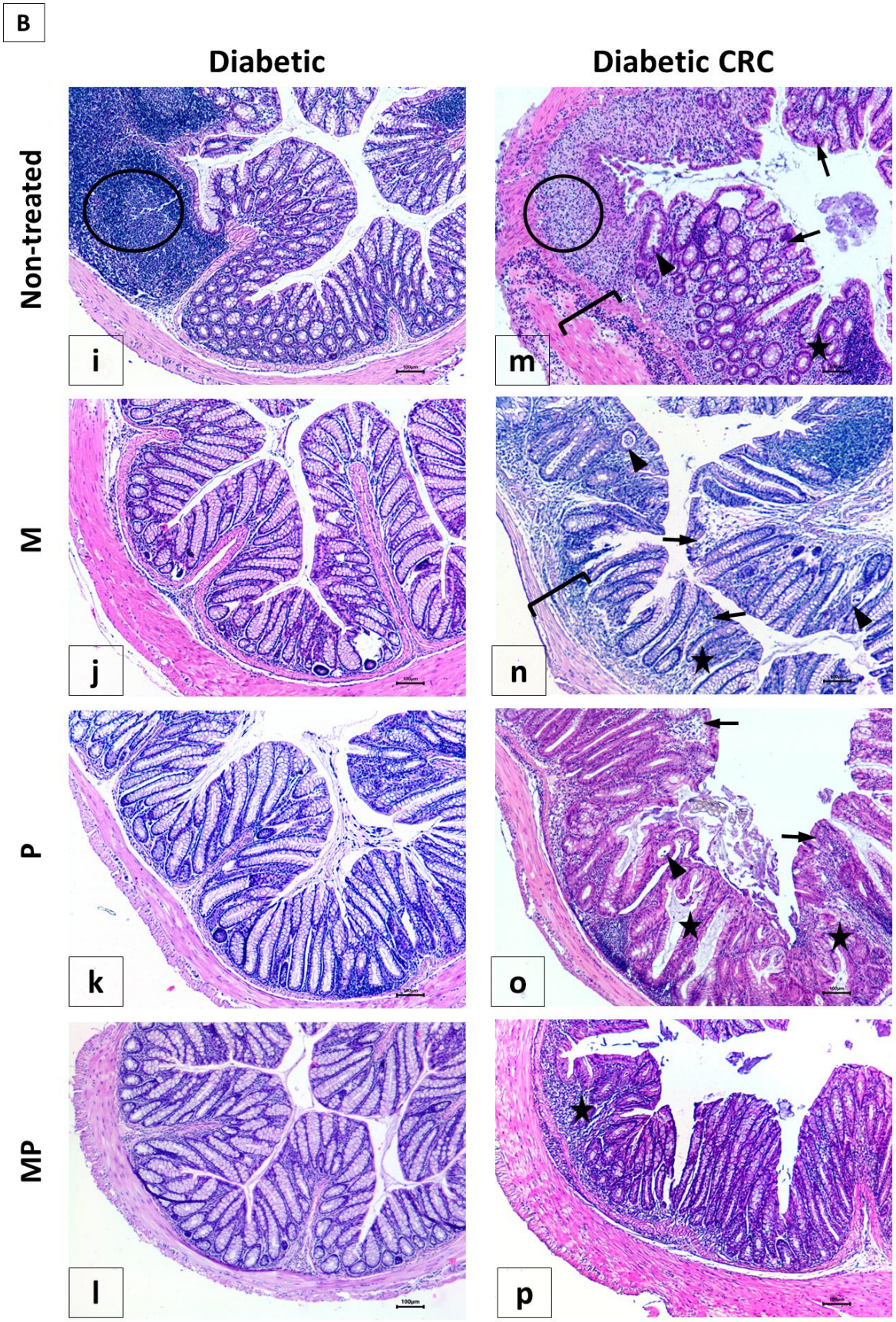
On the other hand, diabetic animals (D group), that were not exposed to CRC induction, showed a histological alteration score of 7.50±1.54, significantly higher than non-diabetic animals (NC group) with $^{\dagger}p<0.05$ (Figure 29C), thus emphasizing on the damage caused by diabetes alone

on the colonic tissue illustrated by the increase in inflammatory cells infiltrates as seen in Panel 29B-i.

Additionally, single drug treatment in groups DCRC+M and DCRC+P was not able to prominently reverse the pathological damage caused by inflammation and dysplasia (Panel 29B-n and 29B-o). However, when probiotics and metformin were administered in combination, the improvement of the histological damage and the reduction in the histological score were statistically significant as noted in group DCRC+MP (Panel 29B-p) with *$p<0.05$ (Figure 29C).

Besides, the structural improvement between treatment with metformin alone (CRC+M, DCRC+M) and metformin with probiotics in CRC+MP and DCRC+MP groups was statistically significant, $^{\dagger}p<0.05$ (Figure 29C). One probable explanation could be that the dysregulated microbiota in CRC could stop metformin from exerting its protective effects on the colonic tissues. Adjustment of this dysbiosis with probiotics showed to be critical in modulating the anti-inflammatory and anticarcinogenic mechanism of action of metformin.


A
Non-diabetic
Non-diabetic CRC
Non-treated
M
P
MP
a
b
c
d
e
f
g
h


B
Diabetic
Diabetic CRC
Non-treated
M
P
MP
i
m
j
n
k
o
l
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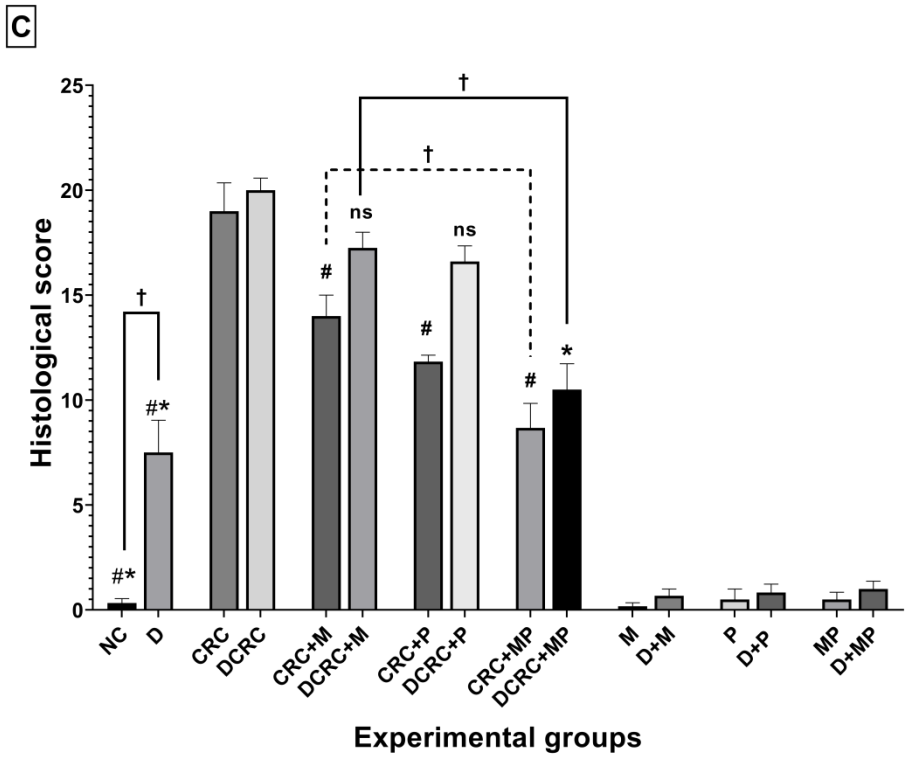

Non-diabetic groups	NC	CRC	CRC+M	CRC+P	CRC+MP	M	P	MP
Mean±SEM	0.33±0.21	19±1.35	14.0±1.00	11.83±0.31	8.67±1.17	0.17±0.17	0.50±0.50	0.50±0.34

Diabetic groups	D	DCRC	DCRC+M	DCRC+P	DCRC+MP	D+M	D+P	D+MP
Mean±SEM	7.50±1.54	20±0.58	17.25±0.75	16.60±0.75	10.5±1.23	0.67±0.33	0.83±0.40	1.00±0.37

Figure 29 Effect of probiotics and metformin on colon histology.

(A-B) Representative images of H&E-stained colon sections illustrating the histological changes in the non-diabetic (Panel A) and diabetic (Panel B) groups.

Note the presence of large inflammatory cells infiltrates (circle), inflammatory cells invading the edematous submucosa (bracket), crypt abcess (black triangle) and cryptitis (black arrow) as well as crypt architecture disarray (star). Significant improvements in the combination treated mice in groups CRC+MP (7A-h) and DCRC+MP (7B-p) were noted. Original magnification 4X; scale bars 100 µm. Photos were adjusted for white balance using Adobe Photoshop®; (C) Histological alterations score. Data is expressed as Average ± SEM (n=6). Significant differences between the groups were determined by one-way ANOVA followed by Tukey's Multiple Comparison Test. Non-diabetic groups (CRC, CRC+M, CRC+P, CRC+MP, M, P and MP) were compared to their experimental CRC control (CRC), significance was expressed by #$p<0.05$. On the other hand, diabetic groups (DCRC, DCRC+M, DCRC+P, DCRC+MP, D+M, D+P and D+MP groups) were compared to their experimental diabetic CRC control (DCRC), significance was expressed by *$p<0.05$. Moreover, when comparing only 2 groups, connecting lines were used to indicate the compared groups with †$p<0.05$, (ns) stands for non-significant.

Lower magnifications of each slide are presented in the figures 30 till 37.

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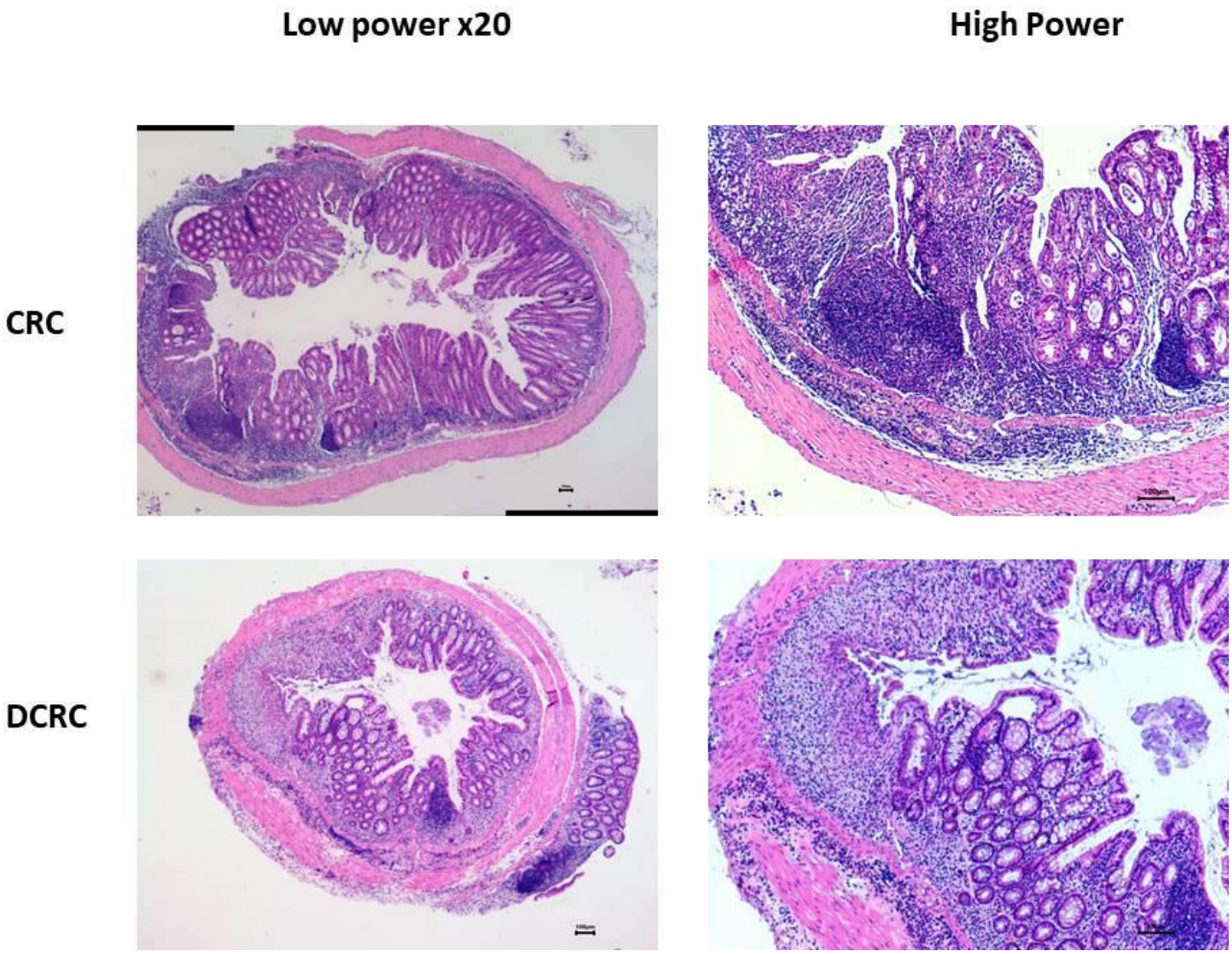


Figure 30: Histology of CRC and DCRC animals.

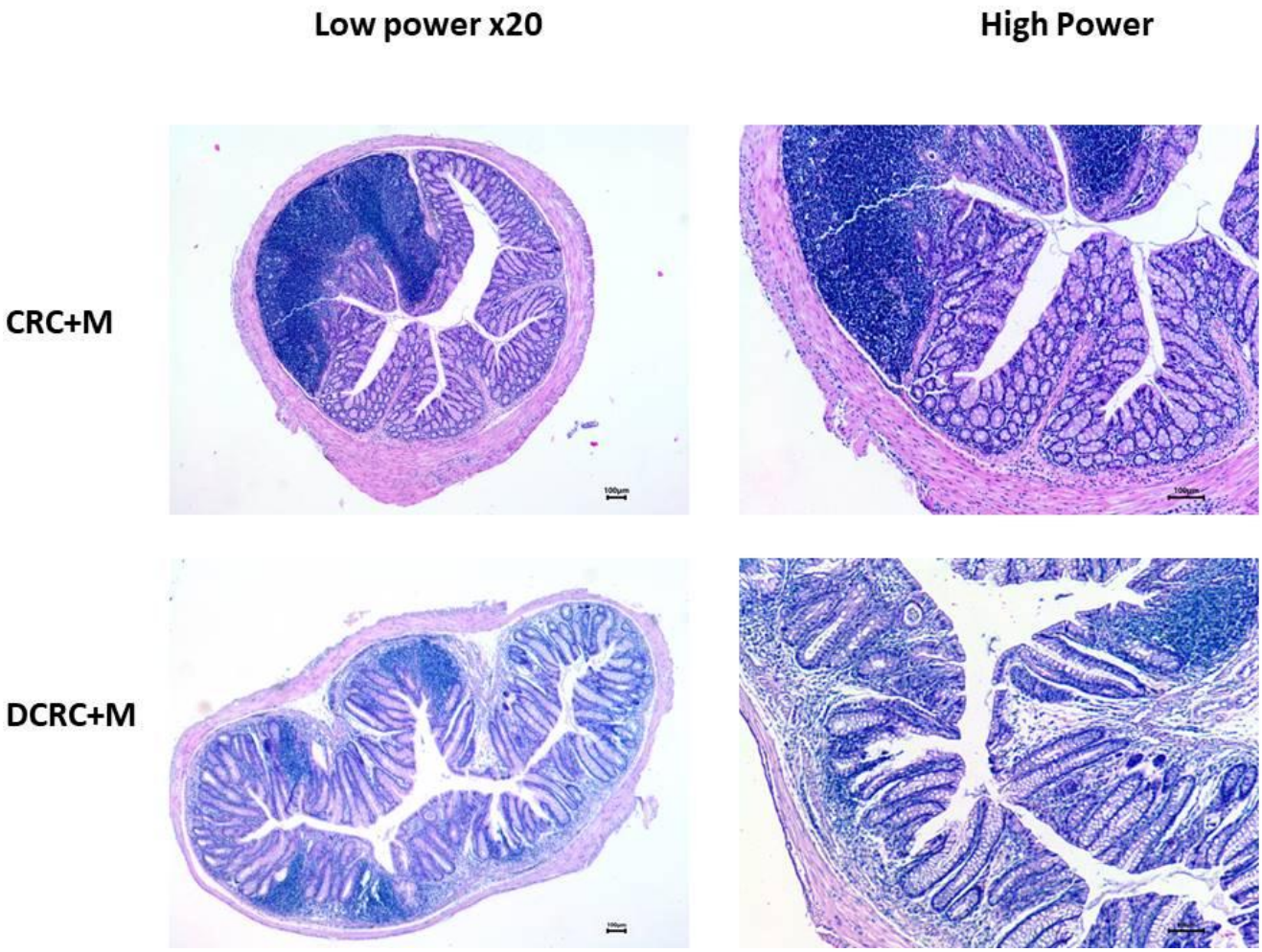


Figure 31: Histology of CRC and DCRC animals treated with metformin alone.

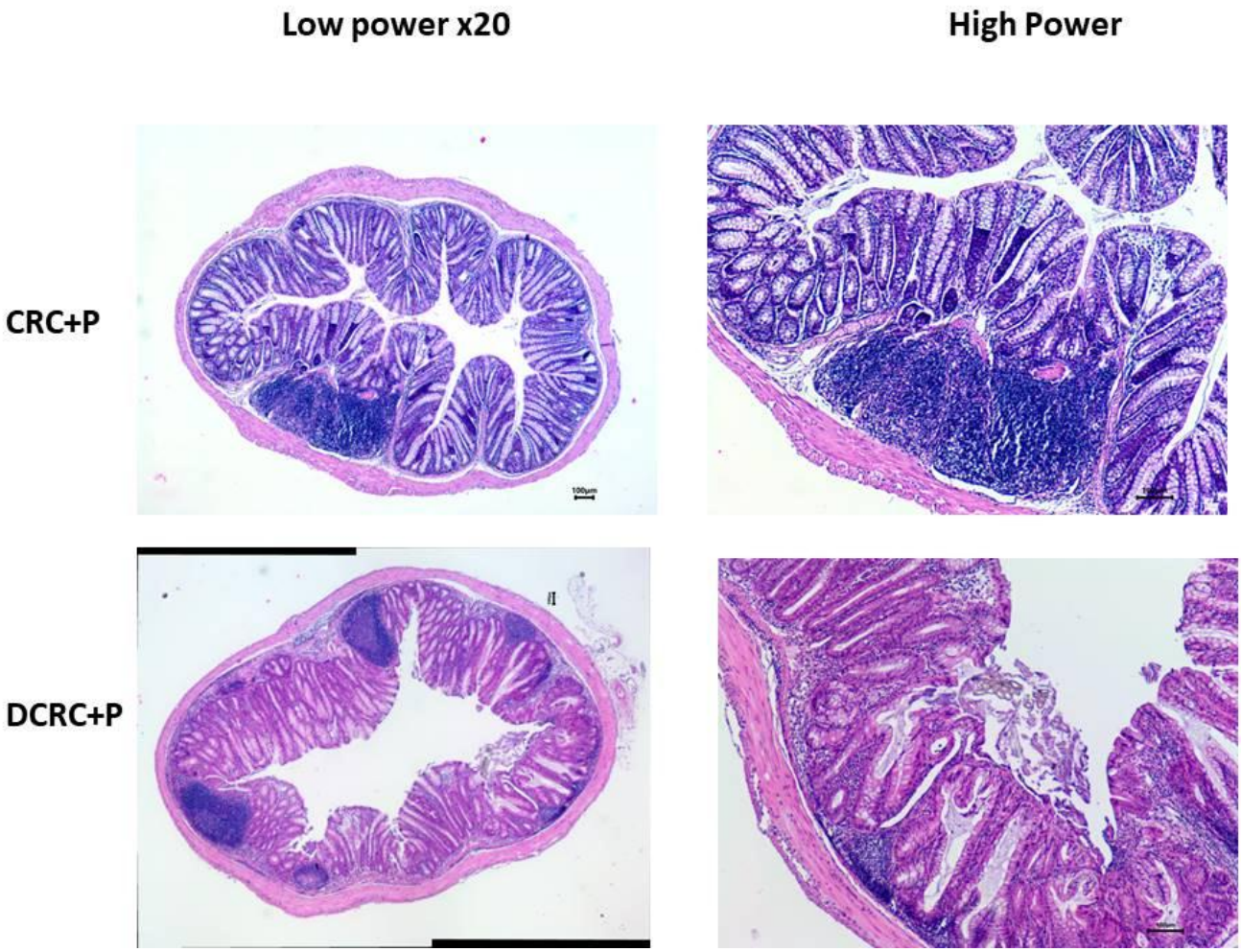


Figure 32: Histology of CRC and DCRC animals treated with probiotics.

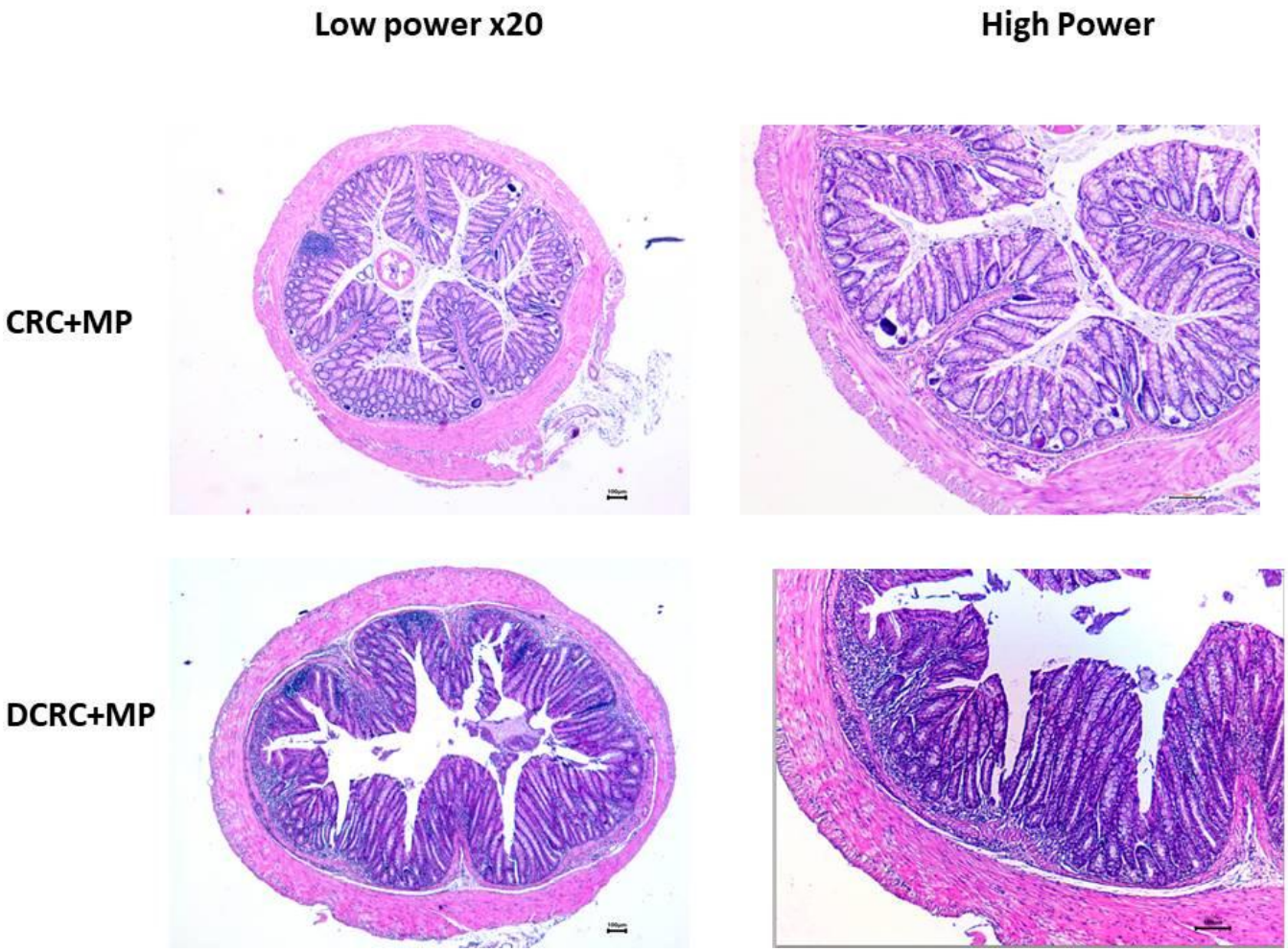


Figure 33: Histology of CRC and DCRC animals treated with the combination therapy**.**

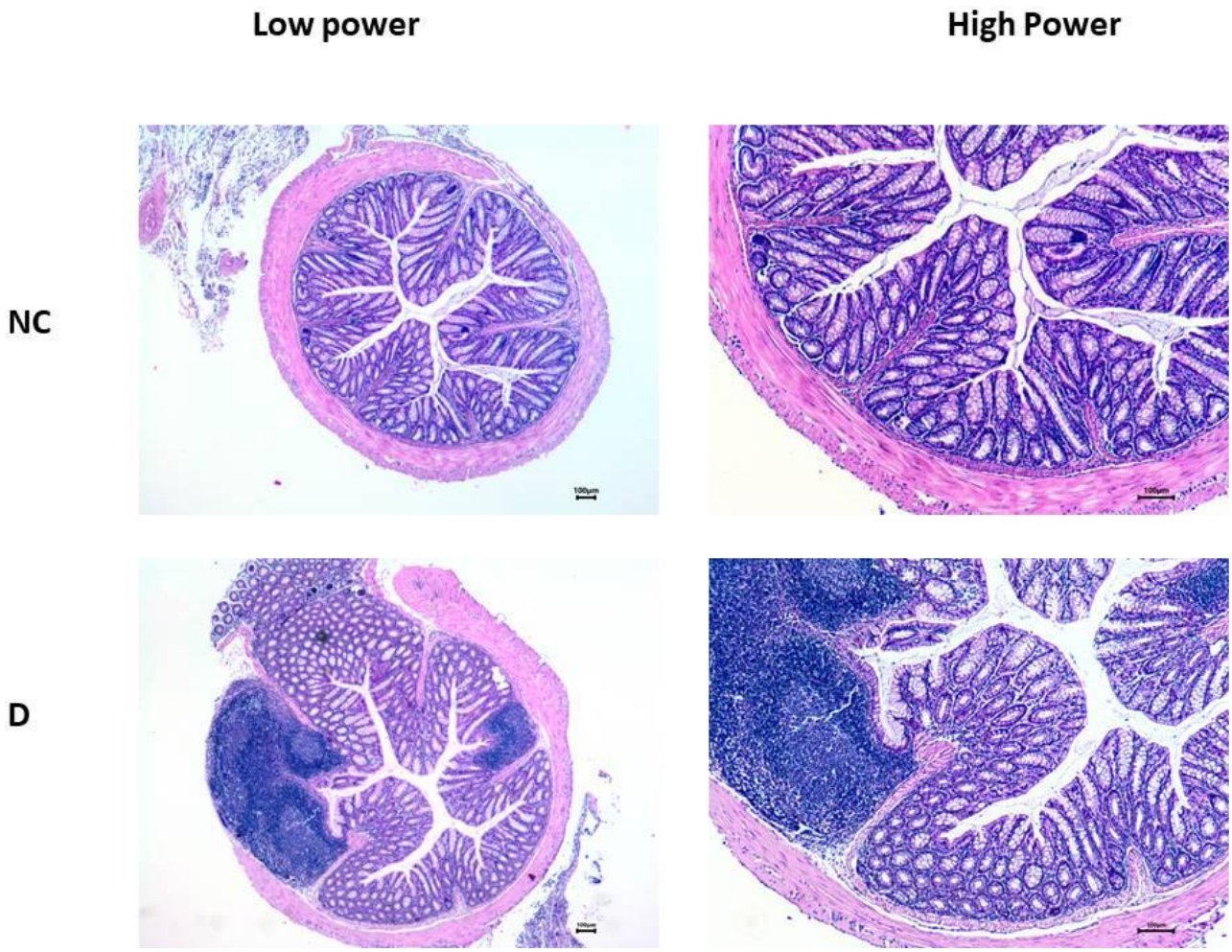


Figure 34: Histology of normal controls and diabetic animals**.**

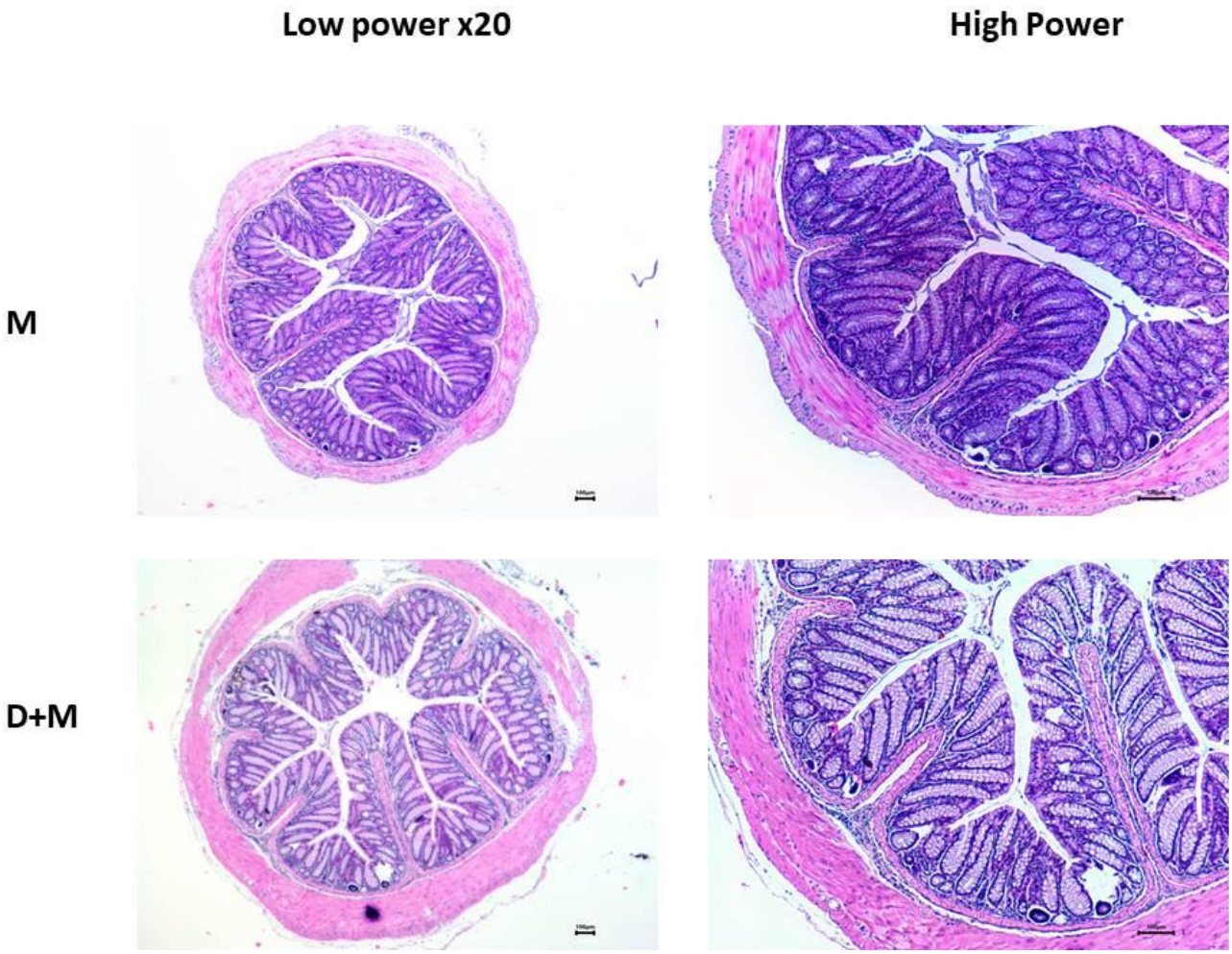


Figure 35: Histology of normal controls and diabetic animals treated with metformin alone.

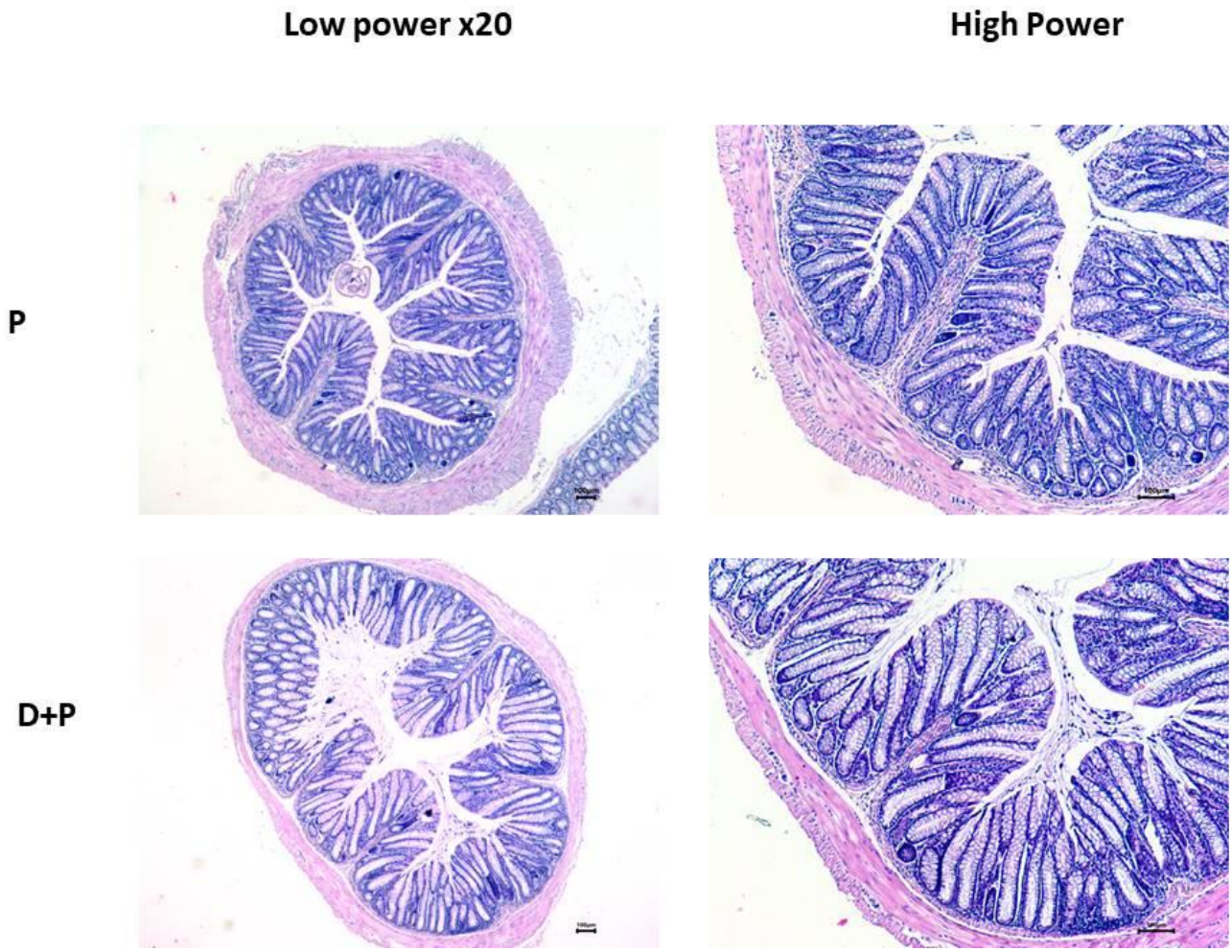


Figure 36: Histology of normal and diabetic animals treated with probiotics alone.

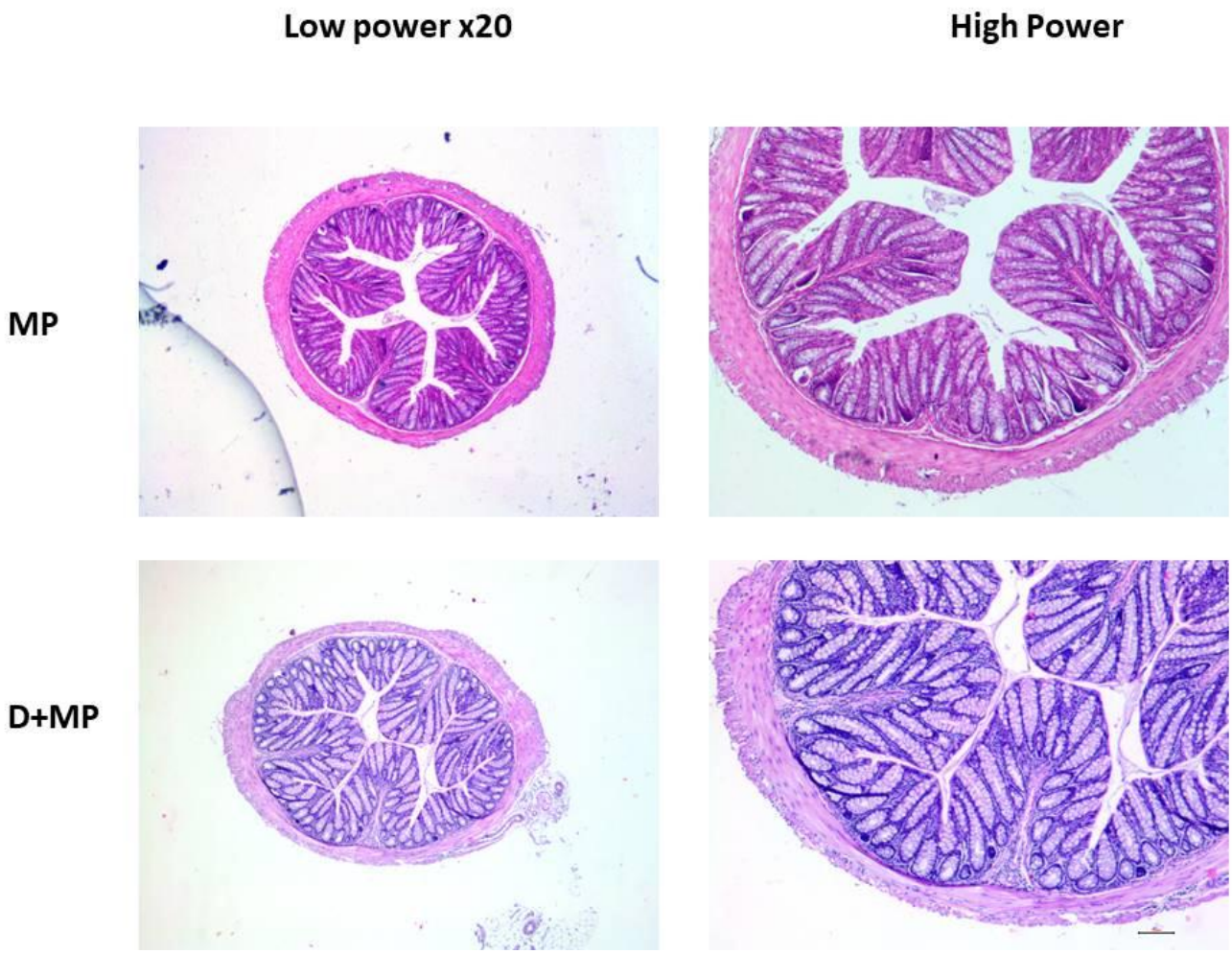


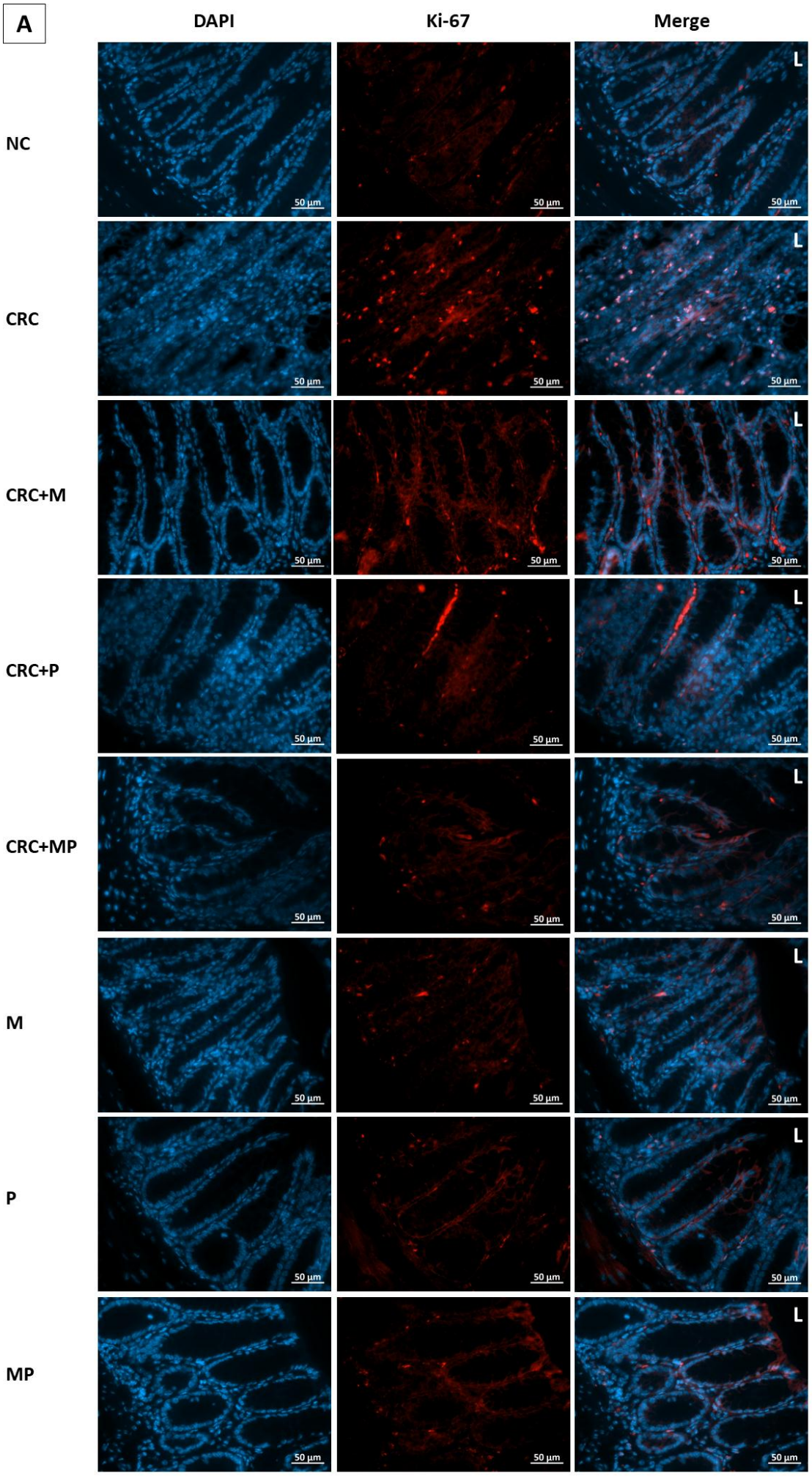
Figure 37: Histology of normal and diabetic animals treated with the MP combination.

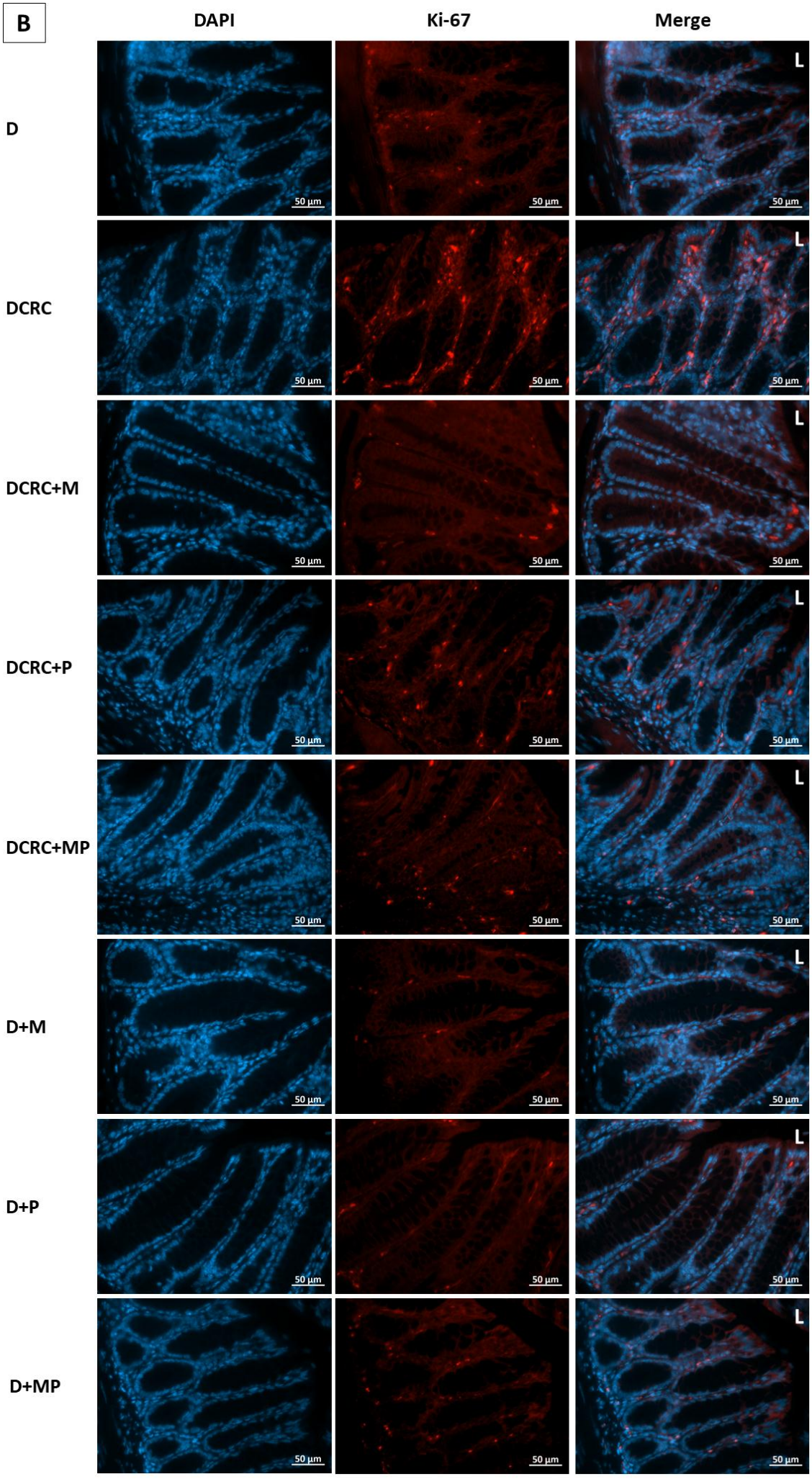
7. Assessment of colonic tissue proliferation

Immunohistological-staining by Ki-67 was performed on paraffin embedded colon tissue to evaluate the proliferation rate in the colon. CRC and DCRC groups showed elevated proliferation indices with the ki-67 positive cells dispersed throughout most of the crypt area, and migrating to the lumen (Figure 38).

Non-diabetic and diabetic CRC animals receiving M alone, had significantly lower proliferation rates as seen in CRC+M and DCRC+M groups ($^{\#}p<0.05$, $*p<0.05$ respectively). Conversely, in CRC+P and DCRC+P groups, when probiotics were administered as single drug, the decrease in proliferation index was not statistically significant (Figure 38C).

The most effective treatment regimen was the MP combination as animals in groups CRC+MP and DCRC+MP had low ki-67 levels, close to that of the normal controls ($^{\#}p<0.05$, $*p<0.05$ respectively). Importantly, we can say that this combination was most likely capable of inhibiting colon cancer cell proliferation (Figure 38).


A
DAPI
Ki-67
Merge
NC
CRC
CRC+M
CRC+P
CRC+MP
M
P
MP
L
50 μm


B
DAPI
Ki-67
Merge
D
DCRC
DCRC+M
DCRC+P
DCRC+MP
D+M
D+P
D+MP
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50 μm

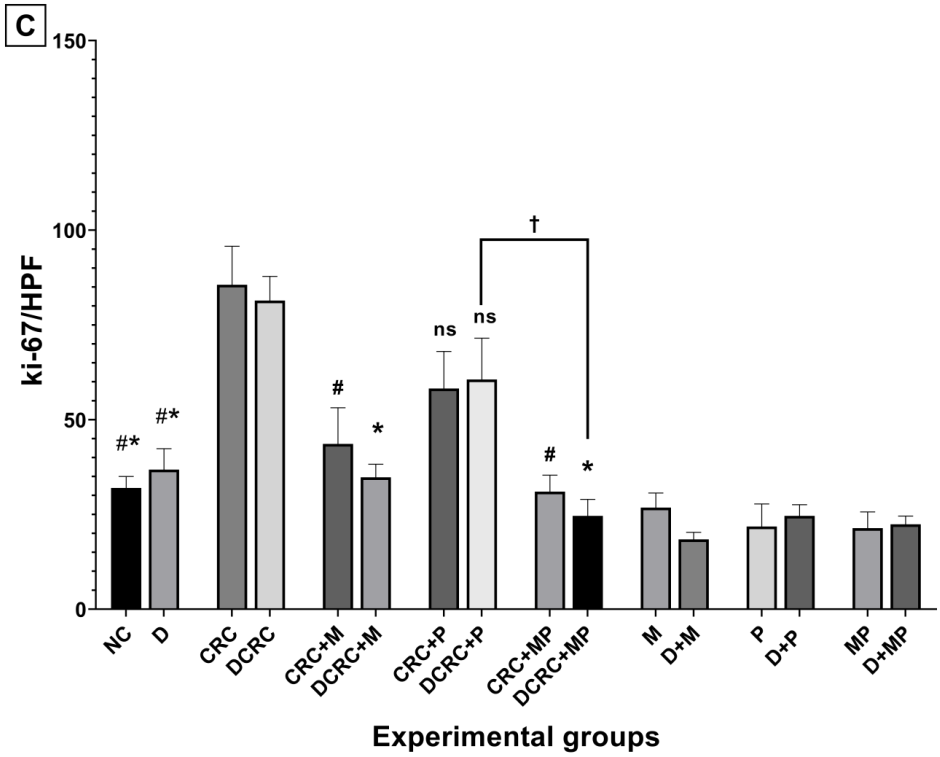


Figure 38: Effect of probiotics and metformin on colon proliferation**.**

(A, B)Panel of representative images of colon section labelled with with Ki-67 (red) and counterstained with DAPI (blue) in non-diabetic (**A**) and diabetic groups (**B**). HPF 40X magnification; scale bars 50 µm. "L" indicates the position of the lumen. (**C**) Proliferation index in the different groups. Data is expressed asmean±SEM (n=5). Significant differences between the groups were determined by one-way ANOVA followed by Tukey's Multiple Comparison Test. Non-diabetic groups were compared to their experimental CRC control , significance was expressed by $^{\#}p<0.05$. On the other hand, diabetic groups were compared to their experimental diabetic CRC control, significance was expressed by $^{*}p<0.05$. Moreover, when comparing only 2 groups, connecting lines were used to indicate the compared groups with $^{\dagger}p<0.05$, (ns) stands for non-significant.

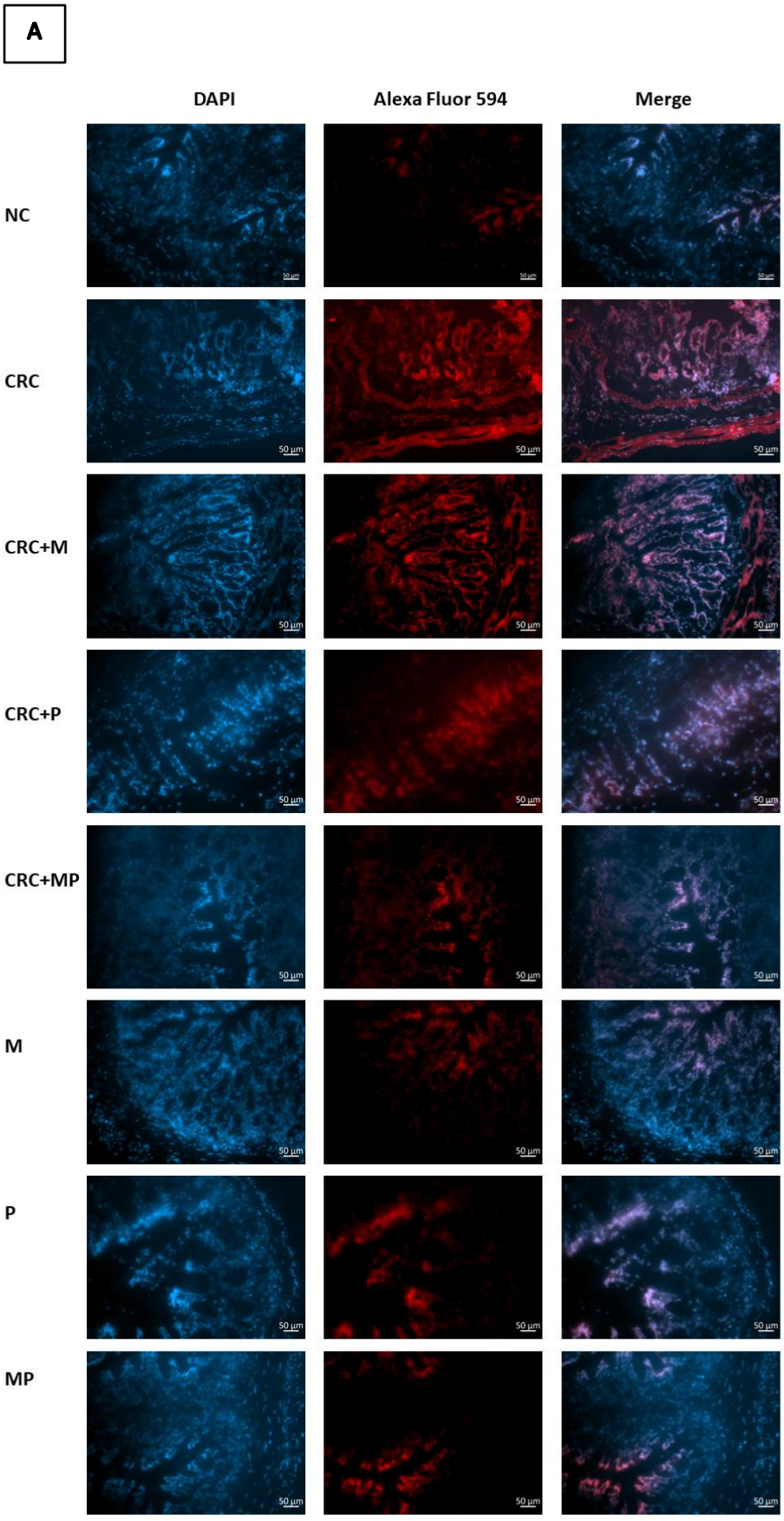
8. Modulation of reactive oxygen species production

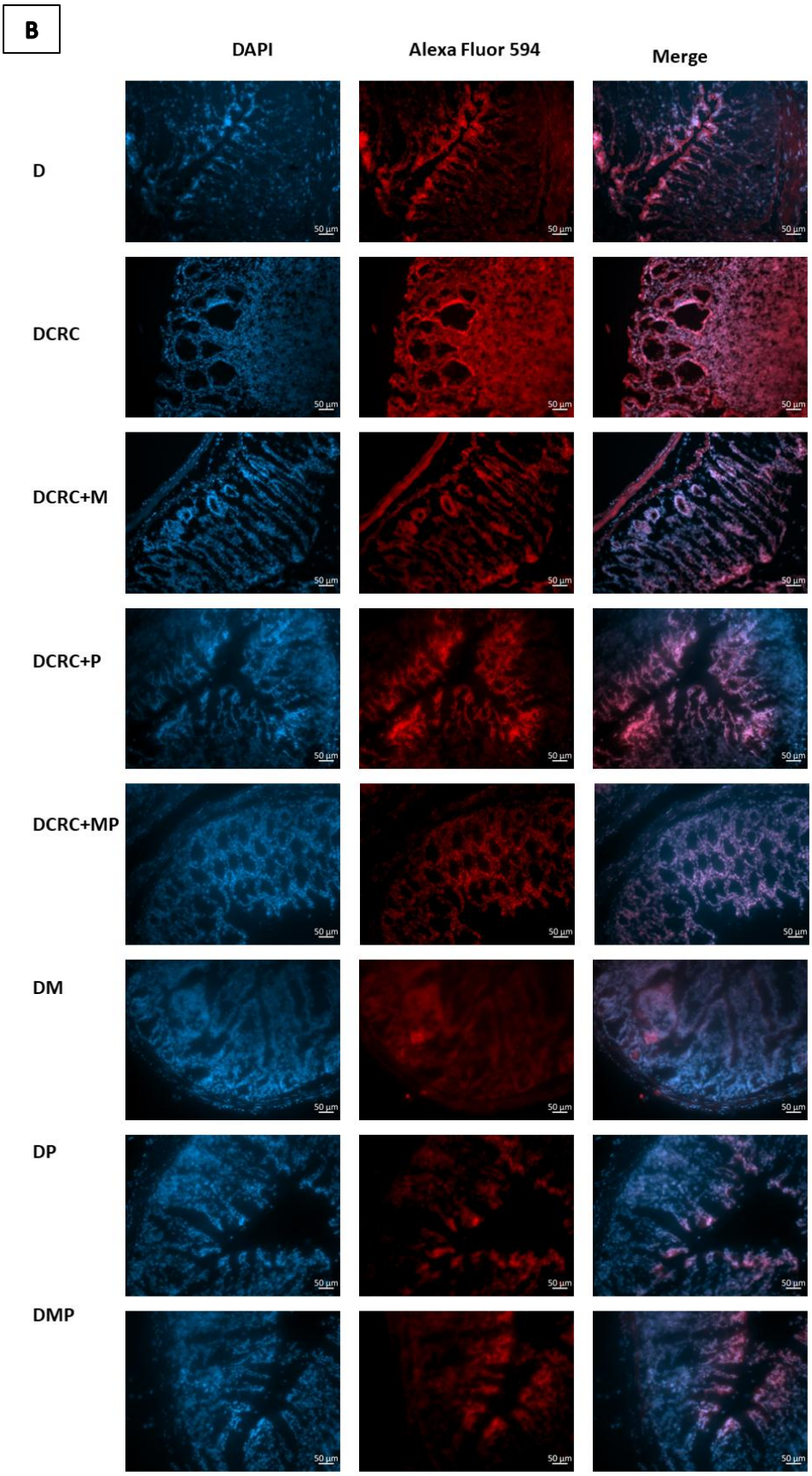
Frozen colon sections were used to evaluate ROS activity, using dihydroethidium (DHE), a ROS responsive stain.

CRC and DCRC groups had the highest ROS levels, Moreover, diabetic animals in group D also had high ROS production with $^{\dagger}p<0.05$ when compared to NC group (Figure 39),thus, demonstrating the increased ROS generation in diabetes and shedding light on the shared oxidative stress increase between CRC, inflammation and diabetes.

In non-diabetics, single drug treatment reduced significantly ROS production, with the lowest DHE/DAPI ratios obtained in CRC+MP group $^{\#}p<0.05$ (Figure 39).

Concerning the diabetic animals, single drug treatment in DCRC animals did not affect the colonic ROS production. However, when the two drugs were combined, a significant reduction in DHE/DAPI ratios was noted in DCRC+MP group (Figure 39).


A
DAPI
Alexa Fluor 594
Merge
NC
CRC
CRC+M
CRC+P
CRC+MP
M
P
MP
50 μm


B
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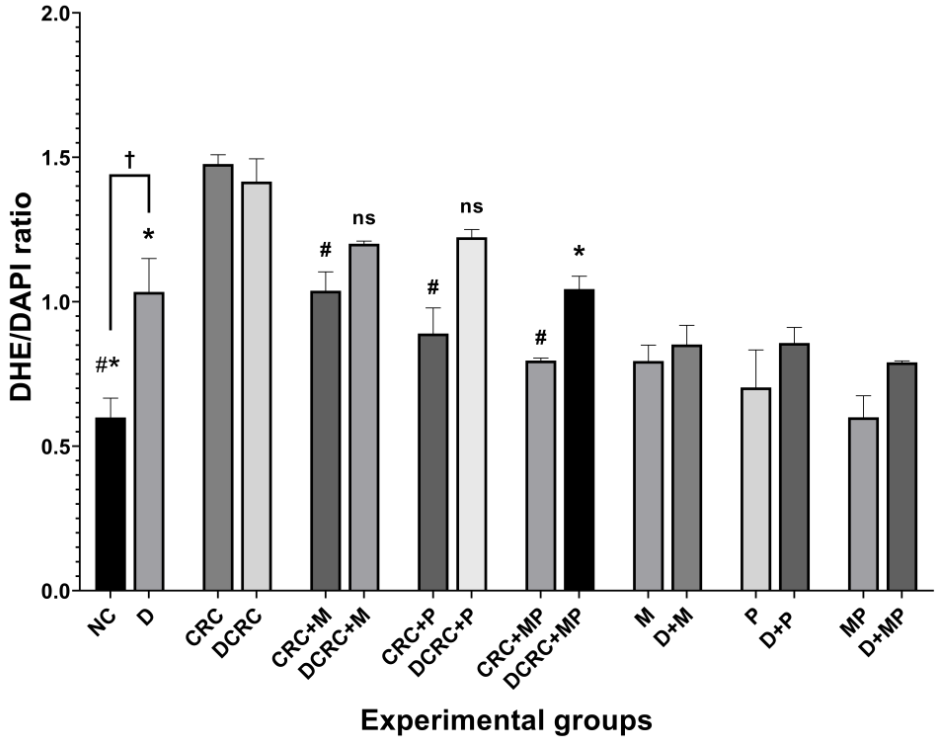


Figure 39: Reactive oxygen species modulation by metformin and probiotics.

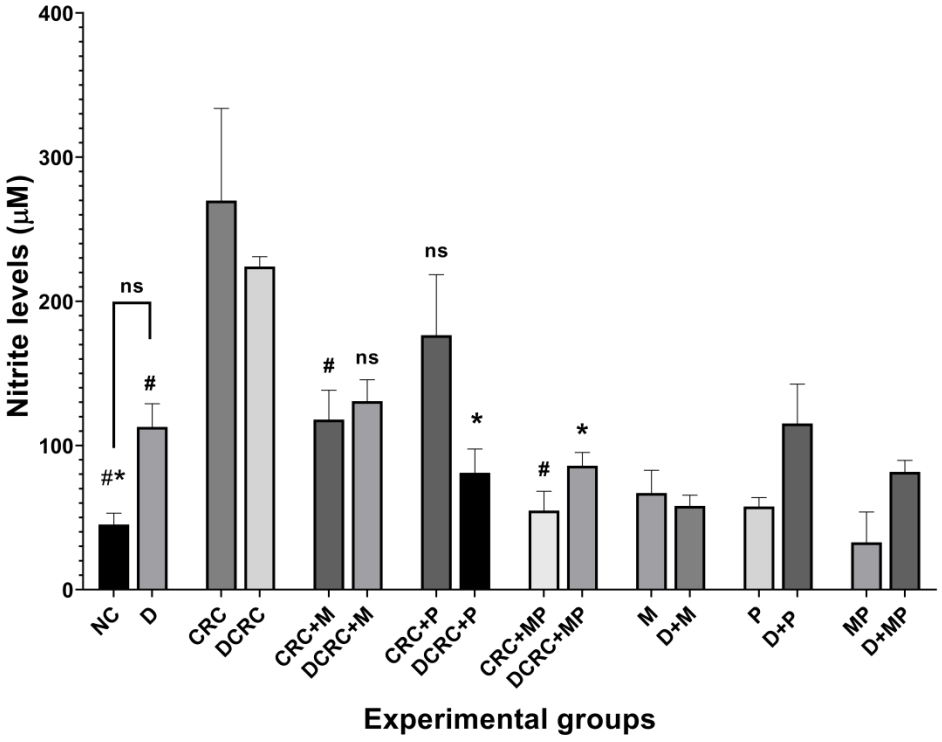
Representative images DHE stained frozen sections from colon tissues of diabetic (A) and non-diabetic (B) animals. (C) Results are expressed as ratios of DHE and DAPI intensity, bars represent averages with SEM as error bars from each group (n=3). Significant differences were determined by one-way ANOVA followed by Tukey's Multiple Comparison Test. Non-diabetic groups were compared to their experimental CRC control, significance was expressed by #$p<0.05$. On the other hand, diabetic groups were compared to their experimental diabetic CRC control, significance was expressed by *$p<0.05$. Moreover, when comparing only 2 groups, connecting lines were used to indicate the compared groups with †$p<0.05$, (ns) stands for non-significant.

9. Modulation of nitric oxide levels with probiotics and metformin

Griess method was used to measure the concentration of nitrite, a stable metabolite of Nitric oxide (Figure 40). Non-diabetic and diabetic CRC mice showed the highest nitrite levels.

The MP combination was effective in both diabetic and non-diabetic CRC groups, whereby a significant suppression of nitrite production in CRC+MP and DCRC+MP groups were noted

(Figure 40). Moreover, diabetic animals in D group, showed elevated levels of nitrite when compared to their non-diabetic counterparts with, however, no statistically significant difference.



Non-diabetic groups	NC	CRC	CRC+M	CRC+P	CRC+MP	M	P	MP
Mean±SEM	45.2±7.9	269.8±63.9	117.9±20.3	176.5±41.9	54.8±13.4	67.0±15.7	57.7±6.2	32.7±21.2
Diabetic groups	**D**	**DCRC**	**DCRC+M**	**DCRC+P**	**DCRC+MP**	**D+M**	**D+P**	**D+MP**
Mean±SEM	112.8±16.1	224.1±6.8	130.7±14.8	81±16.5	85.9±9.2	58.0±7.5	115.2±27.4	81.7±8.0

Figure 40: Nitrite modulation by metformin and probiotics.

Serum nitrite production was measured by Griess assay. Results are expressed as mean±SEM (n=4). Significant differences were determined by one-way ANOVA followed by Tukey's Multiple Comparison Test. Non-diabetic groups were compared to their experimental CRC control, significance was expressed by $^{\#}p<0.05$. On the other hand, diabetic groups were compared to their experimental diabetic CRC control, significance was expressed by $^{*}p<0.05$, (ns) stands for non-significant.

10. Effects of metformin and probiotics on IL-6 and TNF-α production

The analysis of the two cytokines, IL-6 and TNF-α, in the serum and colon extracts of the different animal groups showed interesting variations.

Normal animals in the NC group showed low levels of cytokines, on the other hand, mice in CRC and DCRC groups had significantly higher IL-6 and TNF-α levels in their colons (Figure 41-a, c) and sera (Figure 41-b, d) with $^{\#}p<0.05$ and $^{*}p<0.05$, respectively.

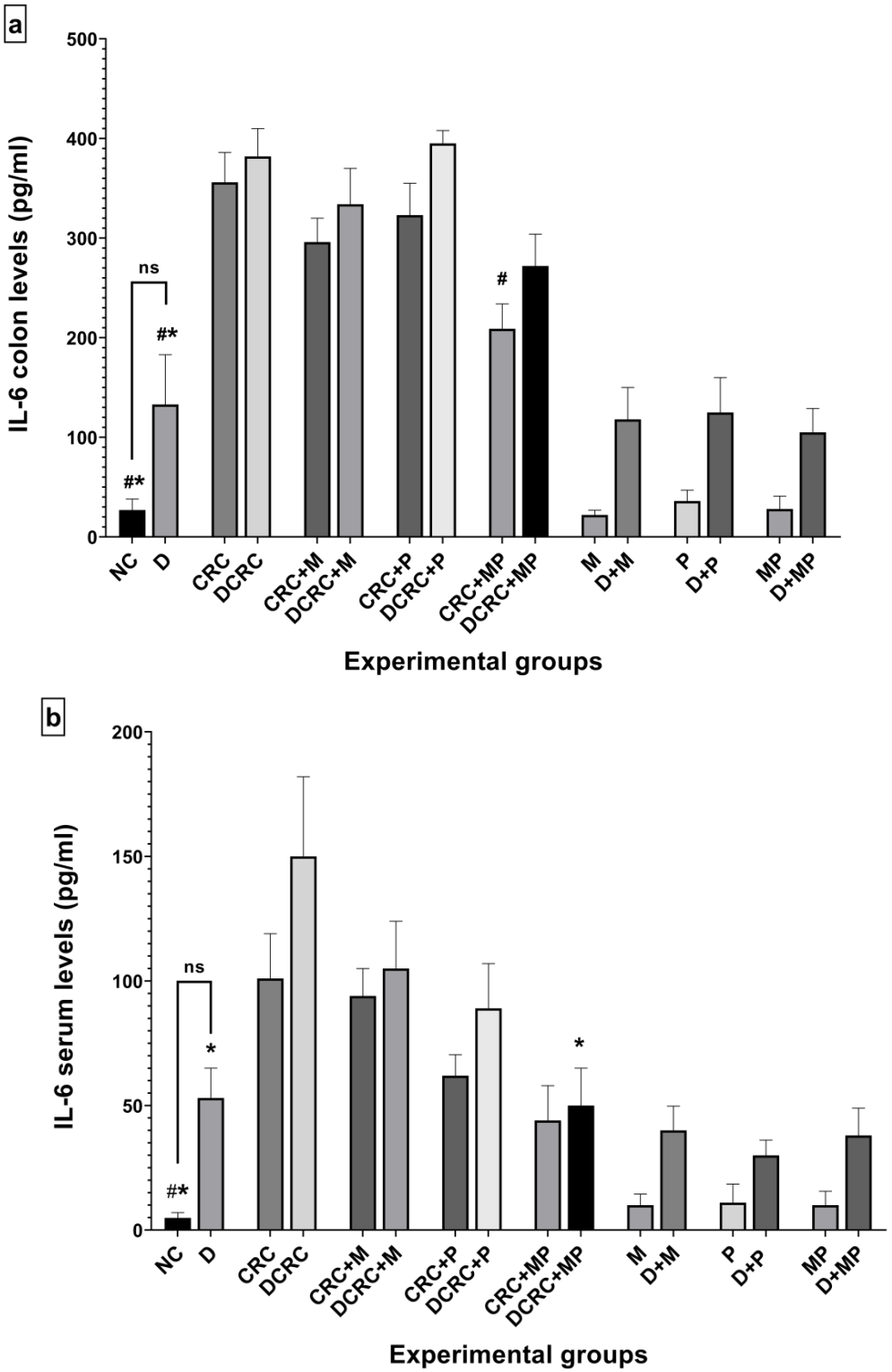
Single drug treatment in diabetic and non-diabetic animals decreased the levels of IL-6 (Figure 41-a,b) and TNF-α (Figure 41-c,d) in both colonic tissue and serum of CRC+M, CRC+P , DCRC+M and DCRC+P animals, with no statistical significance.

However, when the MP combination is administered, a significantly lower level of IL-6 was detected in the colon of non-diabetics (CRC+MP, $^{\#}p<0.05$) and the serum of diabetics (DCRC+MP, $^{*}p<0.05$) as seen in Figures 41-a and 41-b.

In addition, TNF-α levels were also significantly reduced in both colon and serum of CRC+MP group with , $^{\#}p<0.05$ and only in the serum of DCRC+MP group with $^{*}p<0.05$.

Moreover, elevated IL-6 and TNF-α levels were noted in group D, despite the fact that animals in this group were not subjected to CRC induction, shedding light on the inflammatory environment created by diabetes in the colon, which has a major impact on carcinogenesis.

Collectively, metformin and probiotics were shown to work in synergy in the colon and serum of diabetic and non-diabetic CRC groups, whereby the MP treatment reduced the production of IL-6 and TNF-α, two key players in inflammation and tumorigenesis.


a
IL-6 colon levels (pg/ml)
500
400
300
200
100
0
ns
#*
#*
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NC
D
CRC
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CRC+M
DCRC+M
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DCRC+MP
M
D+M
P
D+P
MP
D+MP
Experimental groups
b
IL-6 serum levels (pg/ml)
200
150
100
50
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ns
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CRC
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Experimental groups

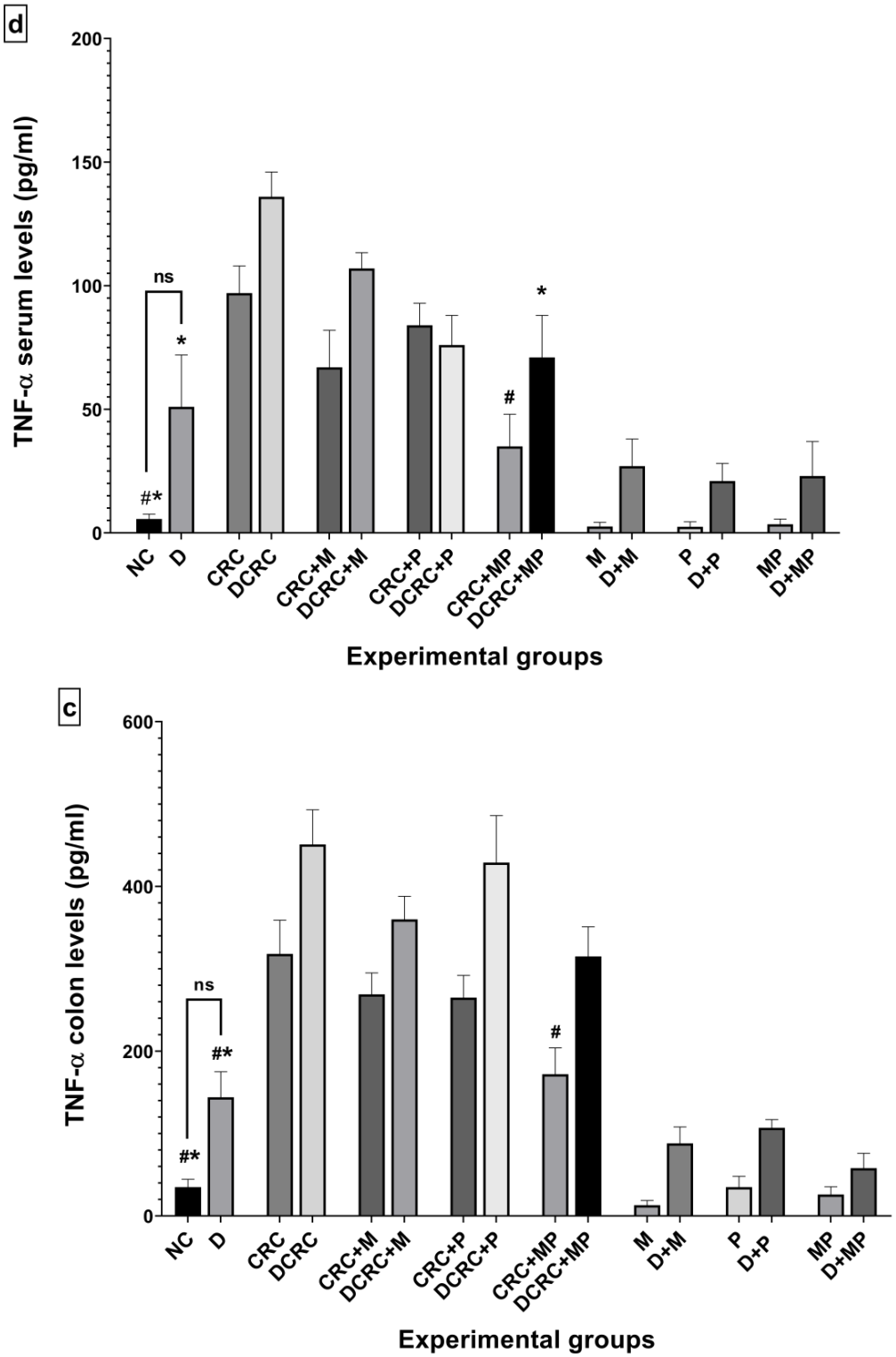

Figure 41: Variation of IL-6 and TNF-α levels from colon extraction (a,c) and serum (b,d) of the different groups.

Results are expressed as mean±SEM (n=4). Error bars represent SEM. Significant differences were determined by one-way ANOVA followed by Tukey's Multiple Comparison Test. Non-diabetic groups were compared to their experimental CRC control , significance was expressed by #p<0.05. On the other hand, diabetic groups were compared to their experimental diabetic CRC control, significance was expressed by *p<0.05, (ns) stands for non-significant.

Chapter 5: Discussion

This work explored further the association between diabetes mellitus and CRC in particular the molecular mechanisms underlying these two disease entities using a well-established experimental model with distinct clinical characteristics.

Protocols using AOM and DSS, in combination or alone, have been frequently used in different mice strains, demonstrating the relevance of the AOM/DSS mouse model in the study of the mechanisms of human colorectal carcinogenesis and its therapeutic targets.

Due to the synergic effects of the tumor-inducing agent (AOM) and the tumor-promoting agent (DSS), the AOM/DSS model replicates colorectal carcinogenesis promoted by an initial acute inflammation phase, showing a shorter latency period than models based on AOM or DSS administration alone and not in combination. Even that they act in synergy, AOM and DSS have divergent mechanisms of action. DSS has a toxic effect on the epithelial lining of the colon and creates a severe colitis, which is characterized by loss of body weight, rectal bleeding and diarrhea [124,125]. This state of chronic inflammation triggered and maintained by DSS plays a major role in colorectal carcinogenesis through different mechanisms such as the alteration of the epithelial cells, the disruption of the gut barrier, as well as through the excessive production of oxidative species such as ROS, cytokines and pro-inflammatory molecules [141].

As for AOM, its hydroxylation results in the formation of the reactive metabolite MAM, a DNA-Alkylating agent that acts through the addition of methyl groups at the O6 or N7 position of guanine (O6-methyl-deoxyguanosine and N7-methyl-deoxyguanosine) of the DNA molecule.

The main advantages of AOM are its low cost, high potency and reproducibility, as well as its simple mode of administration, excellent stability in solution, and low price. Mutations in K-Ras and β-catenin as well as microsatellite instability are commonly present in AOM-induced tumors [142].

Colorectal carcinogenesis is a multistep process in which normal crypts are initiated to form aberrant crypt foci (ACF) that progress into adenocarcinomas. Such multistep tumor development was efficiently reproduced in the AOM/DSS murine model with high reproducibility in several susceptible murine strains. Commonly, 3–10 macroscopic tumors develop in 80%–100% of the animals, with tubular adenoma, dysplasia, and colitis, preferably in male mice of susceptible strains at week 12 from the start of the treatment. Moreover, it is

important to shed light one more time on the fact that tumor development in this model closely mirrors the pattern seen in human CRC [143].

Animals receiving STZ injection have successfully developed diabetes diagnosed by a maintained blood glucose levels above 250 mg/dl. In addition, animals receiving the AOM/DSS combination developed CRC as diagnosed by several clinical and histological parameters.

As for diabetes mellitus, it is a group of metabolic disorders considered as one of the most prevalent and rapidly increasing comorbid disorders.

Medical literature in the past 10 years focused on the relationship between diabetes and CRC, whereby a higher risk on colorectal cancer onset was observed in diabetic patients when compared to their non-diabetic counterparts, Moreover, poor CRC outcomes has been associated with the onset of diabetes [11,144]. These observations parallel our results where cancer was worsened by diabetes; higher histological score and a poorer clinical profile were obtained in diabetic CRC non-treated animals when compared to their non-diabetic counterparts. Moreover, survival rates reflected this observation as the rates in diabetic CRC mice were significantly lower than the non-diabetic CRC animals.

It is well established that hyperglycemia coupled with dysbiosis and an increase in oxidative stress and chronic intestinal inflammation create a favorable environment for the development of the three medical conditions DM, IBD and CRC. Notably, a dysregulated gut microbiota is being recognized as a key player in this crosstalk [10,11]. In this study, probiotics use has been considered an attractive therapeutic target in the management of inflammatory diseases, carcinogenesis as well as diabetes after it has proven in a previous study to reverse the carcinogenic process in solid tumors[9].

In the past years, a large number of experimental studies were conducted, focusing on the protective role of probiotics on a wide spectrum of human disorders, especially colorectal cancer and diabetes. Several possible mechanisms of action of probiotics have been proposed including: improvement of the gastrointestinal mucosa, modifications in the intestinal microbiota and in its metabolic activity, immunomodulation, improvement of glycemia and HbA1c, decrease of cellular proliferation, induction of apoptosis and suppression of inflammatory reactions…among others [145,146].

In our study, a treatment combining a probiotics mixture and metformin was administered to diabetic and non-diabetic Balb/c mice that were exposed to CRC induction. A thorough

analysis of the emanating clinical, histological and molecular data resulted in the beneficial effects of metformin and probiotics on our colitis associated colorectal cancer mouse model.

A large number of previous reports focused on probiotics and metformin and their beneficial effects on diabetes and CRC [111,143,147]. The findings of our work parallel these previous studies as our treatment protocol showed beneficial effects on diabetes and CRC to different extents; whereby treatment with probiotics along with metformin helped in inhibiting the damage caused by the administration of AOM/DSS to Balb/c mice. Less weight loss, a better disease activity index, a lower number of colon polyps and a better colonic architecture were detected in treated animals. Moreover, the inflammatory reaction was suppresses as manifested by a reduction in the secretion of TNF-α and IL-6.
AOM/DSS induced colorectal cancer, is also characterized by a dysregulation of the intestinal barrier, therefore dysbiosis was considered a hallmark in our CRC mouse model which can lead to alterations in the luminal microbiota and consequently to the alteration of the host physiology, thus promoting the development and progression of CRC by different processes. Such processes include the creation of a chronic inflammatory state or hyperactive immune response, altering stem cell dynamics, the biosynthesis of toxic and genotoxic metabolites and dysregulation of the host metabolism [148].

It was documented that dysbiosis in the gut acts as a driving force during the progression from inflammation to carcinogenesis [149,150], thus, probiotics have the ability to inhibit tumor progression mainly through the manipulation of the intestinal microbiota leading probably to homeostatic state or equilibrium. In this study, probiotics reduced glycemia when compared to the untreated diabetic mice, with or without CRC induction, metformin and probiotics single drug treatment significantly reduced blood glucose levels. These results go in parallel with preliminary interventions in humans suggesting that probiotics may ameliorate glucose metabolism, insulin resistance, and HbA1c levels [151,152].

Moreover, our findings suggest a possible chemopreventive effect of probiotics supplementation on CRC, whereby probiotics promoted intestinal homeostasis and regulated the inflammatory response. Non-diabetic and diabetic animals treated with probiotics alone in groups CRC+P and DCRC+P, had a better clinical profile when compared to the untreated DCRC and CRC groups and their DAI scores were improved as well as their survival rates. In addition, occult blood appearance was decreased and delayed; polyp formation, inflammatory

cells infiltration and RONS secretions were significantly decreased with probiotics treatment. These results parallel several previous studies, for instance Mendes et al. showed that probiotics supplementation reduced inflammatory cell infiltration and lowered chemokine expression [150], Another study by Chen et al, described the protective effect of two strains *C. butyricum* and *B. subtilis* on CRC, these probiotics inhibited the proliferation of cancerous cells , induced cell cycle arrest and apoptosis. Their effect was also verified *in-vivo* as they inhibited tumor development in a DMH-induced CRC mouse model [153]. A recent study , conducted in 2020 showed that Probiotics therapy is beneficial on high fat induced carcinogenesis, as it was able to reduce obesity and reverse the microbial imbalance caused by the HFD administration , along with a reduction tumor incidence, thus linking metabolic disorders to carcinogenesis with microbial alterations as a common denominator [154].

Concerning our second drug, metformin, it is widely and commonly used for the treatment of diabetes mellitus. It main advantages are its good safety profile and low cost. Besides its anti-diabetic effects, several properties are being recently associated with metformin, mainly its antitumor, antiaging, cardiovascular and neuroprotective properties [144,155,156].

The efficacy of metformin in decreasing inflammation and oxidative stress as well as in preventing tumorigenesis has been shown to be mediated mainly through the inhibition of various pro-inflammatory mediators and oxidative stress [157].

Moreover, a recent study showed the beneficial effects of Metformin on metastatic colorectal cancer specifically *KRAS*-mutation mCRC patients that usually have limited therapeutic options [158]. Additionally , Wang et al showed that Metformin administration reduced the overall mortality of CRC patients with T2DM[159].

These observations were in line with our results whereby metformin administration to CRC animals ameliorated their clinical profile, their DAI scores and their survival rates. Furthermore, it reduced histological alterations scores and lowered significantly the oxidative stress in diabetic and non-diabetic animals treated with metformin alone, in comparison with the untreated CRC and DCRC animals.

Collectively, our results showed that treatment with metformin alone or probiotics alone had beneficial effects on diabetes and CRC to variable degrees. These variations might be ascribed to the inter-individual difference in the composition of gut microbiota especially with the inflamed microenvironment created by the induction of CRC and diabetes.

Several recent studies have focused on the gut microbiota as a key site of action for metformin. This was supported by old data indicating that the efficacy of metformin is affected by

antibiotics [160]. Additionally, the glucose lowering effects were found to be stronger following an intraduodenal versus intravenous administration of metformin [161].
A meta-analysis conducted recently showed a reduction in HbA1c, FBG and insulin resistance level in T2DM patients treated with probiotics [162]. Moreover, Experimental and clinical evidence showed that that the modulation of gut microbiota through probiotic administration has also a preventive effect against gestational diabetes mellitus [163].
In this study, the addition of probiotics to metformin, helped metformin in excreting its anti-cancerous and anti-inflammatory properties. Possibly, probiotics acted through the correction of dysbiosis which enhanced the activities of metformin. In fact, CRC and DCRC animals that were treated with the combination therapy showed a significant improvement in their diabetic and cancer status, when compared to groups treated with a single drug and to untreated groups.

It is well-established that cellular proliferation is a key factor influencing carcinogenesis onset and development. In the present work, cellular proliferation was evaluated using Ki-67 labeling of colon tissue. High levels of Ki-67 positive cells were detected in the crypts of CRC and DCRC animals with extended labelling to most of the crypt surface. This is in accordance with other studies showing that in CRC, a reversal in the distribution of proliferating cells from the bottom of the crypt into the upper crypt and luminal surface occurs [164]. However, in normal conditions proliferating cells are concentrated at the bottom half of the crypt, and the upper half of the crypt usually consists of non-dividing migrating cells[165]. An inhibition of proliferating colonocytes was induced by metformin and probiotics especially when combined, shedding light again on the protective effect of these 2 compounds on colorectal carcinogenesis.

The beneficial effects granted by metformin and probiotics were also substantiated by the histological analysis of the colonic mucosa. Whereby, the analysis of H&E stained colon tissues showed that the multiple histopathological alterations recorded during the course of the disease were reversed to significant extents. The main alterations observed include surface erosion, inflammatory cells infiltration, submucosal edema, polyp formation and dysplasia. The combination (M + P) attenuated colorectal inflammatory severity, ameliorated colorectal crypt structure and significantly reduced the severity of inflammation as assessed in the histological score. It looks like that the combination treatment (M + P) could alleviate alterations in the intestinal wall through a distinct mechanism that each drug alone. It is very probable that the integrity of the mucosa needed a different mechanism. Thus, through the

balanced microbiota, the junctional complexes of the epithelial cells were maintained; the secretory part of the balanced microbiota could have provided anti-inflammatory elements and restored the eubiosis state.

Moreover, gut barrier dysregulations and pathologies promote the production of pro-inflammatory cytokines mainly TNF-α and IL-6, which in turn activate inflammation and insulin resistance, shedding the light again on the inflammation crosstalk between CRC and diabetes [10]. This work showed an increase in IL-6 and TNF-α in CRC animals with or without diabetes induction in CRC and DCRC groups. The increase in cytokine production was not limited to colon tissue, as the levels of these cytokines were also elevated in the serum of the animals, thus emphasizing on the systemic inflammation occurring in diabetes and CRC. A strong inhibition of these cytokines was detected in the colon of cancerous animals when probiotics and metformin were combined.

In addition, it is clear that a state of chronic inflammation accompanied with inflammatory alterations in the colonic mucosa are characteristic features of CRC. They consist of inflammatory cells aggregates and infiltration and enhanced production of a panel of cytokines [166]. Following recruitment, neutrophils get activated and secrete large amounts of pro-inflammatory mediators mainly IL-1β and IL-6 [167]. These changes, paralleled with elevated levels of reactive oxygen and nitrogen species (RONS) generated in the colon tissue, contribute to destructive mucosal damage, which creates a leaking mucosal barrier that could allow multiple bacteria, including toxic strains to infiltrate and grow leading to a chronic inflammation, an optimal environment for developing colitis associated CRC [39].

In this study, CRC and diabetes induced an upregulation of free radicals production. This was indicated by the increased levels of RONS in diabetic and CRC animals. These levels were restored to normal by the administration of the combination therapy, metformin and probiotics. The increased NO production correlates with the increase in the levels of pro-inflammatory mediators like TNF-α and IL-6, thus leading to exacerbation of the inflammatory chronic reaction, which is at the core of the IBD-CRC etiology [168]. Treatment with metformin in combination with probiotics was able to diminish the production of inflammatory mediators, of RONS, and to reestablish the colonic structure and function of the gut barrier.

This study had potential limitations; first, as with the majority of studies, the design of the current study was subject to limitations as the number of animals per group could be increased, moreover the experiment could have been repeated to validate the results. Second, the study

was only conducted on male mice, both genders could be used to eliminate gender biases and explore the possible different responses between males and females. Third, additional molecular testing could have been done to further explore additional cytokines and molecular pathways involved in the crosstalk between CRC and diabetes.

Chapter 6: Conclusions and future perspectives

Clinical, histological and molecular data emanating from this study, focused on metformin and probiotics as a new therapeutic option for inflammation and its associated carcinogenesis. Inhibition or decrease of cytokines and oxidative stress were the main mechanisms this study focused on, in the context of colitis associated colorectal cancer.

Metformin combined with probiotics were able to significantly prevent AOM/DSS-induced damage through correcting the inflamed microenvironment as well as reducing ROS and cytokines production and cellular proliferation, thus leading to the inhibition of CRC. This protective effect of metformin might relate to its interaction with the balanced microbiota corrected by probiotics (Figure 42). However, very little is currently known about the bacterial targets of metformin. It is possible that the microbiota could affect the host physiology, however, the exact mechanisms are not fully elucidated yet (Figure 42).

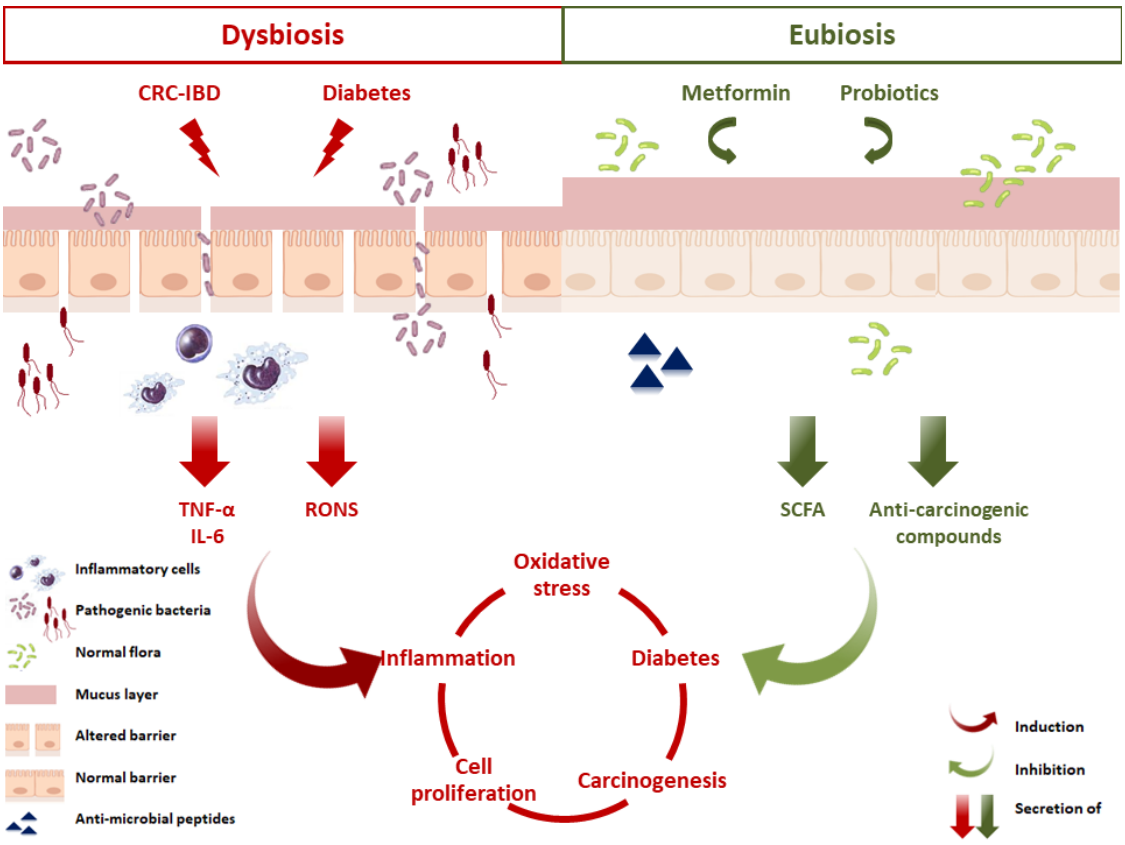

Figure 42: Proposed mechanisms of action elicited by metformin and probiotics in CRC and diabetes.

A dysregulated microbiota in a state of dysbiosis, impairs the functions of the gut barrier by affecting the tight junctions and the mucus layer, thus facilitates the translocation of pathogens and toxins towards lamina propria. This invasion leads to recruitment of inflammatory cells, their activation and the secretion of pro-inflammatory cytokines including IL-6 and TNF-α. In parallel, an increase in RONS production induces a chronic inflammatory state, DNA damage and increased cell proliferation, known as key players in CRC progression. Moreover, inflammation promotes insulin resistance and disturbance in glucose homeostasis thus exacerbating diabetes and enhancing CRC. Probiotics and metformin administration, however, inhibited CRC progression, reduced inflammation and ameliorated diabetes. These beneficial effects are potentially due to a restoration of the gut barrier, production of SCFA, anti-microbial peptides, regulation of hepatic glucose production and modulating the balance between proliferation and apoptosis. The result of such a balanced microenvironment is the preservation of a dynamic intestinal barrier, which controls and maintains homeostasis.

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Publication

Metformin and Probiotics in the Crosstalk between Colitis-Associated Colorectal Cancer and Diabetes in Mice.
Kattar SA, Jurjus R, Pinon A, Leger DY, Jurjus A, Boukarim C, Diab-Assaf M, Liagre B. Cancers (Basel). 2020 Jul 10;12(7):1857. doi: 10.3390/cancers12071857

Impact Factor 2019 = 6.126

cancers

MDPI

Article

Metformin and Probiotics in the Crosstalk between Colitis-Associated Colorectal Cancer and Diabetes in Mice

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Received: 1 June 2020; Accepted: 7 July 2020; Published: 10 July 2020

Abstract: The co-occurrence of colorectal cancer (CRC) and diabetes mellitus along with inflammation and dismicrobism has been frequently reported. Several studies shed light on the antioncogenic potential of metformin on colorectal carcinogenesis. This study aimed to demonstrate that metformin in association with probiotics acts in a synergic effect in breaking the crosstalk, thus inhibiting CRC progression, improving diabetes, and reducing inflammation. Ninety-six male Balb/c mice, 6–8 weeks old, were divided into 16 control and experimental groups to assess the effect of the different treatments and combinations at the clinical, histological, and molecular levels. Metformin and probiotics showed beneficial outcomes on CRC and diabetes, alone and most importantly in combination. Their effects were exerted by inhibiting the inflammatory process whereby a downregulation of IL-6 and TNF-α as well as oxidative stress were depicted. The characterization of the effects of probiotics and metformin on CRC and diabetes sheds light on the role of inflammation and microbiota in this crosstalk. Deciphering the downstream signaling pathways elicited by these compounds will help in developing new effective targeted treatment modalities.

Keywords: colorectal cancer; microbiota; inflammation; diabetes; oxidative stress; probiotics

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide with 1.8 million new cases and almost 881,000 deaths in 2018, according to the World Health Organization GLOBOCAN database [1]. In Lebanon, CRC accounts for 8.5% of all cancers. It is one of the highest incidence rate in the MENA region, as 1109 cases of CRC were diagnosed in 2018 in a population of almost 6 million [1,2].

It is well established that the etiology of CRC is multifactorial, encompassing genetic, environmental, and lifestyle-related factors, including westernized diet, alcohol consumption, and obesity among others. However, chronic inflammation, in particular inflammatory bowel disease (IBD) and dysbiosis in enteric microbiota, remain the key players in this process [3–6]. Additionally, a consensus statement by the American Diabetes Association and the American Cancer Society in 2010

concluded that there is a higher risk of CRC among patients with diabetes mellitus, shedding more light on diabetes as a risk factor for CRC [7].

Diabetes mellitus is a group of metabolic disorders characterized by chronic hyperglycemia and dysregulated metabolism of carbohydrates, lipids, and proteins induced by insulin insufficiency [8]. According to the International Diabetes Federation, diabetes affected 451 million people worldwide in 2017; this number is expected to rise to 693 million by 2045. This rising trend has been promoted by a shift into urban lifestyle, the spread of western style diet, and lack of physical activity [9].

A role of microbiota in the development of diabetes mellitus has been recognized; several studies showed that in diabetic humans, there is a lack of uniformity in gut microbiota profiles, as these individuals show differences in both human host markers and gut microbiome signatures when compared with healthy people [10,11]. It has also been suggested that there might be an inflammation-triggering effect of the intestinal microbiota in the development of autoimmune diabetes [11,12]. Previous publications from our group and others supported the possible pathogenic links among CRC and diabetes, whereby an altered glucose metabolism, an oxidative stress, and a chronic inflammatory state, triggered by an imbalance in the intestinal microbiota, are potential mediators [3,13,14].

It has been also reported that inflammation is a common denominator between CRC and diabetes mellitus and a crucial factor contributing to the development of these disease entities, as subclinical systemic inflammation has been observed in patients with diabetes and in patients with CRC. It is characterized by elevated circulating levels of inflammatory parameters, including C-reactive protein and inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-Alpha (TNF-α), among others [15]. IL-6 and TNF-α, are the main MAPK-activated protein kinase 2 mediated cytokines. They are strongly associated with inflammation-driven cancers, including CRC and contribute to tumor growth and invasion [16]. Moreover, these inflammatory disease entities are characterized by excessive reactive oxygen species (ROS) production that triggers inflammation by driving the production of proinflammatory cytokines [3,17].

On the other hand, the human gut microbiota is a large and complex microbial community, comprising different populations of microorganisms that live in the intestinal lumen: bacteria, viruses, fungi, archaea, bacteriophages, and protozoans, with bacteria being the most abundant. Approximately 100 trillion bacteria inhabit the human intestine, a count ten times greater than the total number of cells in human body, with at least 1000 different species of known bacteria whose genome is 150 times greater than that of humans [18,19]. Microbial distribution varies spatially and temporally from the mouth to the rectum during the individual's lifetime, and several host factors are able to shape the microbial community, including genetics, the mode of delivery at birth, and antibiotics use, among others [19].

However, a perturbation of the normal gut microbial balance, defined as dysbiosis, can disrupt the intestinal barrier and its associated immune function, thus leading to autoimmunity and chronic inflammation [5,20]. This chronic inflammatory state promotes cell proliferation, angiogenesis, and apoptosis, along with the production of an array of proinflammatory cytokines, growth factors, and reactive oxygen and nitrogen species that result in the generation of a carcinogenic microenvironment; in addition to extra intestinal manifestations, affecting the host metabolism and promoting obesity, insulin resistance, and diabetes [5,21–23].

A series of recent publications support the fact that inflammation paralleled with dysfunctional interactions between gut microbiota and the mucosal immune system are well defined risk factors for IBD, CRC, and other inflammatory and metabolic diseases such as diabetes [3,5,13]. On this basis, the recommendation of probiotics as part of the management protocols has been widely considered [23,24].

The World Health Organization defines probiotics as live microorganisms that when administered in appropriate amounts, confer a health benefit on the host [25]. In the last few years, a growing interest in studying and using probiotics has been observed for the treatment of gastrointestinal

diseases and the improvement of overall human health [26]. The leading mechanism of probiotics action primarily includes alteration in the composition of gut microbiota, maintaining epithelial barrier function, competition with harmful gut flora for nutrients and adhesion to the epithelium of the gastrointestinal tract, as well as the production of antimicrobial peptides that were proven to exhibit anticarcinogenic action, anti-inflammatory effects and anticholesterol activities [4,25,27].

On the other hand, metformin, a widely prescribed antihyperglycemic agent of the biguanide family, is one of the most extensively recognized metabolic modulators with well-documented anticancer properties [28,29]. Metformin has a high oral bioavailability and its mechanisms in targeting the host for the treatment of metabolic diseases have been well studied. However, recent studies revealed that the gut microbiota might play an important role in its efficacy, shedding light on the fact that the gastrointestinal tract is a key site in the action of metformin [30,31]. Thus, the modulation of the gut microbiota may be one of the mechanisms contributing to the antidiabetic and anticarcinogenic effects of metformin.

The challenge to treat cancer still lies in the inability to identify new treatment strategies beyond chemotherapy, immunotherapy, and surgery to inhibit tumor progression. Given the importance of the gut microbiota and dysbiosis in the crosstalk between CRC and diabetes, our present study evaluated the effect of the addition of a microbiota modulator on metformin in a well-established model of colitis-associated CRC that recapitulates the progression from chronic inflammation to dysplasia and adenocarcinoma in humans, with an emphasis on the role of diabetes in this process. This work explored the molecular mechanisms of metformin and probiotics while focusing on their chemopreventive properties and the importance of microbiota in this strategy.

2. Results

2.1. Effect of Metformin and Probiotics on Glycemia

All the data, clinical, histological, immunohistochemical, and molecular, were analyzed with a focus on the effect of the combination therapy of metformin (M) and probiotics (P) in treating CRC in nondiabetic and diabetic animals. Diabetes was successfully induced in all intended animals. Blood glucose level was measured weekly as a direct indicator of diabetic status.

As expected, glycemia levels had increased beyond 250 mg/dL one week after an IP injection of 150 mg/kg Streptozotocin (STZ). These high levels were successfully maintained in all diabetic untreated mice (group D and DCRC) throughout the experimental period (Figure 1b).

All animals belonging to the groups where diabetes was not induced, i.e., in groups NC, CRC, CRC + M, CRC + P, CRC + MP, M, P, and MP, had glycemia levels below 250 mg/dL at all time points and the difference between these groups was not statistically significant (ns) as shown in Figure 1a. However, in the CRC group, whose animals were not subjected to diabetes induction by STZ, high glycemia peaks were observed in weeks 3, 5, 8, and 11 in which Dextran sulfate sodium (DSS) was administered. These blood sugar fluctuations were only significant at week 8 when comparing normal controls (NC) to the CRC group. The metformin and probiotics (MP) combination significantly reduced the peaks at week 8 (CRC + MP versus CRC, $^{\#}\ p < 0.05$) as shown in Figure 1a.

The highest glycemia values were obtained in DCRC and D groups at all time points with no statistical difference (ns) between the two groups showing that CRC did not affect glycemia levels in these animals. In diabetic animals, P and M administration alone or in combination significantly reduced glucose levels during the experimental period. In comparison with the untreated diabetic mice, with CRC induction (DCRC group), M and P single drug treatment in DCRC + M and DCRC + P groups decreased significantly blood glucose levels (Figure 1b, $^{*}\ p < 0.05$). Interestingly, M and P combination showed a significant effect, greater than either drug alone, as depicted when comparing DCRC + M versus DCRC + MP groups ($^{\dagger}\ p < 0.05$) and DCRC + P versus DCRC + MP groups ($^{\dagger}\ p < 0.05$), indicating that P helped M in alleviating the hyperglycemic phenotype induced by STZ (Figure 1b).

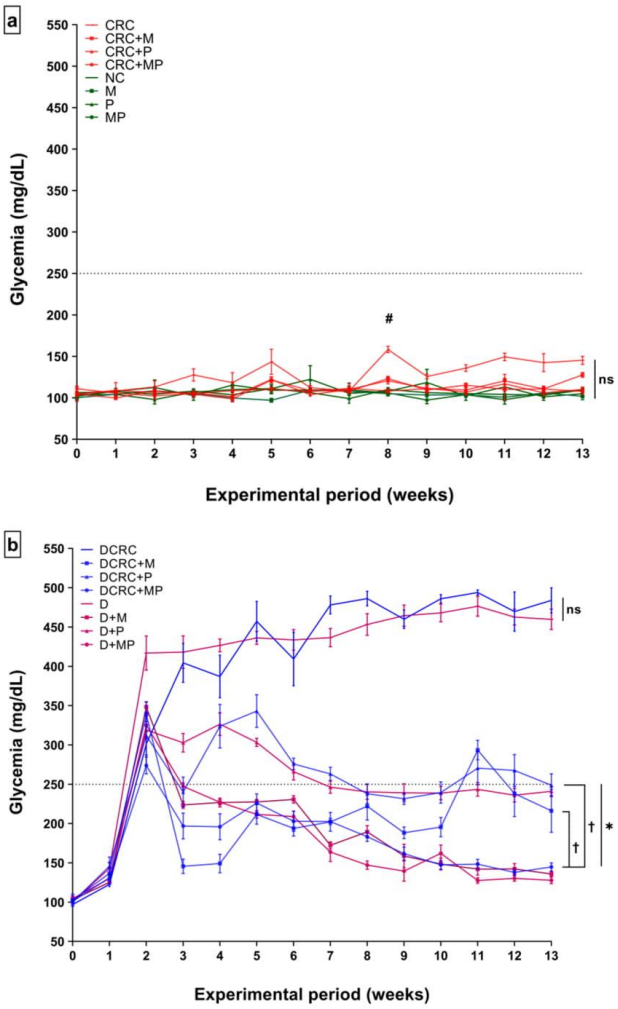


Figure 1. Effects of probiotics and metformin on glycemia levels in nondiabetic (**a**) and diabetic (**b**) Mice. Statistical significance was expressed by $^{\#}$ $p < 0.05$ in nondiabetics when compared to their experimental colorectal cancer control (CRC group) and * $p < 0.05$ in diabetics when compared to their experimental control, the diabetic colorectal cancer mice (DCRC group) with $n = 6$ animals per group. Moreover, when comparing only two groups, connecting lines were used to indicate the compared groups with † $p < 0.05$, (ns) indicates nonsignificant.

2.2. Metformin and Probiotics Modulate CRC Induction in Balb/c Mice

The optimal procedure adopted for CRC induction was successful in all mice. DSS concentration and its number of cycles were determined by pilot studies using different concentrations of DSS ranging from 1% to 3%, whereby treatment of male Balb/c mice with 1.5% DSS in their drinking water for four cycles, in addition to an injection of 10 mg/kg Azoxymethane (AOM), resulted in clinical signs and symptoms and gross and histological alterations associated with CRC.

Animals that were subjected to this optimized DSS/AOM protocol showed signs of sickness starting the first cycle, and these signs were aggravated by each successive DSS cycle. The main observed alterations were weight loss (Figure 2), loose stools, diarrhea, and rectal bleeding (Table 1), as calculated in the disease activity index (DAI). These animals presented signs of discomfort and sickness such as bad posture, hunched back, decreased grooming, and low mobility and responsiveness.

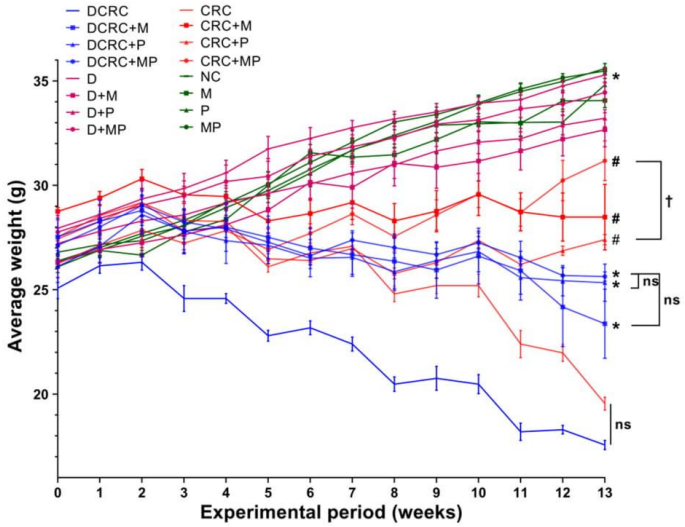


Figure 2. Weight changes in the different animal groups during the experimental period. Data are shown as mean ± SEM with $n = 6$ animals per group. Statistical significance was expressed at week 13 by $^{\#}$ $p < 0.05$ in nondiabetics when compared to their experimental CRC control (CRC) and by * $p < 0.05$ in diabetics when compared to their experimental diabetic CRC control (DCRC). Moreover, when comparing only two groups, connecting lines were used to indicate the compared groups with † $p < 0.05$.

Nondiabetic and diabetic animals that were not subject to CRC induction had an increase in their average weight as seen in the NC, M, P, MP, D, D + M, D + P, and D + MP groups. On the other hand, weight loss was detected in groups with CRC; the lowest weight averages were recorded in CRC and DCRC groups, with no statistically significant difference between these two groups. Treatment with metformin or probiotics alone and, most importantly, in combination, had a positive effect in preventing the weight loss in the nondiabetic and diabetic CRC animals with $^{\#}$ $p < 0.05$ and * $p < 0.05$ respectively (Figure 2).

When looking at fecal occult blood, normal controls and groups that were not subjected to CRC induction (NC, D, M, D + M, P, D + P, MP and D + MP groups) showed negative occult blood for all animals at all time points. Groups that were subjected to CRC induction showed positive results for fecal blood to varied degrees. CRC and DCRC animals were the first to show blood in their stools in 17% of the animals starting week 3 (on the first DSS administration), to reach 100% by the end of the experiment (Table 1).

In line with the improvements encountered in the glycemia levels and body weight, treatment with P and M alone or in combination reduced the frequency of blood in the stools and delayed their appearance until week 5. The best scores were obtained with the combination treatment in nondiabetic and diabetic CRC animals (CRC + MP and DCRC + MP groups) whereby appearance of blood was observed in only 67% of the animals at week 5 and the positive rates were 50% and 33%, respectively,

at week 13 (Table 1). Moreover, when comparing single drug treatment in nondiabetic and diabetic CRC animal groups (CRC + M, CRC + P, DCRC + M and DCRC + P) to the combination groups (CRC + MP and DCRC + MP), lower percentages in the combination treatment were encountered (Table 1). Treatments triggered a decrease of the blood in the stools, thus indicating a recovery in the mucosa.

Table 1. Effects of Metformin and Probiotics on Fecal Occult Blood (FOB). FOB was Assessed Weekly; Percentages were Obtained by Calculating the Number of "Positive" Animals in Each Group. A Color Scale Ranging from Strong to Faint Red is Used, whereby the Shade of the Red Color Represents the Value of the Cell.

Group	Experimental Period (Weeks)													
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
NC	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
D	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
CRC	0%	0%	0%	17%	33%	100%	100%	50%	100%	100%	100%	100%	100%	100%
DCRC	0%	0%	0%	17%	17%	100%	100%	80%	100%	100%	100%	100%	100%	100%
CRC + M	0%	0%	0%	0%	0%	100%	83%	33%	100%	83%	67%	100%	100%	100%
DCRC + M	0%	0%	0%	0%	0%	100%	33%	17%	100%	50%	33%	100%	100%	100%
CRC + P	0%	0%	0%	0%	0%	83%	33%	33%	100%	100%	67%	100%	100%	100%
DCRC + P	0%	0%	0%	0%	0%	100%	50%	33%	100%	33%	33%	100%	100%	60%
CRC + MP	0%	0%	0%	0%	0%	67%	17%	0%	67%	50%	33%	67%	67%	50%
DCRC + MP	0%	0%	0%	0%	0%	67%	17%	17%	100%	17%	17%	100%	100%	33%
M	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
D + M	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
P	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
D + P	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
MP	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
D + MP	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

In the CRC and DCRC groups, animals had the highest disease activity indexes (Figure 3). Treatment of CRC animals with M alone or P alone ameliorated the clinical profile of the animals and decreased DAI, but not significantly. Importantly, the combined MP treatment induced a statistically significant decrease in DAI levels in nondiabetic CRC and diabetic CRC ($^{\#}$ $p < 0.05$ and * $p < 0.05$, respectively), as these groups (CRC + MP and DCRC + MP) had the lowest DAI along with a better mobility, fur shape, and responsiveness. The addition of P to M induced a significant amelioration in its action on decreasing DAI in nondiabetics (CRC + M versus CRC + MP, † $p < 0.05$) and in diabetics (DCRC + M versus DCRC + MP, † $p < 0.05$) at week 13 (Figure 3).

Normal DAI levels close to zero were obtained in normal controls (NC group) and in all of the other groups that were not subject to AOM/DSS CRC induction (M, P, MP, D, D + M, D + P and D + MP groups). Treatment of normal mice with P alone, M alone, and their combination did not affect the animals, their DAI was similar to those of normal mice at all time points. Diabetic animals showed more signs of discomfort and sickness than their nondiabetic counterparts; however, their diabetic status did not seem to affect their DAI score and no significant differences between diabetics and nondiabetics were observed (Figure 3).

2.3. Effect of Metformin and Probiotics on the Survival Rates

Based on our experience and the reported literature, week 13 was selected as the terminal time point. Survival rates fluctuated between the different groups based on treatments. In diabetics, the survival rate of diabetic animals with CRC induction was the lowest at 50% (DCRC group). Treatment with M and P improved survival rates to 67% and 83% in the DCRC + M and DCRC + P groups, respectively. Moreover, the MP combination in the DCRC + MP group improved and raised the survival rate to 100%.

Nondiabetic animals that were subjected to CRC induction had survival rates of 67%; while the same animals treated with M and P alone or in combination (CRC + M, CRC + P, and CRC +

MP groups) had 100% survival rates, similar to normal animals in the normal controls (NC) and in nondiabetic M, P, and MP groups (Figure 4).

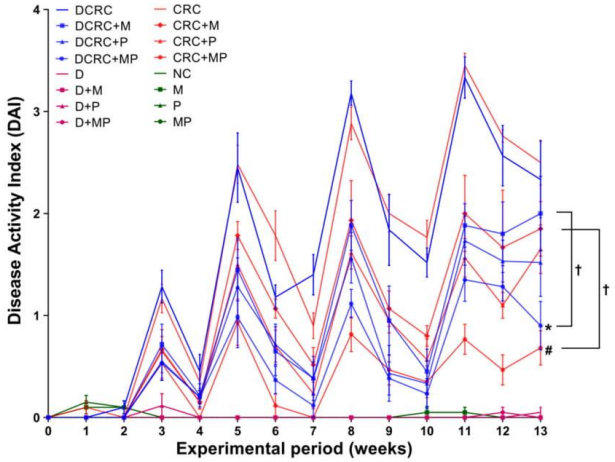


Figure 3. Weekly Disease Activity Index (DAI) variations among the different groups. Note that animals that were not subject to the induction of CRC had very low indexes, close to zero. However, an increase in DAI was obtained in the groups with CRC induction, with peaks obtained at week 3, 5, 8, and 11. M and P treatments elicited a reduction in DAI. Statistical significance was expressed by $^{\#}$ $p < 0.05$ in nondiabetics when compared to their experimental CRC control (CRC), and by * $p < 0.05$ in diabetics when compared to their experimental diabetic CRC control (DCRC). Moreover, when comparing only two groups, connecting lines were used to indicate the compared groups with † $p < 0.05$.

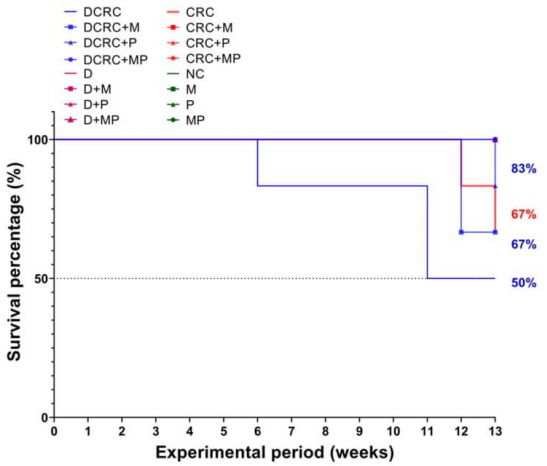
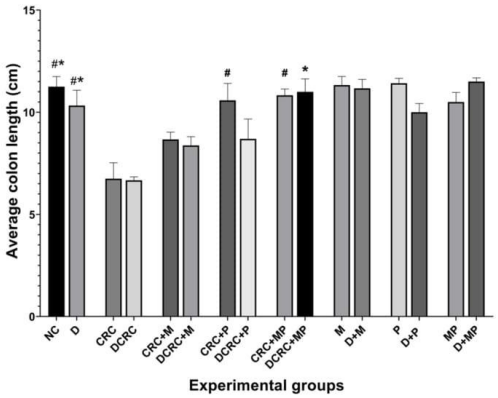


Figure 4. Kaplan–Meier survival curves of animals in the different groups. Diabetic animals with CRC had the worst survival rates of 50% (DCRC group); treatment with M alone or P alone (DCRC+M and DCRC + P groups) raised the survival percentages to 67% and 83%, respectively. In nondiabetic CRC animals, the CRC group had a survival rate of 67%, and no deaths were observed in all other groups.

2.4. Metformin and Probiotics Restore Normal Colon Length

Colon length was measured upon dissection at week 13 as colon shortening is often considered a visual index that reflects the severity of colorectal inflammation. Mice treated with AOM/DSS (CRC and DCRC groups) had the shortest colons with 6.75 ± 0.78 cm and 6.67 ± 0.17 cm, respectively, (Figure 5).



Nondiabetic groups	NC	CRC	CRC + M	CRC + P	CRC + MP	M	P	MP
Mean ± SEM	11.25 ± 0.50	6.75 ± 0.78	8.67 ± 0.36	10.58 ± 0.83	10.83 ± 0.31	11.33 ± 0.42	11.42 ± 0.24	10.5 ± 0.47
Diabetic groups	**D**	**DCRC**	**DCRC+M**	**DCRC+P**	**DCRC+MP**	**D+M**	**D+P**	**D+MP**
Mean ± SEM	10.33 ± 0.75	6.67 ± 0.17	8.37 ± 0.43	8.70 ± 0.97	11.0 ± 0.63	11.17 ± 0.44	10.0 ± 0.43	11.5 ± 0.18

Figure 5. Colon length variation. Average colon length (cm) was recorded on the day of sacrifice at week 13 Nondiabetic groups were compared to their experimental CRC control (CRC), significance was expressed by $^{\#}$ $p < 0.05$. On the other hand, diabetic groups were compared to their experimental diabetic CRC control (DCRC), and significance was defined as * $p < 0.05$, with $n = 6$ animals per group.

In nondiabetic animals that were subject to CRC induction (CRC group), mice had significantly shorter colons than the normal controls (NC) ($^{\#}$ $p < 0.05$). Administration of M in the CRC+M group increased the colon length, but this was not statistically significant. However, administration of P alone in (CRC + P) group or in combination with metformin (CRC + MP) significantly increased colon length to reach 10.58 ± 0.83 cm and 10.83 ± 0.31 cm, respectively, with $^{\#}$ $p < 0.05$ (Figure 5), close to that of the normal control (NC) group.

Diabetic mice with CRC (DCRC group) had significantly shorter colons than diabetic animals (D group), * $p < 0.05$. The administration of M or P alone ameliorated the colon length, but not significantly in the DCRC + M and DCRC + P groups. However, administration of MP in combination restored the normal length of the colon, whereby animals in DCRC + MP group had colon length of 11 ± 0.63 cm (* $p < 0.05$) (Figure 5).

Colon length was not affected by diabetes alone, as the difference between nondiabetic and diabetic animals in all subgroups was not statistically significant.

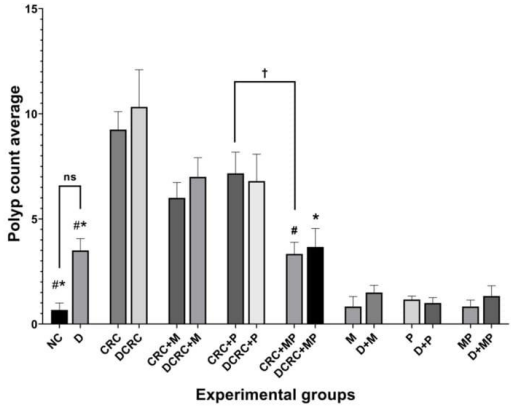
2.5. Effect of Metformin and Probiotics on Polyp Formation

Polyp formation was assessed by quantifying the number of formed nodules within the colon from the ileocecal junction to the distal end of rectum.

Animals in the CRC and DCRC groups had an average of 9.25 ± 0.85 and 10.33 ± 1.76 polyps, respectively. This number was significantly higher than the negligible number obtained in the normal controls (0.67 ± 0.33), with $^{\#}$ $p < 0.05$ and * $p < 0.05$, respectively.

Treatment with either drug alone in nondiabetic and diabetic CRC animals reduced polyp formation; however, this reduction was not statistically significant. On the other hand, combining MP

reduced significantly the number of nodules in the CRC+MP and DCRC+MP groups to 3.33 ± 0.56 and 3.67 ± 0.88 polyp ($^{\#}$ $p < 0.05$, * $p < 0.05$), respectively (Figure 6).



Nondiabetic groups	NC	CRC	CRC + M	CRC + P	CRC + MP	M	P	MP
Mean ± SEM	0.67 ± 0.33	9.25 ± 0.85	6 ± 0.73	7.17 ± 1.01	3.33 ± 0.56	0.83 ± 0.48	1.17 ± 0.17	0.83 ± 0.31
Diabetic groups	**D**	**DCRC**	**DCRC + M**	**DCRC + P**	**DCRC + MP**	**D + M**	**D + P**	**D + MP**
Mean ± SEM	3.5 ± 0.56	10.33 ± 1.76	7.0 ± 0.91	6.8 ± 1.25	3.67 ± 0.88	1.5 ± 0.34	1.0 ± 0.26	1.33 ± 0.49

Figure 6. Polyp count variation in the different groups. Average polyp number was recorded on the day of sacrifice at week 13. Nondiabetic groups were compared to their experimental CRC control (CRC). Significance was expressed by $^{\#}$ $p < 0.05$. On the other hand, diabetic groups were compared to their experimental diabetic CRC control (DCRC). Significance was expressed by * $p < 0.05$. Moreover, when comparing only two groups, connecting lines were used to indicate the compared groups with † $p < 0.05$, (ns) stands for nonsignificant, with $n = 6$ animals per group.

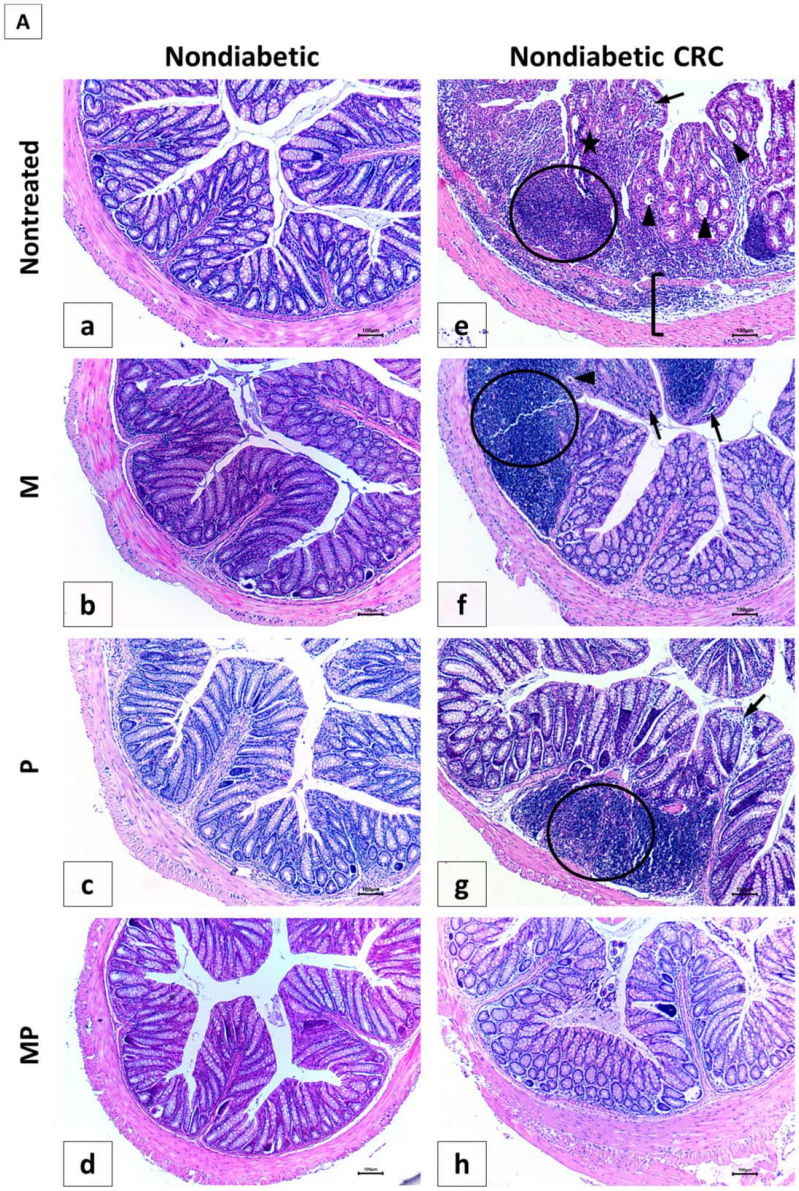
It is worth noting that the addition of metformin to probiotics treatment had a better effect in reducing polyp formation, as the total number of polyps in the MP treated groups was significantly lower compared to the P-treated group in nondiabetic CRC animals with † $p < 0.05$ (Figure 6).

Diabetic mice that were not subject to colorectal cancer induction (D group), had a higher number of polyps than their nondiabetic counterparts (NC group), with 3.5 ± 0.56 compared to 0.67 ± 0.33, but this difference was not statistically significant (Figure 6).

2.6. Histological Alterations of the Colon Due to Metformin and Probiotics Treatment

The histology of the colon in normal control mice (NC group) showed no alterations; there were straight crypts reaching the muscularis mucosa, intact lining epithelium, normal goblet cells, as well as unremarkable changes in inflammatory cells infiltration (Panel 7A-a) with a score of 0.33 ± 0.21.

In contrast, all AOM/DSS-treated animals, nondiabetic and diabetic (CRC and DCRC groups), showed remarkable histological changes in the colon, indicating inflammation and dysplasia. Animals in these groups had the highest histological alterations, with 19.0 ± 1.35 and 20.0 ± 0.58, respectively (Figure 7C). The main changes detected include epithelial ulceration, cryptitis, crypt abscesses, crypt architecture disarray, inflammatory cells infiltration in the mucosa and submucosa, interruption in muscularis mucosa, and goblet cells depletion, as depicted in Panels 7A-e and 7B-m for CRC and DCRC, respectively.



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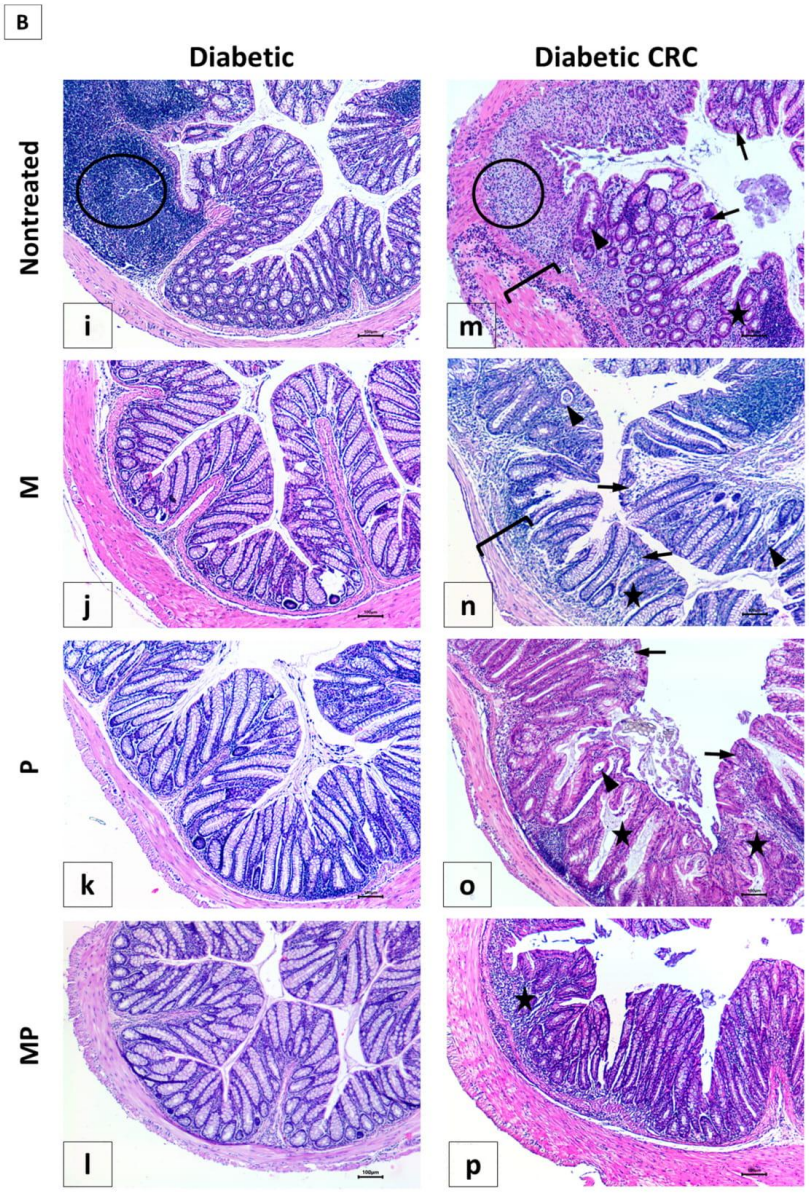

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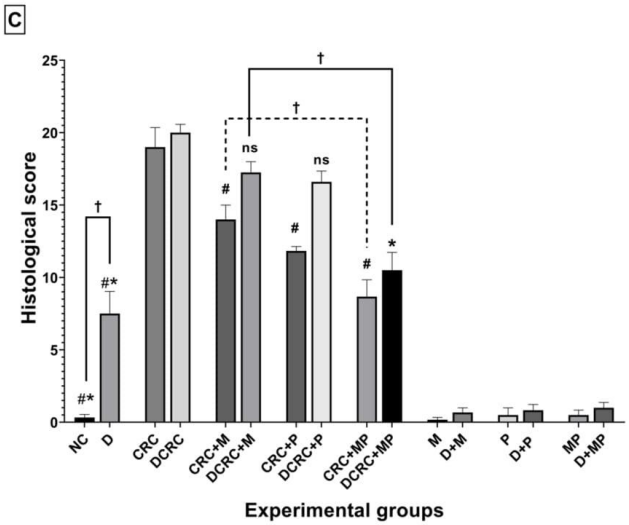


Nondiabetic groups	NC	CRC	CRC + M	CRC + P	CRC + MP	M	P	MP
Mean ± SEM	0.33 ± 0.21	19.0 ± 1.35	14.0 ± 1.00	11.83 ± 0.31	8.67 ± 1.17	0.17 ± 0.17	0.50 ± 0.50	0.50 ± 0.34
Diabetic groups	**D**	**DCRC**	**DCRC + M**	**DCRC + P**	**DCRC + MP**	**D + M**	**D + P**	**D + MP**
Mean ± SEM	7.50 ± 1.54	20.0 ± 0.58	17.25 ± 0.75	16.60 ± 0.75	10.5 ± 1.23	0.67 ± 0.33	0.83 ± 0.40	1.00 ± 0.37

Figure 7. Effect of probiotics and metformin on colon histology. (**A**,**B**) Representative images of hematoxylin and eosin (H&E)-stained colon sections illustrating the histological changes in the nondiabetic (**Panel A**) and diabetic (**Panel B**) groups. The worst alterations were encountered in the nontreated CRC (7A-e) and diabetic CRC (7B-m). Note the presence of large inflammatory cells infiltrates (circle), inflammatory cells invading the edematous submucosa (bracket), crypt abcess (black triangle) and cryptitis (black arrow), as well as crypt architecture disarray (star). Significant improvements in the combination treated mice in groups CRC + MP (7A-h) and DCRC+MP (7B-p) were noted. Original magnification: 4×; scale bars 100 µm. Photos were adjusted for white balance using Adobe Photoshop®; (**C**) Histological alterations score. Data is expressed as average ± SEM (n = 6). Nondiabetic groups were compared to their experimental CRC control (CRC), significance was expressed by $^{\#}$ $p < 0.05$. On the other hand, diabetic groups were compared to their experimental diabetic CRC control (DCRC), significance was expressed by * $p < 0.05$. Moreover, when comparing only two groups, connecting lines were used to indicate the compared groups with † $p < 0.05$; (ns) stands for nonsignificant.

The analysis of the various histological scores indicated a significant effect of the combination therapy in improving the colonic tissue, both in nondiabetic and diabetic groups.

In nondiabetic animals, single drug administration in group CRC + M (Panel 7A-f) and CRC + P (Panel 7A-g) ameliorated colorectal crypt structure and significantly reduced the histological score to reach 14.0 ± 1.0 and 11.83 ± 0.31, respectively, with $^{\#}$ $p < 0.05$ when compared to untreated CRC (Figure 7C). Treatment with a combination of metformin and probiotics in group CRC + MP (Panel 7A-h) had an effect that was better than either drug alone, and recorded the lowest histological score (8.67 ± 1.17, $^{\#}$ $p < 0.05$) with significantly reduced alterations.

On the other hand, diabetic animals (D group) showed a histological alteration score of 7.50 ± 1.54, significantly higher than nondiabetic animals (NC group) with † $p < 0.05$ (Figure 7C), despite the fact that they were not subject to CRC induction, thus shedding light on the damage caused by diabetes alone on the colonic tissue, particularly the increase in inflammatory cells infiltrates, as seen in Panel 7B-i.

Moreover, in diabetic CRC animals, the administration of either metformin alone or probiotics alone in the DCRC + M and DCRC + P groups was not able to noticeably reverse the pathological damage (Panel 7B-n and 7B-o), as the respective histological scores were lowered with no statistical significance (ns, Figure 7C). However, when probiotics were combined with metformin, a significant amelioration of the histological alterations was observed in the DCRC + MP group (Panel 7B-p) with * $p < 0.05$ (Figure 7C).

The structural improvement between treatment with metformin alone (CRC + M, DCRC + M) and metformin with probiotics in the CRC + MP and DCRC + MP groups was statistically significant, † $p < 0.05$ (Figure 7C). One possible explanation could be that the dysregulated microbiota in CRC could prevent metformin from exerting its protective effects. Correction of this dysbiosis with probiotics proved to be crucial in regulating the anti-inflammatory and anticarcinogenic mechanism of action of metformin.

2.7. Proliferation Assessment in Colonic Tissue

To assess the effect of metformin and probiotics on cell proliferation, immunostaining by Ki-67 was performed on paraffin-embedded colon tissue. The highest proliferation indices were observed in CRC and DCRC groups, whereby the ki-67 staining was distributed throughout most of the crypt area, towards the lumen (Figure 8).

Metformin administration in nondiabetic and diabetic CRC animals was able to significantly reduce the cellular proliferation in CRC + M and DCRC + M groups ($^{\#}$ $p < 0.05$, * $p < 0.05$ respectively). However, the decrease in proliferation index with probiotics administration alone in the CRC + P and DCRC + P groups was not statistically significant (Figure 8C).

When combined, metformin and probiotics (in the CRC + MP and DCRC + MP groups) were the most effective in bringing ki-67 count close to that of the normal controls ($^{\#}$ $p < 0.05$, * $p < 0.05$, respectively), indicating that this combination was most likely able to suppress tumor cell proliferation in the AOM/DSS-induced CRC model (Figure 8).

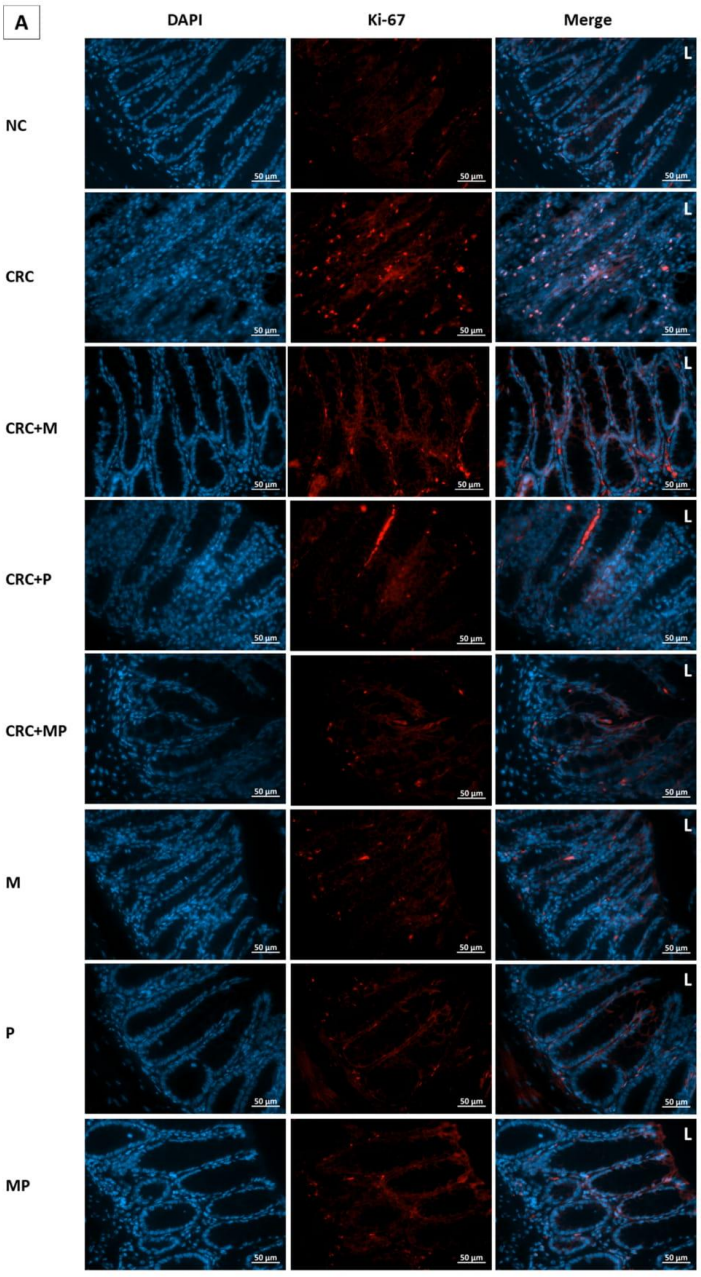
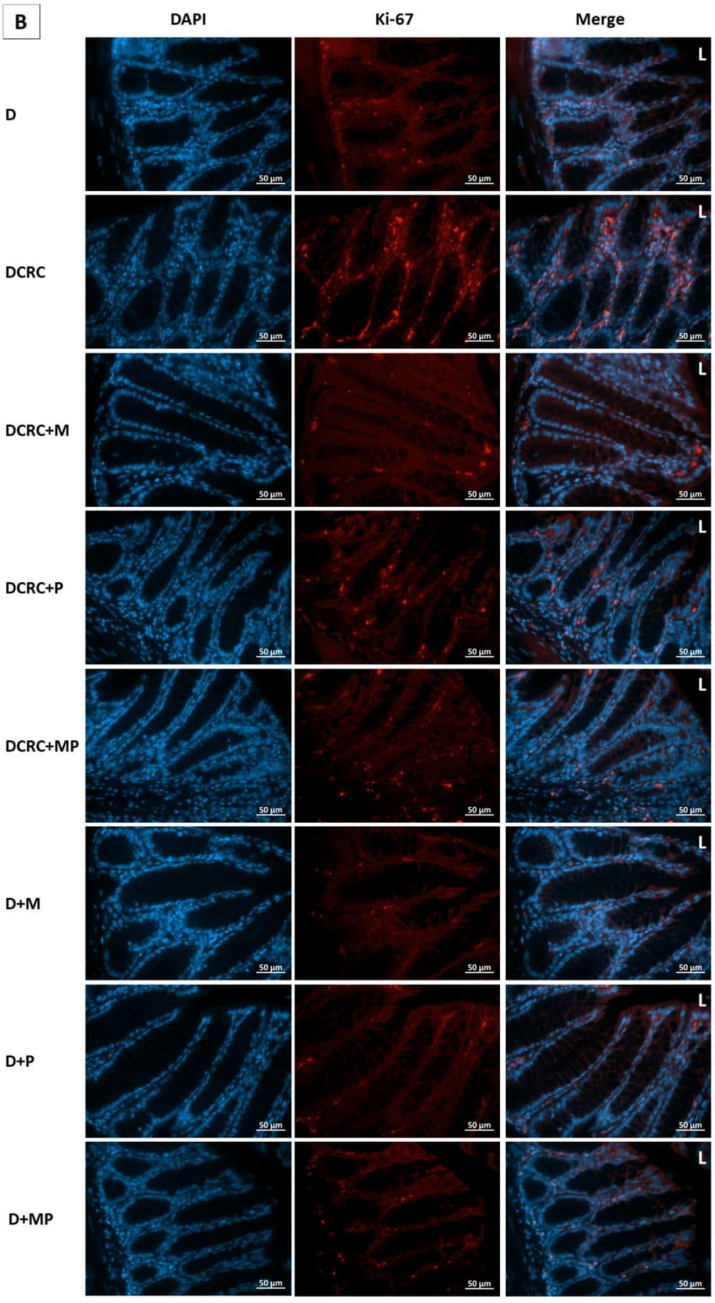


Figure 8. *Cont.*



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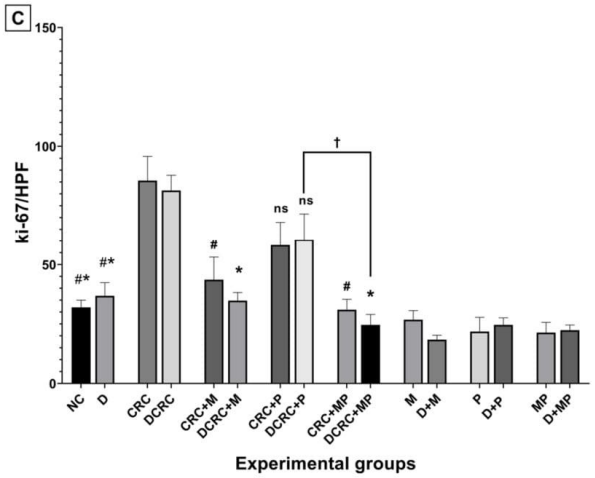


Figure 8. Effect of probiotics and metformin on colon proliferation. (**A**,**B**) Panel of representative images of colon section labelled with with Ki-67 (red) and counterstained with DAPI (blue) in nondiabetic (**A**) and diabetic groups (**B**). HPF 40× magnification; scale bars 50 µm. "L" indicates the position of the lumen. CRC and DCRC groups showed the highest positive nuclear Ki-67 staining throughout the crypts, with increased staining intensity from the midcrypt region to the lumen. A decrease proliferating cells was observed with probiotics and metformin administration to different extents. Normal controls and animals that were not subject to CRC induction had low proliferation indices; (**C**) Proliferation index in the different groups. Data is expressed as mean ± SEM (n = 5). Nondiabetic groups were compared to their experimental CRC control (CRC), significance was expressed by $^{\#}$ $p < 0.05$. On the other hand, diabetic groups were compared to their experimental diabetic CRC control (DCRC); significance was expressed by * $p < 0.05$. Moreover, when comparing only two groups, connecting lines were used to indicate the compared groups with † $p < 0.05$; ns stands for nonsignificant.

2.8. Modulation of Reactive Oxygen Species Production

To analyze ROS levels within the intestinal epithelium, frozen colon sections were stained with the ROS-responsive dye dihydroethidium (DHE). A significant increase, reaching its highest staining intensity, was obtained in untreated CRC animals in both nondiabetic and diabetic groups (CRC and DCRC) as depicted in Figure 9. Moreover, diabetic animals without CRC induction (group D) showed high ROS production, with † $p < 0.05$ when compared to the normal controls in the NC group (Figure 9), thus indicating the increased ROS generation in the colon epithelium in diabetes and shedding light on the common oxidative stress increase linking CRC, inflammation, and diabetes.

In nondiabetics, treatment of cancerous animals with metformin alone, probiotics alone, or their combination significantly reduced ROS production, with the lowest DHE/DAPI ratios obtained in the CRC + MP group, $^{\#}$ $p < 0.05$ (Figure 9).

On the other hand, diabetic animals with CRC showed a significant reduction in their DHE/DAPI ratios only when the combination protocol was adopted in the DCRC+MP group (Figure 9).

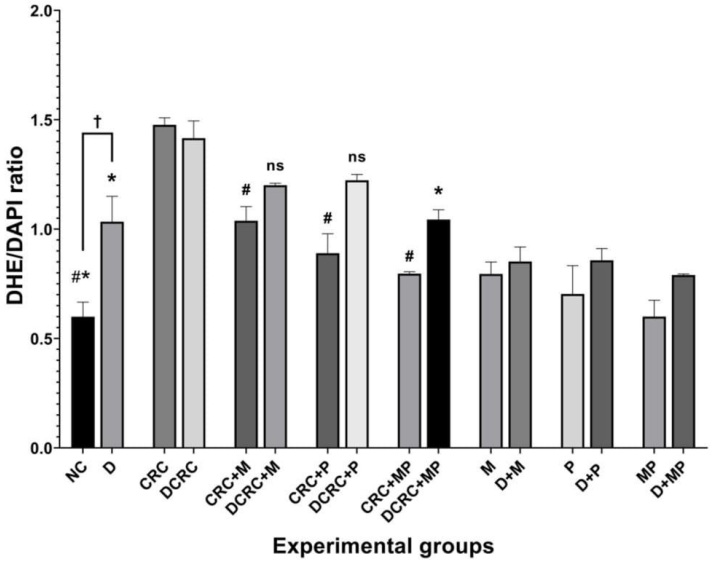
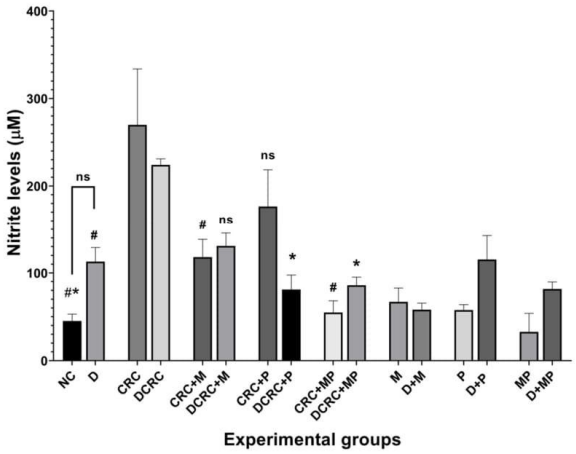


Figure 9. Reactive oxygen species modulation by metformin and probiotics. Results are expressed as ratios of DHE and DAPI intensity ($n = 3$). Nondiabetic groups were compared to their experimental CRC control (CRC group); significance was expressed by $^{\#}$ $p < 0.05$. On the other hand, diabetic groups were compared to their experimental diabetic CRC control (DCRC group); significance was expressed by * $p < 0.05$. Moreover, when comparing only two groups, connecting lines were used to indicate the compared groups with † $p < 0.05$; ns stands for nonsignificant.

2.9. Modulation of Nitric Oxide Levels with Probiotics and Metformin

The serum nitric oxide concentration was determined by measuring nitrite concentration, a stable metabolite of NO (Figure 10). Nitrite levels were significantly upregulated in both nondiabetic and diabetic CRC mice. This upregulation was modulated by the different treatments to various extents. In the nondiabetic subgroups, treatment with metformin alone or its combination with probiotics were able to significantly reduce nitrite levels in the CRC + M group and, most importantly, in the CRC + MP group with $^{\#}$ $p < 0.05$, whereby the combination of the two drugs had additive effects in suppressing nitrite production to a level close to that of normal animals (NC group) (Figure 10).

However, in diabetic CRC animals, probiotics administration alone in the DCRC + P group or in combination with metformin in the DCRC + MP group significantly reduced nitrite levels (* $p < 0.05$), without a significant additive effect (Figure 10). On the other hand, diabetic animals (D group), had high levels of nitrite when compared to the normal controls (NC group); however, the difference was not statistically significant. These diabetic animals treated with metformin alone, probiotics alone, or their combination in groups D + M, D + P and D + MP expressed no significant difference and they had comparable nitrite levels among the groups that were higher than normal controls in group NC (Figure 10).



Nondiabetic groups	NC	CRC	CRC+M	CRC+P	CRC+MP	M	P	MP
Mean ± SEM	45.2 ± 7.9	269.8 ± 63.9	117.9 ± 20.3	176.5 ± 41.9	54.8 ± 13.4	67.0 ± 15.7	57.7 ± 6.2	32.7 ± 21.2
Diabetic groups	**D**	**DCRC**	**DCRC+M**	**DCRC+P**	**DCRC+MP**	**D+M**	**D+P**	**D+MP**
Mean ± SEM	112.8 ± 16.1	224.1 ± 6.8	130.7 ± 14.8	81 ± 16.5	85.9 ± 9.2	58.0 ± 7.5	115.2 ± 27.4	81.7 ± 8.0

Figure 10. Nitrite modulation by metformin and probiotics. Results are expressed as mean ± SEM (n = 4). Nondiabetic groups were compared to their experimental CRC control (CRC), significance was expressed by $^{\#}$ $p < 0.05$. On the other hand, diabetic groups were compared to their experimental diabetic CRC control (DCRC); significance was expressed by * $p < 0.05$; ns stands for nonsignificant.

2.10. Modulation of IL-6 and TNF-α Production by Metformin and Probiotics

IL-6 and TNF-α, two critical inflammatory mediators involved in the stimulation of tumor microenvironment, were measured in both serum and colon extracts.

AOM/DSS treated animals in nondiabetic and diabetic groups (CRC and DCRC) showed elevated IL-6 and TNF-α levels in their colons (Figure 11a,c) and sera (Figure 11b,d). On the other hand, normal controls (NC group) presented significantly lower levels of cytokines when compared to CRC and DCRC, with $^{\#}$ $p < 0.05$ and * $p < 0.05$, respectively.

Treatment with metformin alone or probiotics alone decreased the levels of IL-6 (Figure 11a,b) and TNF-α (Figure 11c,d) in both colonic tissue and serum of cancerous nondiabetic and diabetic animals, as seen in groups CRC + M, CRC + P, DCRC + M and DCRC + P; however, no reduction was observed in the colonic tissues of group DCRC + P. Such reductions were not statically significant.

Importantly the MP combination significantly reduced the IL-6 levels in the colon of nondiabetics (CRC + MP, $^{\#}$ $p < 0.05$) and the serum of diabetics (DCRC+MP, * $p < 0.05$), as seen in Figure 11a,b.

In addition, TNF-α levels were also significantly reduced in both colon and serum of nondiabetics with CRC as seen in Figure 11c,d (CRC + MP, $^{\#}$ $p < 0.05$), and only in the serum of diabetics (DCRC + MP, * $p < 0.05$).

Furthermore, the control diabetic group that did not receive any treatment (group D) also showed high levels of IL-6 and TNF-α production in colon tissue and serum compared to the NC group, shedding light on the inflammatory state created by diabetes in the colon, which will in turn affect the carcinogenic process.

The synergistic effect between metformin and probiotics was remarkable to various extents in the colon and serum of CRC + MP and DCRC + MP groups. Interestingly, supplementation of MP recovered the healthy levels of these two cytokines, resulting in a significant improvement in the inflammatory response and a consequently lower likelihood of carcinogenesis.

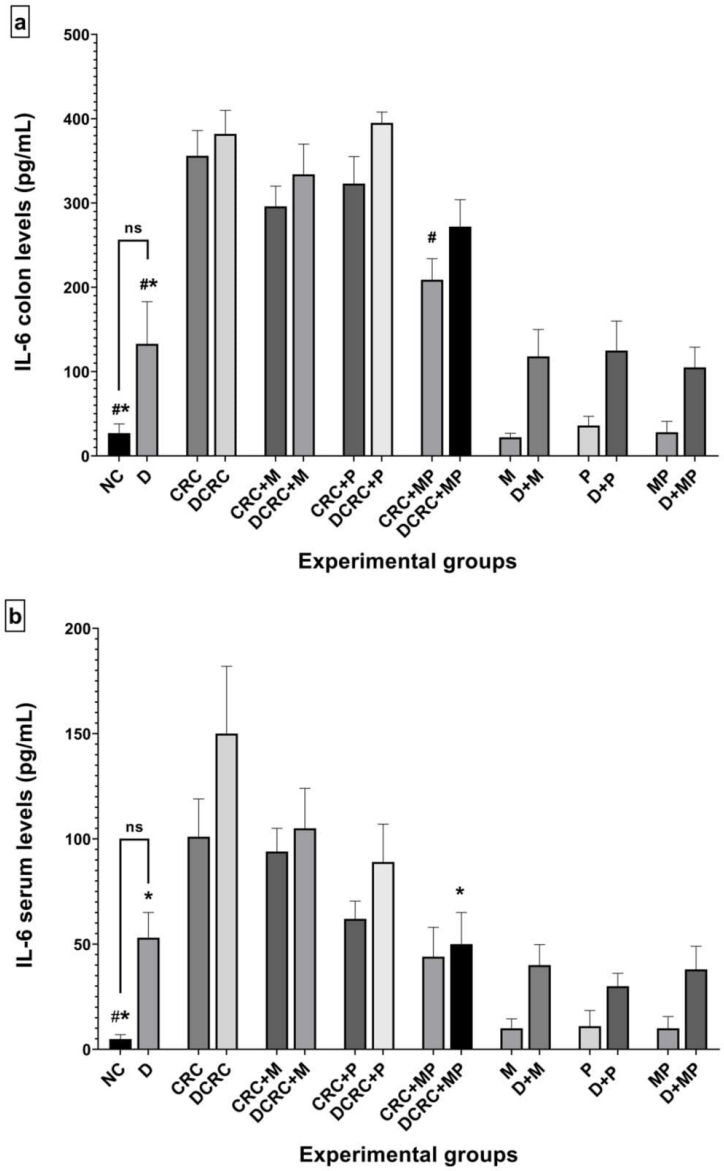


Figure 11. *Cont.*

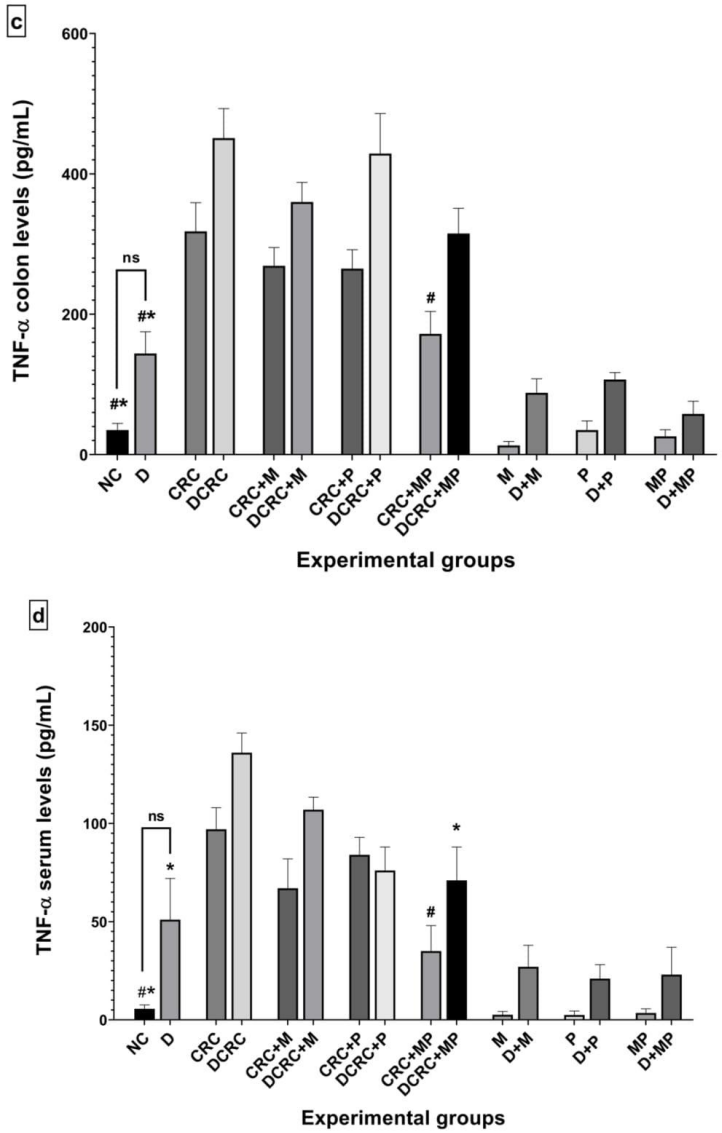


Figure 11. Variation of IL-6 and TNF-α levels from colon extraction (**a**,**c**) and serum (**b**,**d**) of the different groups. Results are expressed as mean ± SEM ($n = 4$). Nondiabetic groups were compared to their experimental CRC control (CRC); significance was expressed by $^{\#}$ $p < 0.05$. On the other hand, diabetic groups were compared to their experimental diabetic CRC control (DCRC group); significance was expressed by * $p < 0.05$; ns stands for nonsignificant.

3. Discussion

A growing body of evidence sheds light on the association between diabetes mellitus and CRC. The mechanisms underlying these two medical conditions are not fully elucidated yet; however, hyperglycemia coupled with an increase in oxidative stress and chronic inflammation create a favorable environment for the progression of diabetes, IBD, and CRC. Importantly, an altered gut microbiota is being recognized as a key player in this crosstalk [3,32].

In recent years, increasing attention has been given from the scientific community to experimental and clinical studies supporting the role of probiotics in the management of colorectal carcinogenesis and diabetes. Along this line, numerous potential mechanisms of action of probiotics have been proposed, including amelioration of the gastrointestinal mucosa, changes in the intestinal microbiota and in its metabolic activity, modulation of the immune responses, improvement of glycemic parameters, inhibition of cellular proliferation, induction of apoptosis, and exerting anti-inflammatory and antioxidative effects, among others [33,34].

This study used the combination of probiotics and metformin in nondiabetic and diabetic Balb/c mice that were subject to CRC induction. Analysis of the emanating data from clinical, histological, and molecular sources pointed to the beneficial effects of the therapy in the various groups, to different extents, in reestablishing homeostatic equilibrium and in actively preventing the inflammatory and carcinogenic processes.

Concerning the protocol, it is worth mentioning that the intended animals successfully developed diabetes based on multiple clinical criteria: high serum glucose >250 mg/dL and excess urination. Moreover, selected groups of these diabetic as well as nondiabetic mice underwent successful CRC induction by AOM/DSS as documented by clinical and histological data. This animal model has been widely used by our group and by other investigators in testing drug efficacy to screen new therapeutic treatments relevant to human IBD and CRC. It is also well established that this model provides reproducible and long-lasting colonic damage that mimics the human colitis-associated CRC process [35] and that DSS-induced chronic inflammation of the colon plays a major role in colorectal carcinogenesis by altering multiple parameters, including the microstructure of the gastrointestinal tract, the intestinal barrier integrity and function, the production of ROS and its metabolites, and the secretion of specific mediators and cytokines. Such changes happen in parallel with a modification in the intestinal microbiota and the creation of a state of dysbiosis [36].

On the other hand, diabetes mellitus is one of the most prevalent and rapidly increasing comorbid conditions. For more than 10 years, medical literature has shed light on the relationship between diabetes and CRC, connecting diabetes onset to poor cancer outcomes as comorbid diabetes worsens the course of chronic inflammatory diseases and complicates its management [32,37]. This is in agreement with our results where cancer was exacerbated by diabetes; higher histological score and a worse clinical profile were obtained in nontreated animals that were subject to diabetes induction along with CRC than their nondiabetic counterparts. Moreover, survival rates in diabetic CRC mice (DCRC group) were significantly lower than their nondiabetic counterparts (CRC group).

The administration of a mixture of probiotics along with metformin helped in inhibiting the damage caused by the administration of AOM/DSS to Balb/c mice, through preventing weight loss, ameliorating the DAI, reducing the production of polyps, ameliorating colon histology, and regulating the secretion of the proinflammatory cytokines, thus reducing or inhibiting the inflammatory pathways in the colon. These results are in agreement with numerous previous reports focusing on probiotics and metformin and their beneficial effects on diabetes and CRC [29,31]. These health benefits could be due to the multiple mechanisms involved in such a mutualistic approach of probiotics and the intestinal barrier, resulting in the inhibition of the dynamics of initiation and development of IBD and CRC.

It was proven that dysbiosis in the gut acts as a driving force during the progression from inflammation to carcinogenesis [38,39]; thus, probiotics have the possibility of retaining tumor progression by manipulating the intestinal microbiota and improving multiple related parameters. In this study, metformin or probiotic single drug treatment significantly decreased blood glucose

levels reduced glycemia, in comparison to the untreated diabetic mice, with or without CRC induction. These results are in line with preliminary interventions in humans suggesting that probiotics may improve glucose metabolism, insulin, and HbA1c levels [40,41].

In addition, our results suggest a potential chemopreventive effect of probiotics supplementation on CRC, whereby probiotics promoted intestinal homeostasis and regulated the inflammatory response. Animals treated with probiotics alone (CRC + P and DCRC + P) had a better clinical profile when compared to the untreated (CRC and DCRC groups) animals, and their DAI scores were ameliorated with better survival rates. In addition, occult blood appearance was decreased and delayed; polyp formation, inflammatory cells infiltration, and Reactive oxygen and nitrogen species (RONS) secretions were reduced when probiotics were administered. These results parallel those of Mendes et al. where probiotics supplementation reduced inflammatory cell infiltration and lowered the inflammatory response [39].

On the other hand, metformin, one of the most prescribed molecules in the drug market is widely used for the treatment of diabetes mellitus. Metformin is being now recognized as a complex drug possessing antitumor and antiaging effects, as well cardiovascular and neuroprotective properties [37,42,43].

The efficacy of metformin in alleviating inflammation and oxidative stress as well as prevention of CRC has been shown to be mediated mainly through the inhibition of various proinflammatory mediators and oxidative stress [44]. These observations were in parallel with our results, whereby metformin administration to CRC animals ameliorated their clinical profile, their DAI scores, and their survival rates. Furthermore, it reduced histological alterations scores and significantly lowered the oxidative stress in CRC + M and DCRC + M animals when compared to the untreated CRC and DCRC groups.

Collectively, our results showed that the administration of metformin alone or probiotics alone had beneficial effects on the diabetic and CRC phenotypes to variable extents. These variations might be explained by the interindividual differences in the composition of gut microbiota, especially in the inflamed microenvironment created by the induction of CRC and diabetes.

Several recent studies have shed light on the gut microbiota as a key site of action for metformin. This was supported by old data indicating that the efficacy of metformin is affected by antibiotics [45]. Moreover, the glucose-lowering effects were found to be stronger following intraduodenal versus intravenous administration of metformin [46].

In the present study, the combination of probiotics with metformin helped metformin in potentiating its anticancerous and anti-inflammatory effects. One possible mechanism might be through the correction of dysbiosis by probiotics, which enhanced the activities of metformin. In fact, animals with CRC and DCRC that were treated with the combination therapy showed a significant amelioration of their diabetic and cancer status when compared to groups treated with a single drug and to untreated groups.

Cell proliferation is considered as a crucial factor influencing carcinogenesis progression. In our study, immunohistochemical analysis of colon tissue from the different groups showed high levels of Ki-67-labelled cells in the crypts of CRC and DCRC animals; moreover, the ki-67 labelling extended to most of the crypt surface. This is in accordance with other studies showing that in CRC, a reversal in the distribution of proliferating cells from the bottom of the crypt into the upper crypt and luminal surface occurs [47]. However, in normal conditions, proliferating cells are concentrated at the bottom half of the crypt, and the upper half of the crypt usually consists of nondividing migrating cells [48]. Metformin and probiotics administration induced an inhibition of proliferating colonocytes especially when combined, shedding light again on their protective effect on colorectal carcinogenesis.

The beneficial effects granted by metformin and probiotics were also substantiated by the histological analysis of the colonic mucosa. Histopathologic analysis of colorectal tissues showed that the multiple histopathological alterations recorded during the course of the disease were reversed to significant extents, including surface erosion, inflammatory cells infiltration, submucosal edema, polyp

formation, and dysplasia. The MP combination attenuated the severity of colorectal inflammation and ameliorated colorectal crypt structure as evaluated in the histological score. It looks like the combination treatment could stabilize the intestinal wall through a mechanism that is different from that of metformin alone or probiotics alone. It is very likely that the integrity of the mucosa needed a different mechanism. Thus, through the balanced microbiota, the junctional complexes of the epithelial cells were maintained; the secretory part of the balanced microbiota could have provided anti-inflammatory elements and protected the mucosal barrier.

Moreover, gut barrier dysregulations and pathologies promote the production of proinflammatory cytokines (IL-6 and TNF-α), which in turn trigger subclinical inflammation and insulin resistance, shedding light again on the inflammation loop between CRC and diabetes [3]. In our study, there was an upregulation of IL-6 and TNF-α in CRC animals with or without diabetes induction in the CRC and DCRC groups. This upregulation was not restricted to colon tissue, as the levels of these cytokines were also upregulated in the serum of the animals, thus emphasizing the systemic inflammation occurring in diabetes and CRC. A strong inhibition of these cytokines was detected in the colon of cancerous animals when probiotics and metformin were combined. It is well established that inflammatory changes in the colonic mucosa are characteristic features of CRC that include infiltration of inflammatory cells and enhanced production of a panel of cytokines [49]. Following recruitment, neutrophils get activated and produce large amounts of proinflammatory mediators, mainly IL-1β and IL-6 [50]. These changes, coupled with elevated levels of RONS generated in the colon tissue, contribute to destructive mucosal damage, which will create a leaking mucosal barrier that could allow multiple bacteria, including toxic strains, to infiltrate and grow, leading to chronic inflammation, an optimal environment for colitis-associated CRC [51].

In the present work, excessive generation of free radicals was indicated by the increased levels of RONS following CRC and diabetes induction in Balb/c mice. These levels were restored to normal by the administration of the combination therapy of metformin and probiotics. The increased nitric oxide (NO) production correlates with the increase in the levels of proinflammatory mediators, such as TNF-α and IL-6, thus leading to exacerbation of the inflammatory chronic reaction at the core of the IBD-CRC etiology [52]. Treatment with metformin in combination with probiotics was able to reduce the release of inflammatory mediators alongside its antioxidant effects, and to reestablish the colonic structure and function of the intestinal wall.

4. Materials and Methods

4.1. Animals

In this study, a total of 96 six-week-old male Balb/c mice were grouped by body weights and were housed in medium sized polysulfone cages at a constant temperature (21 °C ± 2 °C) with an alternating 12 h light/dark cycle. Animal chow (Teklad-Envigo) and water were provided ad libitum. All animal experiments adhered strictly to institutional and international ethical guidelines of the care and use of laboratory animals, and personnel handling animals were qualified. The experimental protocol was approved by the Institutional Animal Care and Use Committee, American University of Beirut, Lebanon (IACUC#16-04-370).

4.2. Experimental Design

The animals were randomly divided into two main groups, diabetics and nondiabetics, and two other subgroups, CRC and non-CRC. The animals were then distributed according to the different treatment combinations to form a total of 16 subgroups: Nontreated, treated with metformin (M) alone, probiotics (P) alone, and a combination of the two treatments (MP), as illustrated in Table 2. Mice were individually labelled for tracking, and the average group weight was equilibrated to eliminate any significant weight difference between groups.

Table 2. Experimental Design. Balb/c Male Mice were Divided into two Main Groups, Nondiabetics and Diabetics, and Two Other Subgroups, CRC and non-CRC. They were Then Divided According to the Different Treatment Combinations to form a Total of 16 Subgroups.

Nondiabetic Animals	
With CRC Induction	**Without CRC Induction**
CRC (CRC)	Normal controls (NC)
CRC + metformin (CRC + M)	Metformin (M)
CRC + probiotics (CRC + P)	Probiotics (P)
CRC + metformin and probiotics (CRC + MP)	Metformin and probiotics (MP)
Diabetic Animals	
With CRC Induction	**Without CRC Induction**
Diabetic CRC (DCRC)	Diabetic (D)
Diabetic CRC + metformin (DCRC + M)	Diabetic + metformin (D + M)
Diabetic CRC + probiotics (DCRC + P)	Diabetic + probiotics (D + P)
Diabetic CRC + metformin and probiotics (DCRC + MP)	Diabetic + metformin and probiotics (D + MP)

4.3. Induction of CRC

Azoxymethane (AOM)/Dextran sulfate sodium (DSS)-induced colon cancer is a well-established model commonly used in experimental colitis-associated CRC studies. An optimized concentration of the proinflammatory agent DSS (Sigma-Aldrich, Thermo Fisher Scientific, Villebon-sur-Yvette, France) was prepared in autoclaved water and administered to animals in their drinking water. Each DSS cycle consisted of seven days of DSS followed by two weeks normal drinking water. Pilot studies were conducted in order to determine the optimal concentration and needed number of DSS cycles since the colitogenic effect of DSS is affected by several environmental, batches used, and strain-related factors [53]. The carcinogen, AOM (Sigma-Aldrich, Thermo Fisher Scientific, Villebon-sur-Yvette, France), was injected intraperitoneally at the Maximum Tolerated Dose (MTD) of 10 mg/kg body weight.

4.4. Induction of Diabetes

Streptozotocin (STZ) (Sigma-Aldrich, Fisher Thermo Fisher Scientific, Villebon-sur-Yvette, France) at a single dose of 150 mg/kg was used to induce diabetes mellitus. Immediately before administration, STZ was suspended in citrate buffer (pH 4.4–4.5) and injected intraperitoneally [54,55].

4.5. Probiotics and Metformin Administration

The probiotic (P) used is a mixture of seven strains of lactic-acid-producing bacteria: *Lactobacillus rhamnosus*, *Saccharomyces boulardii*, *Bifidobacterium breve*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, and *Lactobacillus reuteri*. It was administered with daily dose of 10^8 CFU per animal. Metformin (Glucophage) treatment was provided at a dosage of 150 mg/kg body weight. Continuous treatments were administered via drinking water since day 1 until the end of the experiment. Their consumption was measured on a daily basis and fresh solutions were administered twice a week.

4.6. Clinical Course Assessment

During the experimental period, body weight, stool consistency, and gross bleeding scores were recorded. A previously validated clinical disease activity index (DAI) ranging from 0 to 4 was calculated based on the following parameters: stool consistency (0, normal; 2, loose; 4, diarrhea), gross bleeding (0, absence; 2, blood stained; 4, presence) and weight loss (0, none; 1, 1–5%; 2, 5–10%; 3, 10–20%; 4, >20%) as per the formula: DAI = (Stool consistency + Fecal bleeding + Weight loss)/3 [56].

4.7. Blood Glucose Determination

Blood glucose levels (BGL) were measured in tail vein blood using an Accu-Chek® Performa blood glucose meter system. The range for the Accu-Chek is 10–500 mg/dL and any value >500 mg/dL registers as "HI" (Readings of "HI" were recorded as 500 mg/dL). Measurements were done prior to diabetes induction and weekly after STZ injection. Diabetes was diagnosed with BGL >250 mg/dL. All blood glucose measurements were taken in the fed state early in the morning to eliminate variability in blood glucose levels caused by feeding patterns of the mice [57].

4.8. Fecal Occult Blood Measurement

Collection of feces was done by placing a single mouse in an empty cage without bedding material for few min; feces were collected and occult blood was measured using Guaiac fecal occult blood test kit, as per the manufacturer instructions [58].

4.9. Blood and Serum Collection

On the day of sacrifice, bleeding was performed by cardiocentesis in accordance with approved institutional animal ethical protocols. The blood was collected in BD Microtainer tubes and centrifuged at 2500 rpm for 10 min. The separated serum was stored at −20 °C.

4.10. Dissection

At the indicated time point, animals were sacrificed; their colon was isolated, quickly flushed with cold phosphate-buffered saline (PBS) on ice to remove feces and blood. A portion of it was fixed in 10% buffered formalin and the other portion was stored in liquid nitrogen for further analyses.

4.11. Histological Studies

Formalin-fixed descending colons biopsies were paraffin embedded, cut into 5 µm sections on a glass slide, and stained with hematoxylin and eosin (H&E) for general morphology. These protocols were performed in accordance with the standard histology procedures developed by our team [59]. The different sections were photographed using an Olympus CX41 microscope. Histologic scoring was performed on H&E stained colon tissue on a scale adapted and modified from Hussein et al. where seven parameters were evaluated, as listed in Table 3. Each parameter had four scores based on the degree of structural change, accordingly the measures ranged from 0 (normal) to 21 (severe alterations) [60].

Table 3. Histological Changes. A Scale Adapted and Modified from Hussein et al. is Used to Calculate the Scores, Where Seven Listed Parameters were Evaluated. Each Parameter has Four Scores from 0 (normal) to 3 (altered) Based on the Degree of Structural Changes [60].

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

4.12. Cellular Proliferation by Immunohistochemistry Using Ki-67 Stain

Immunohistochemistry was performed on paraffin-embedded sections. For antigen retrieval, slides were immersed in citrate buffer (pH 6). After washing with TBST, the slides were incubated with a primary antibody (Ki-67, EnCor Biotechnology Inc, Gainesville, FL, USA, 1/1000) at 4 °C overnight. After washing the slides three times with TBST, the sections were incubated with Goat anti-Rabbit IgG Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (Thermo Fisher Scientific, Villebon-sur-Yvette, France, 1/1000) in TBST with 5% BSA for 2 h at room temperature. The nuclei were counterstained with DAPI and slides were photographed using Zeiss Axio microscope. Sections were evaluated focusing on longitudinally oriented crypts and the number of Ki-67-positive cells per HPF (40× objective) were counted [61,62].

4.13. Reactive Oxygen Species Measurement by Dihydroethidium (DHE)

DHE was performed on frozen tissues. Colon rings were demarcated with a solvent-resistant pen. DHE solution (Thermo Fisher Scientific, Villebon-sur-Yvette, France, 1/1000) was dispensed over the tissue. The slides were placed for 30 min at 37 °C and then the DHE residues were removed, slides counterstained with DAPI in a mounting medium, coverslipped, and stored at 4 °C (light sensitive) until microscopic evaluation and quantification using Zeiss Zen 2.3 software (Zeiss, Ulm, Germany).

4.14. Determination of Nitrite Levels

Serum nitrite concentrations were measured using the classic colorimetric Griess reaction. One hundred microliter serum samples were pipetted into 96 well microtiter plates, 100 µL Griess reagent (equal volumes of 1% sulphanilamide and 0.1% and naphthylethylenediamine dihydrochloride) was added. After incubation in dark for 10 min at room temperature, absorbance was measured at 550 nm. Nitrite concentration (µM) was calculated from a sodium nitrite standard curve freshly prepared in distilled water [63,64].

4.15. Assessment of Cytokine Levels

The levels of IL-6 and TNF-α were measured in both plasma and colon extraction using ELISA assay performed according to the manufacturer's instructions (Thermo Fisher Scientific, Villebon-sur-Yvette, France). The optical density was measured at a wavelength of 450 nm using a microtiter plate reader (Multiskan Ascent 96/384 plate reader, Thermo Fisher Scientific, Villebon-sur-Yvette, France). The final results were expressed as pg/mL and the limit of detection of IL-6 and TNF-α were 4–500 pg/mL and 8–1000 pg/mL, respectively.

4.16. Statistical Analysis

Statistics were performed using GraphPad Prism 8.0.1, San Diego, CA, USA and data were expressed as a mean ± SEM. Significant differences were evaluated using the one-way ANOVA followed by Tukey–Kramer multiple comparisons test. A value of $p < 0.05$ was considered significant [65].

5. Conclusions

Data in this study lead to multiple conclusions that can assist in the development of targeted therapies in the presence of metformin and probiotics. Data suggest that selective cytokine inhibition, as well as ROS and NO inhibition might be an important strategy for the prevention of CRC. Metformin combined with probiotics prevented AOM/DSS-induced damage through attenuating the inflammation pathway in colorectal mucosal cells and reducing tumor cell proliferation, thus leading to the inhibition of colitis-associated CRC. The mechanism supporting these inhibitory effects of metformin might relate to its interaction with the balanced microbiota in the presence of probiotics (Figure 12). However, very little is currently known about the bacterial targets of metformin and it is possible that the microbiota

could regulate some of its effects on host physiology via unknown mechanisms. This undoubtedly warrants further investigation.

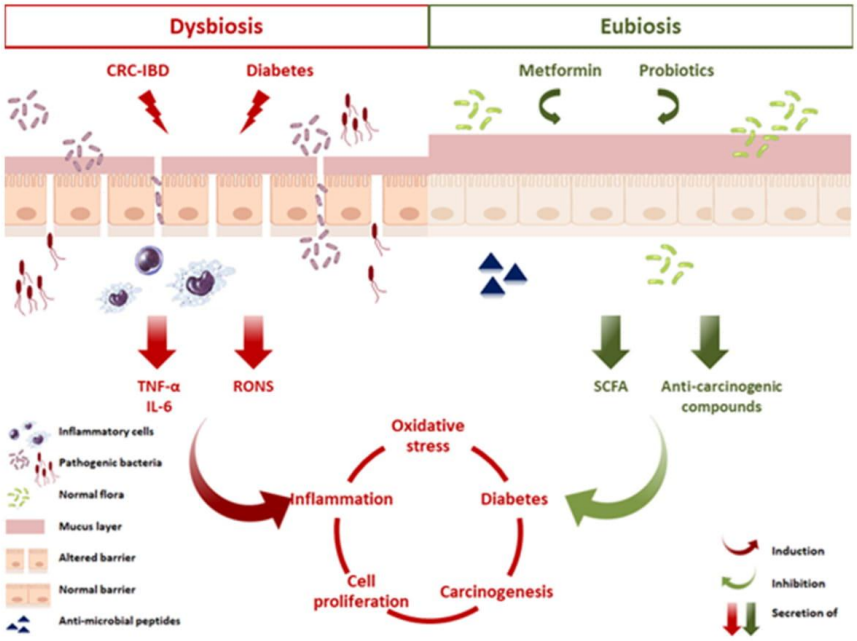


Figure 12. Proposed mechanisms of action elicited by metformin and probiotics in CRC and diabetes. An altered microbiota in a state of dysbiosis impairs the functions of the gut barrier by affecting the tight junctions and the mucus layer, thus facilitating the translocation of pathogens and toxins into the lamina propria. This invasion leads to recruitment of inflammatory cells, their activation, and the secretion of proinflammatory cytokines, including IL-6 and TNF-α. In parallel, an increase in RONS production induces a state of chronic inflammation, DNA damage, and increased cell proliferation, known as key players in CRC progression. Moreover, this state of chronic inflammation promotes insulin resistance and disurbance in glucose homeostasis, thus exacerbating diabetes and enhancing CRC. Probiotics and metformin administration, however, inhibited CRC progression, reduced inflammation, and ameliorated diabetes. These benefical effects are potentially linked to a restoration of the gut barrier, production of SCFA, antimicrobial peptides, regulation of hepatic glucose production, and modulating the balance between proliferation and apoptosis. The result of such a balanced miroenvironment is to preserve a dynamic intestinal barrier that controls and maintains homeostasis.

Author Contributions: S.A.K. performed, analyzed the experiments and wrote the paper; A.J. and B.L. conceptualized the work; A.P. provided technical assistance; D.Y.L., A.P., M.D.-A., R.J., A.J. and B.L. reviewed and edited the paper; C.B. and R.J. supervised the work; R.J. and B.L. acquired the funding. All authors have read and agreed to the published version of the manuscript.

Funding: The expenses of this work were defrayed in part by the Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation through the European Fund of Regional Development FEDER 2014–2020, by the Conseil Régional Nouvelle Aquitaine, and by the Ligue contre le Cancer; and by the American University of Beirut, Seed grant.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ANOVA	analysis of variance;
AOM	azoxymethane;
CFU	colony-forming unit;
CRC	colorectal cancer;
DAPI	4′6-diamidino-2-phenylindole;
DHE	Dihydroethidium;
DSS	dextran sulfate sodium;
H&E	hematoxylin and eosin;
HPF	high power field;
IBD	inflammatory bowel disease;
IL-6	interleukin-6;
M	metformin;
MP	metformin and probiotics;
NO	nitric oxide;
P	probiotics;
PBS	phosphate-buffered saline;
RONS	reactive oxygen and nitrogen species;
ROS	reactive oxygen species;
SEM	standard error of the mean;
STZ	Streptozotocin;
TBST	tris-buffered saline and tween;
TNF-α	tumor necrosis factor-alpha.

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