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DEPARTMENT OF PHYSIOLOGY

# THE INVESTIGATION OF HIGH-CARBOHYDRATES DIET EXPOSURE AND EFFECTS IN COLON IN SPRAGUE DAWLEY RATS DURING MATERNAL AND MATURATION PERIODS.

MASTER THESIS

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APPROVAL
This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated 01.09.2021 and numbered 2021/09-22

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## DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

20.08.2021

Shyma Osama F. Musa

## DEDICATION

This thesis work is dedicated to my parents, Osama and Asma, who have always supported me and loved me unconditionally. This work is also dedicated to my friend Lama and my sister Rana, who have been a constant support and encouragement during the challenges of graduate school and life.

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| AhR | Aryl Hydrocarbon Receptor |
| ARNT | Aryl Hydrocarbon Receptor Nuclear Translocator |
| ER | Endoplasmic Reticulum |
| ETC | Electron Transport Chain. |
| Foxo1 | Forkhead Box Protein O1 |
| G+L | Gestation-Lactation |
| GLUT | Facilitative Glucose Transporter |
| GI | Glycemic Index |
| (GL) | Glycemic Load |
| HAT | Histone Acetyltransferase |
| HFCS | High-Fructose Corn Syrup |
| ICA | Ileocolic Artery |
| IL-6 | Interleukin 6 |
| IMA | Inferior Mesenteric Artery |
| IR | Insulin Resistance |
| K/HDAC | Lysine/Histone Deacetylase |
| KHK | Cetohexazines |
| LCA | Left Colic Artery |
| MAPK | Mitogen-Activated Protein Kinase |
| MCA | Middle Colic Artery |
| MCP-1 | Monocyte Chemoattractant Protein-1 |
| PLC | Phospholipase C |
| Rab11a | Ras-Related Protein-In-Brain 11a |
| RCA | Right Colic Artery |
| ROS | Reactive Oxygen Species |
| SGLT1 | Na+-Dependent Glucose Transporter 1 |
| SMA | Superior Mesenteric Artery |
| TF | Transcription Factor |
| TLR4 | Toll-Like Receptor 4 |
| TAS | Total Oxidant Stress |
| TOS | Total Oxidant Stress |
| XRE | Xenobiotic Response Element |

## ABSTRACT

**MUSA, S.O.F (2021). The Investigation of High-Carbohydrate Diet Exposure and Effects in Colon in Sprague Dawley Rats During Maternal and Maturation Periods. Yeditepe University, Institute of Health Sciences, Department of Physiology, MSc Thesis, Istanbul.**

Nutrition has an important role in shaping the gut microbiome thus the health of the colon. The effects of carbohydrates on the human health have been widely investigated but the exposure of high carbohydrates on colon tissue during pregnancy has not been fully illustrated. The aim of the present study to investigate high carbohydrate intake exposure in colon tissue during maternal and maturation period.

Rats were fed with high carbohydrate diet during the first stage of the study (gestation and lactation) and in the second stage of study (maturation). The experiment was terminated by removing the proximal colon tissue and feces of the rats under anesthesia after 21 days G+21 days L and 120 days M. The effect of diet during diet on proximal colon morphology was measured by hematoxylin-eosin (HE). H2AX and RAD51 DNA repair biomarker proteins were determined by immunofluorescence (IF). The production of reactive oxygen species and the causes of colon distress, total oxidant status (TOS) and total antioxidant status (TAS) in proximal colon tissue was determined by Rel Assay.

High exposure of carbohydrate diet cause mild disturbance in colon crypt, a tendency increase in TOS for the maternal HCD-SFD group while it shows a tendency decrease in TAS for the maternal and maturation HCD-HCD group. OSI presented only a tendency increase in the HCD-HCD group. High maturation diet (SFD-HCD) and high carbohydrate diet (HCD-HCD) shows a tendency increase of RAD51 expression levels.

In conclusion, high carbohydrate diet may have a possible effect in disturbing the colon tissue during maternal and maturation period.



**Keywords:** Maternal/Maturation periods, RAD51, H2AX, Proximal colon, Reactive oxygen species.

## ABSTRACT (Turkish)

**Sprague Dawley Sıçanlarda Maternal ve Matürasyon Dönemlerinde Yüksek Karbonhidrat Diyetine Maruz Kalmanın Kolondaki Etkilerinin Araştırılması. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Fizyoloji Anabilim Dalı, Yüksek Lisans tezi, İstanbul.**

Hamilelik sırasında, annenin hemen hemen her organ sisteminin fizyolojisi, yavrularının oluşumu ve gelişimini destekleyen değişiklikler içerir. Bağırsak mikrobiyomunun, dolayısıyla kolonun sağlığının şekillenmesinde beslenmenin önemli bir rolü vardır. Karbonhidratların insan sağlığı üzerindeki etkileri geniş çapta araştırılmış, ancak hamilelik sırasında kolon dokusunun yüksek karbonhidratlara maruz kalmasının etkileri tam olarak gösterilmemiştir. Bu çalışmada, maternal ve maturasyon dönemlerinde, yüksek karbonhidrat alımı sonucu kolonda meydana gelen değişiklikleri araştırdık. Bu amaçla çalışmanın ilk aşamasında (maternal dönem/gebelik ve laktasyon) ve çalışmanın ikinci aşamasında (matürasyon) sıçanlar yüksek karbonhidratlı diyetle beslendi. 21 gün G+21 gün L ve 120 gün M sonrasında anestezi altında sıçanların proksimal kolon dokusu ve dışkısı alınarak deney sonlandırıldı. G+L ve M dönemlerindeki diyetlerin proksimal kolon üzerine etkilerini belirlemek amacıyla morfoloji, hematoksilen-eozin (HE) boyama tekniği uygulandı. Ayrıca proksimal kolon dokusu, H2AX ve RAD51 sırasıyla DNA hasarı ve onarım biyobelirteç proteinleri immünofloresan (IF) tekniği ile belirlendi. Böylece, farklı gelişim dönemlerinde kolon epitelinde yüksek karbonhidratlı diyet içeriğinin neden olabileceği DNA hasarı ve onarım koşullarındaki değişiklikler tespit edildi. Proksimal kolon dokusunda reaktif oksijen türlerinin üretimi ve toplam oksidan durum (TOS) ve toplam antioksidan durum (TAS) seviyeleri belirlendi. Sonuçlarımız, karbonhidrat diyetine maruz kalmanın proksimal kolon kriptinde hafif bir bozulmaya, TOS, OSI ve RAD51 ekspresyonunda artış eğilimine neden olurken, TAS'da ise bir azalma eğilimi gözlendiğini ortaya koymaktadır. Sonuç olarak, yüksek karbonhidratlı bir diyet, maternal ve maturasyon dönemlerinde kolon dokusunun bütünlüğünü bozucu olbir etkiye sahip olabilir.



**Anahtar kelimeler:** Maternal/Maturasyon dönemleri, RAD51, H2AX, Proksimal kolon, Reaktif oksijen türleri.

## 1. INTRODUCTION and PURPOSE

The human colon has a diverse combination of bile, desquamated epithelial cells, mucus, numerous microbes and their fermentation products, undigested or unabsorbed food, and its metabolic products such as salts, metals, mutagens, toxins, dissolved gases and carcinogens. Intestinal mucosa is in constant challenge with diet- and bacterial-derived oxidants and carcinogens. Rapid growth of free radicals, DNA damage and redox imbalance could be a consequence of chronic exposure of such challenging conditions [1].

The gut microbiome plays a vital role in human health and influences chronic diseases ranging from metabolic disease to gastrointestinal disorders and colorectal cancer [2].

In previous studies, nutritive patterns plus the environmental factors were found vastly effective when it comes to the gut microbiome, thus the colon's health. Despite the dearth of research that administered the effect of a high intake of carbohydrates on the gut microbiota or the colon, some researchers illustrated that higher intakes of total carbohydrates were most strongly associated with decreased microbiome diversity. It was found that high consumption of total carbohydrates was most strongly associated with decreased microbiome diversity.

Moreover, the transmission of bacteria from mother to her offspring is vital for developing a healthy embryonic microbiome. Therefore, emerging studies commenced exploring the importance of maternal diet during pregnancy and its impact on the premature neonatal microbiome. That is because of the transmission of microbiota from mother to progeny before delivery. Therefore, the importance of nutrition in the pregnancy period came to light for developing the neonatal microbiome.

On the other hand, fat and refined sugar-rich diet is associated with increased oxidative stress characterized by reactive oxygen species (ROS). It is believed that increased production of ROS upsurges the inflammatory reaction and subsequently causes tissue damage.

During the past 2 decades, renewed interest in nutritional carcinogenesis has developed. Although the diet and nutrition effects on cancer dilemma is not up to the minute, in recent times their relationship has remarkably caught little detailed attention.

Literature shows that carbohydrates play an essential role in supporting the growth of fetus during pregnancy by providing energy through placenta. We have hypothesized that excessive intake of carbohydrates may disturb the colon tissue and cause in distress

that may lead to DNA damage. For this purpose, we have investigated each of Gamma-H2AX and RAD51 DNA damage and repair biomarker proteins during gestation-lactation (G+L) and maturation (M) periods. Besides, Total Oxidant Stress (TOS) and Total Oxidant Stress (TAS) effect on the mother's proximal colon and the fetus' gut microbiota.

# 2. LITERATURE REVIEW

## 2.1. Colon Overview

### 2.1.1. Colon Anatomy:

The human colon serves to absorb water and electrolytes, store intraluminal contents until elimination is socially convenient, and salvage nutrients after bacterial metabolism of carbohydrates that have not been absorbed in the small intestine. The colorectum consist of three segments: the proximal colon (cecum, ascending colon, and transverse colon), the distal colon (descending colon, sigmoid colon) and the rectum. From the embryological and anatomical perspective, the proximal and distal colons are located in the peritoneal cavity, while the rectum rests within the pelvis.

Moreover, the proximal colon develops from the midgut embryologically, while the distal colon and rectum arise from the hindgut[3]. Anatomically, Branches of the superior mesenteric artery via the ileocolic and right colic arteries supply the proximal colon, whereas tributaries of the inferior mesenteric artery supply the distal colon and rectum[4] as shown at Figure 2.1.

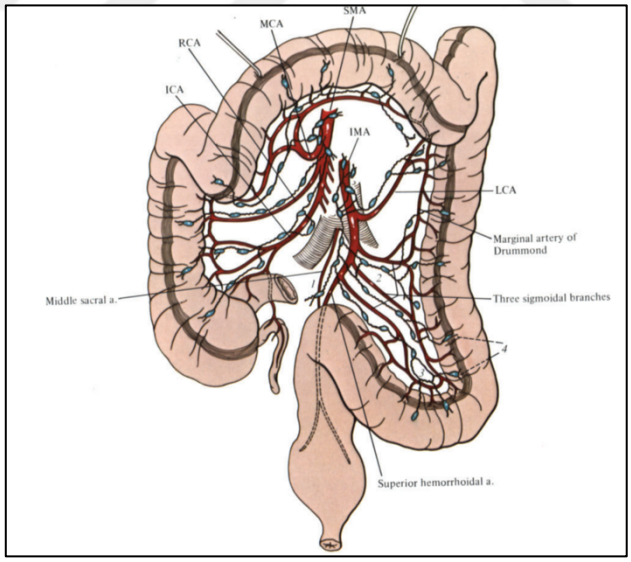



**Figure 2.1.** Arterial supply and lymph nodes of the colon[4]. (ICA, ileocolic artery; RCA, right colic artery; MCA, middle colic artery; SMA, superior mesenteric artery; IMA, inferior mesenteric artery; LCA, left colic artery; 1, principal nodes; 2, intermediate nodes; 3, paracolic nodes; 4, epibolic node).

### 2.1.2. Colon Nutrition:

In the proximal colon, bacteria ferment organic carbohydrates to SCFAs, predominantly acetate, propionate, and butyrate [5]. There is a low, normal rate of SCFA production from malabsorbed (up to 10% of ingested) carbohydrates; diets high in fiber, beans, resistant starches, and complex carbohydrates increase the production of SCFA. SCFA are rapidly absorbed from the colon, augment sodium, chloride, and water absorption and constitute the preferred metabolic fuel for colonocytes. SCFA may also serve to regulate proliferation, differentiation, gene expression, immune function, and wound healing in the colon [6].

In healthy individuals, the small intestine absorbs more than 90% of a regular diet and distributes nutrients to sustain overall body health. Most of the residues that reach the colon are complex carbohydrates (fiber), but they also comprise protein residues and primary bile acids released by the liver in response to fat ingestion. Therefore, these residues play an important role in colonic health maintenance because they determine the composition and metabolic activity of the colonic microbiota, which maintains mucosal and colonic health through fermentation [7] as shown in figure 2.2.

In addition, Saccharolytic fermentation of carbohydrates is dominant in a balanced diet, creating short-chain fatty acids, mainly butyrate, which is the preferred energy source for colonocytes and possesses anti-inflammatory and antineoplastic characteristics via the actions described in (Figure 2.2). Also, protein fermentation and bile acid deconjugated residues have proinflammatory and preneoplastic effects in an imbalanced high-fat, high-meat, low-fiber diet, increasing the risk of colon cancer.

However, Experiments have shown, that microbial fermentation products (such as butyrate) and microbial activated phytochemicals (such as polyphenols) have a wide range of antineoplastic effects that counteract tumorigenic signaling pathways and mount epigenetic mechanisms such as histone deacetylase inhibition that promote apoptosis, suppress proliferation, and arrest neoplastic transformation [8-14].

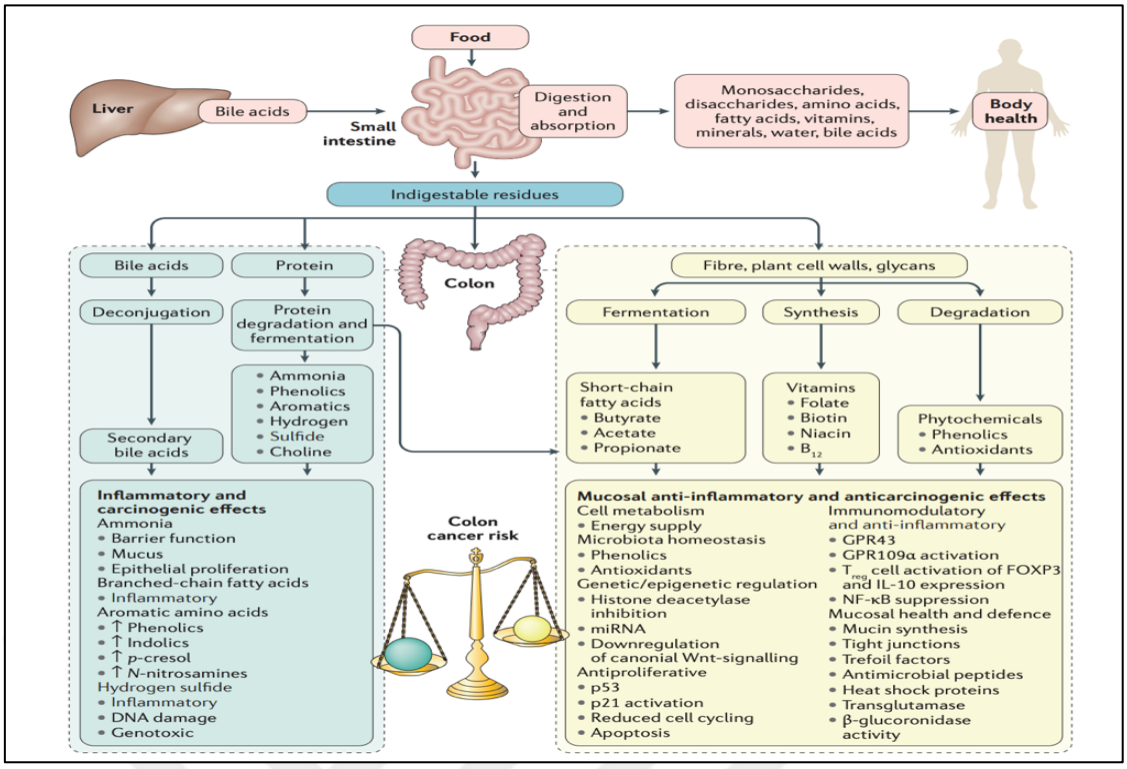



**Figure 2.2.** The role of food residues and the colonic microbiota on colon cancer risk[7].

## 2.2. Carbohydrates

### 2.2.1. Carbohydrate Introduction:

Carbohydrates are one of the most common types of organic compounds found in nature. Sugar and sugar products, as well as glucose and starch products, form the foundation of many significant businesses or parts of industries[15].Furthermore, almost in all living cells, the carbohydrate supports the high energy necessities of the brain, muscular system, mechanical work, and chemical reaction. Moreover, adenosine triphosphate and associated substances which are carbohydrate derivatives are considered a key substance in energy storage and transfer. In addition to the phosphate esters of the sugar and their significance in such transformations[15].

Digestion and microbial activity are the characterization of the nutritional quality of carbohydrates. Man-kind have thrived on diverse amounts and forms of carbohydrates. Specialists recommend a carbohydrates consumption of 150 g per day to support muscle and brain physiology in adults thus no minimal requirement has been established yet [16,17]. However, it is thought that the increased consumption and reliance on low-quality carbohydrates has caused an increase in chronic diseases incidence[17]. The gut bacteria

ferment the unabsorbed carbohydrates into lactic acid, SCFAs (acetate, propionate, and butyrate), and hydrogen, succinate, methane, and carbon dioxide gases [18].

Nutritive carbohydrates are majorly classified as digestible and nondigestible. Energy is obtained via the degradation of digestible carbohydrates by digestive enzymes[19].Nondigestible carbohydrates are classified as fermentable or nonfermentable fibers. Fermentable fibers such as inulins, β-glucans, β-fructans, pectins, oligosaccharides, and some resistant starches are fermented by the intestinal microbiota producing Short-chain fatty acids (SCFAs) along with several beneficial substances [20,21]. Furthermore, nondigestible and digestible carbohydrates have different glycemic indexes (GI) and glycemic loads (GL)[22].

### 2.2.2. Metabolic Disorders Linked to Carbohydrates:

High carbohydrates intake is linked to metabolic syndrome rate upsurge since the modern industries made concentrated carbs consumption easy [23,24]. Starches are known as the storage carbs of plants and made up by linked sugars. Starches, particularly in agricultural areas, obtained from grains and tubers are the main energy source while Mono- and disaccharides are carbohydrates, obtained naturally from fruit, composed of one and two sugar molecules, respectively. Generally, previous studies reported that food rich in starch is associated with incidence of Crohn's disease and ulcerative colitis [25]. In addition, recent studies illustrated a direct relationship between excessive exposure of carbs obtained from starchy food and metabolic syndrome in addition to hyperlipidemia[26].

Sugars include monosaccharides (glucose, galactose, and fructose) and disaccharides (lactose, sucrose, maltose and trehalose) consumption are linked with metabolic disorder, pancreatic cancer as well as an increased risk of Crohn's disease, and ulcerative colitis. Moreover, fructose, is considered as one of dietary catalysts of metabolic disorder via gut dysbiosis stimulation [27]. Changes in levels of dietary fructose profoundly affect the gut microbiota, resulting in the acquisition of a microbiome with altered metabolic capacity [28]. This implies that changes in intestinal fructose absorption and luminal concentrations affect the microbiome.

Artificial sweeteners were advertised as natural sugar healthy replacement. However, their consumption was linked with increased risk of obesity, metabolic

syndrome, hyper-tension, cardiovascular events, while contrariwise they are associated with the risk of breast and ovarian cancers[29].

Even though carbohydrates are essential for mankind by providing energy, excessive carbohydrate intake affect metabolic disorder, the Gut microbiota, and after long investigation, the colon. In table 2.1 below illustrate how carbohydrates are associated with metabolic disorders.

**Table 2.1.** Metabolic disorders associated with carbohydrates[29].

| Types of Carbohydrates | Details | Effects on Human Health |
|---|---|---|
| Starches | Carbohydrates from starchy foods | Risk of metabolic disorders and hyperlipidemia ↑ |
| | Carbohydrates from starchy foods | Visceral fat and serum TG level ↑ |
| | Carbohydrates from starchy foods | HDL-C level ↓ |
| | Total carbohydrate, starch and refined sugar | Risk of Crohn's disease and ulcerative colitis ↑ |
| | Refined grains and tubers | Risk of glycemia and insulin resistance ↑ |
| Mono- and disaccharides | Sugar-sweetened beverages | Risk of type 2 diabetes ↑ |
| | Disaccharides<br>Fructose | Risk of cardiovascular disease ↑ |
| | Fructose | Lipogenesis, dyslipidemia, and visceral adiposity ↑ |
| | Fructose | Insulin resistance ↑ |
| | Sugar | Risk of Crohn's disease ↑ |
| | Sugar<br>Total sugars, sucrose | Risk of ulcerative colitis ↑ |
| | Total sugars, sucrose, fructose | Risk of pancreatic cancer ↑ |
| Artificial sweeteners | Saccharin and acesulfame potassium | Adipogenesis ↑ and Lipolysis ↓ |
| | Saccharin, sucralose, and aspartame | Insulin resistance ↑<br>Risk of dysbiosis and metabolic abnormalities ↑ |
| | Diet soda | Risk of type 2 diabetes and cardiovascular disease ↑ |
| | Artificially sweetened beverages | Risk of hypertension ↑ |
| | Saccharin/cyclamate aspartame/acesulfame-K | Risk of urinary tract tumor and laryngeal cancer ↑ |
| | Saccharin/cyclamate aspartame/acesulfame-K | Risk of breast and ovarian cancer ↓ |
| | Artificially sweetened beverages | Risk of stroke and dementia ↑ |
| Nondigestible carbohydrates | Additional 10 g of dietary fibers/day | Risk of cardiovascular disease ↓ |
| | Cereal fibers<br>Total dietary fibers and cereal fibers | Risk of type 2 diabetes ↓ |
| | Dietary fibers | Risk of colorectal cancer ↓ |
| | Dietary fibers (Particularly fruits)<br>Wheat bran cereal | Risk of Crohn's disease ↓ |
| | Cereal fibers | Risk of gastric cancer ↓ |

### 2.2.3. Carbohydrate Digestion:

Sugars and starches account for the vast majority of carbohydrates consumed. Sugars are composed of monosaccharides (such as glucose, galactose, and fructose) and disaccharides (e.g. Lactose, sucrose, maltose and trehalose). As previously illustrated, starches are plant storage carbohydrates made up primarily of sugars linked together.

Lactase and sucrase hydrolyze lactose and sucrose to monosaccharides, respectively. Moreover, Salivary, and pancreatic amylase are in control of the initial breakdown of starches, which is then followed by further digestion by mucosal enzymes (such as amylase, sucrase, and isomaltose) responsible for the final digestion to glucose.

### 2.2.4. Carbohydrate Absorption Mechanism:

The SGLT1 transporter is responsible for glucose and galactose transport in the intestine and is expressed on the brush border membrane of the enterocytes in the upper third of the small intestinal villi [30] as shown in figure 2.3.

Additionally, it is now thought to oversee Na+-dependent sugar transport, which drives the upward transport of glucose and galactose from the lumen of the gut into the enterocyte[31].

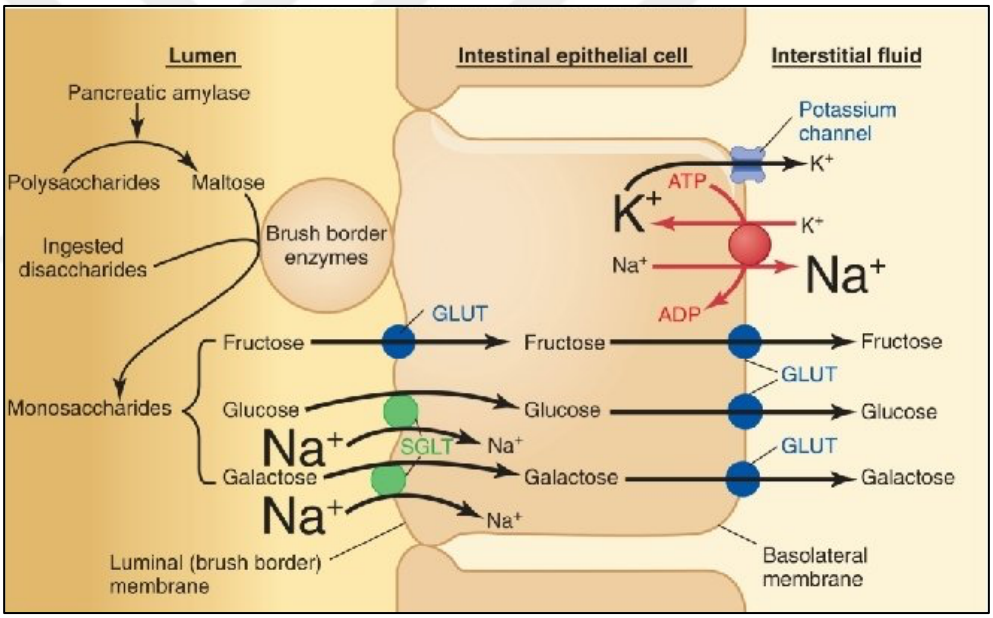



**Figure 2.3.** Carbohydrate's digestion and absorption[30].

Furthermore, the fundamental driving force is the Na+ electrochemical gradient across the brush border membrane. On the other hand, the basolateral Na+/K+ ATPase pump maintains the Na+ gradient. Each sugar molecule delivered into the cells is thought to be accompanied by two Na+ ions[32].

Moreover, SGLT1 transports Na+ and glucose in a 2:1 ratio against a glucose gradient via a Na+ gradient. Each sugar molecule is co-transported with Na+ throughout the cell in each cycle, which is accompanied by 260 water molecules[32].

In contrast, Fructose is transported through the brush border membrane by GLUT5 – another member of the GLUT family of transporters27 and will be discussed in more detail later.

## 2.3. Maternal and Maturation Nutrition:

### 2.3.1. Nutrition in Pregnancy:

Environmental changes (nutritional or non-nutritional) experienced during critical periods of an organism's life (for example, during early development) are widely acknowledged to cause long-term changes in metabolism and physiology, which is referred to as developmental programming, or metabolic programming when modifying metabolism[33]. Hence, such adaptations frequently manifest as persistent changes in metabolic signaling pathways or gene expression patterns, and they may persist later in life even if the environmental stimulus that triggered them is no longer present[34].

The Developmental Origins of Health and Disease (DOHaD) theory suggests that adverse conditions in utero can influence developmental pathways in early life, resulting in long-term changes in disease susceptibility in offspring, emphasizes the importance of nutrition in pregnancy to neonatal health[35].For that, the biological significance of maternal nutrition has long been recognized. Not only is it the only way for the fetus to get the nutrients it needs, but it also affects the maternal metabolic adjustment capacity to the placental hormones that affect the metabolism of all nutrients[36].

The impact of mother diet on the offspring gut microbiome and associated health outcomes is anticipated to extend beyond pregnancy and into the postnatal period via breastfeeding. Indeed, many research, including several of the ones cited above, cannot or do not separate dietary affects during gestation and lactaion periods; consequently, when interpreting these findings, the contribution of the postnatal period to long-term health outcomes in offspring should be considered[33].

Exogenous factors that influence the early colonization and succession of bacteria in the gut may cause gut maturation and development to be delayed. Asthma, allergies, diabetes, inflammatory bowel disease, and obesity have all been linked to disruptions in microbial networks during this critical window of development [37,38]. However, the impacts of early-life programming may lead to an increased susceptibility to disease later in life[39], as it shows in (Figure.2.4).

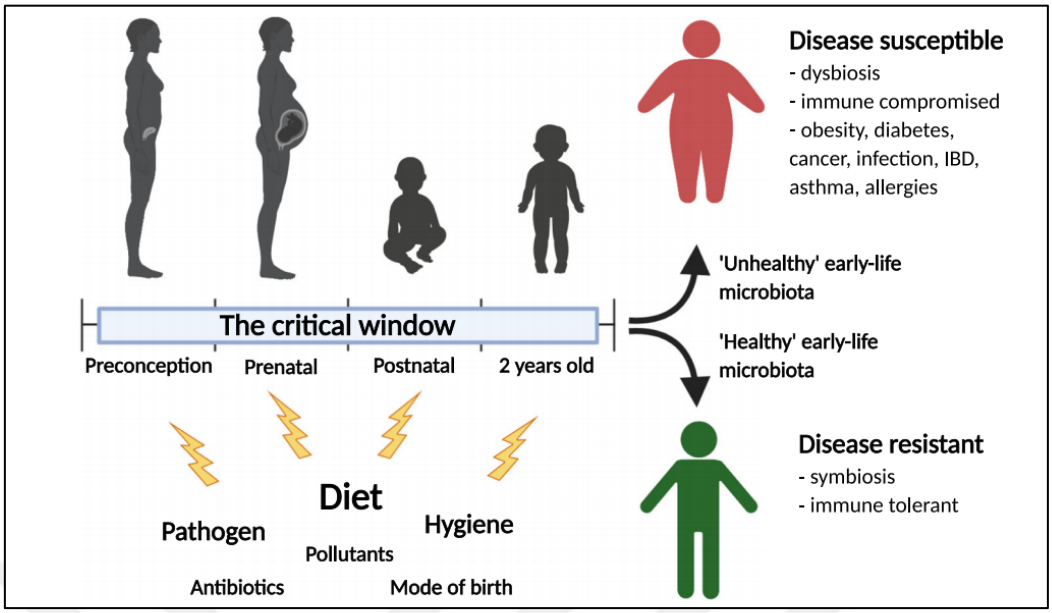



**Figure 2.4.** Exogenous elements that impact the microbiome[39]. Exogenous elements that impact the microbiome, such as antibiotics, food, hygiene, infections, mode of birth, and pollutants, might alter immunological and physiological programming throughout the perinatal period[39].

### 2.3.2. Microbial Dysbiosis:

Dysbiosis is an imbalance between the numerous organisms that live in the human gut. Furthermore, it can be caused by inherited factors, antibiotics drugs, chemotherapy, radiotherapy, and age, but it is usually caused by inadequate nutrition and physical and psychological stress, which results in immune system dysregulation [40-42].

All of these predispose to the onset of conditions as type 2 diabetes, liver disease and especially inflammatory bowel disease (IBD) and colorectal cancer (CRC)[43].

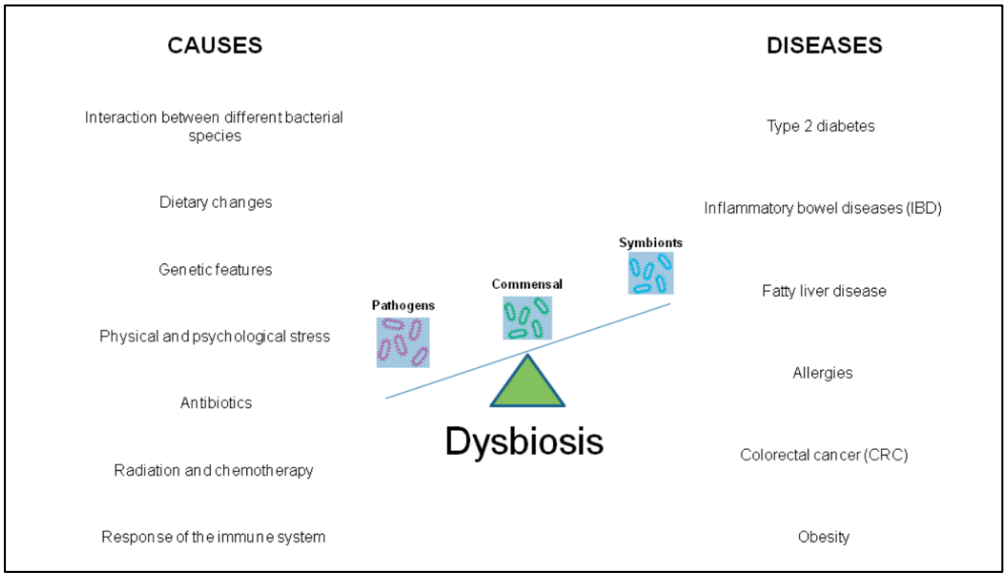



**Figure 2.5.** Causes of dysbiosis and related diseases[43].

In the study of malnutrition and the microbiome, like in many other cases, there is an ongoing conflict between cause and effect in terms of microbial dysbiosis and disease outcome. Although it is unknown what role the microbiota plays in disease, it is reasonable to believe that both gut microorganisms and the environment play a role in the etiology of malnutrition and undernutrition[39]. Diet plays a significant role in the pathophysiology of malnutrition and undernutrition. Nutrient quality and quantity can influence host immunity both directly and indirectly through changes in gut microbial populations (Figure 2.6).

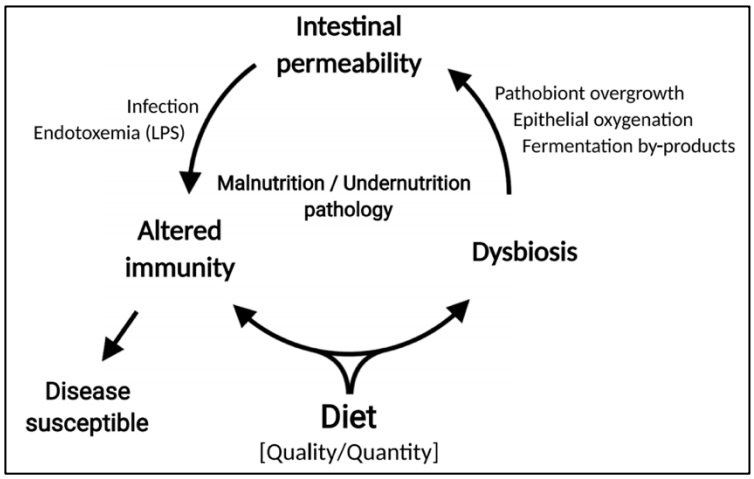



**Figure 2.6.** Food fermentation byproducts, pathobiont overgrowth, and epithelial oxygenation, dysbiosis contributes to the malnutrition cycle [39.] Through food fermentation byproducts, pathobiont overgrowth, and epithelial oxygenation, dysbiosis contributes to the malnutrition cycle. These circumstances change intestinal permeability, increasing pathogen susceptibility and endotoxemia, which impairs host immunity and makes disease more likely [39].

### 2.3.3. Microbial Transition from Mother to Fetus:

Microbial transmission of bacteria to offspring is essential for the formation and development of a healthy embryonic microbiome which is widely acknowledged by its effect on the infant growth[44-46]. Moreover, a rising evidence has pointed out that microbiol transmission from mother to her offspring may occur before delivery, which shed light to the significance of the pregnancy period to the maturity of the neonatal microbiome.

The human body is colonized with vast number microbial population, known as human microbiome. As we know that the fetal gut is sterile and during pregnancy, bacterial colonization and microbiota acquisition occur during birth[43]. The neonatal intestine is colonized by the maternal, vaginal flora, or anal, which contains bacteria such as Bacteroidetes, Bifidobacterium, Prevotella, and Lactobacillus spp. During the birth canal transit [47,48].

A prospective rat study attempted to determine the impact of prenatal and lactation dietary difficulties on the offspring's eating behavior, obesity, circulating glucose and insulin levels, triglyceride, and cholesterol concentrations from birth through adolescence[49]. Junk-food diet that is rich in fat, sugar and salt through maternal feeding causes increased adiposity, elevated circulating glucose, triglyceride, insulin, and cholesterol by the end of rat offspring's adolescence when compared to offspring of mothers who fed a balanced chow diet during pregnancy and lactation[48]. For that, the

junk-food diet is similar to the standard Western diet in terms of fat content and GI. Moreover, these studies render carbohydrate quality as an important component of the maternal diet.

### 2.3.4. Glycemic Response in Maternal CH Intake:

Contradictory evidence emerged when the effect of total carbohydrates intake was examined on insulin sensitivity. Even though maternal glycemia and insulinemia is determined in terms of offspring development and growth, the direct effect of carbohydrates on the metabolic profile is until now not fully illustrated[50].

Furthermore, up to date dietary intervention obtained that among obese individuals and after six months on low-carbohydrates diet and high fat diet exposure, insulin sensitivity developed[51]. However, insulin action may differentially be stimulated by the source and the quality of dietary carbohydrates, and thereby influence the degree of insulin resistance (IR). The GI is a glycemic index that has been used to qualitatively classify dietary carbohydrates by measuring the glycemic response to various carbohydrate-containing foods[52], hence each type of carbohydrate's glycemic and insulinemic effect is revealed. Furthermore, the GI is defined as the area under the glucose response curve to a carbohydrate-containing item when compared to either a certain glucose dose or a specific amount of white bread [53,54]. Noting that the lower the glucose and insulin response, the larger the particle size while a higher glucose response of a specific carbohydrate displays with a greater level of processing and refining.

### 2.3.5. Glycemic Index and Glycemic Load During Pregnancy:

High carbohydrate diets influence on the host metabolism is dependent on the glycemic index (GI) and glycemic load (GL). In addition, high carbohydrate diets rich with processed grains with high GI and GL exacerbate dyslipidemia[55]. Moreover, the higher the GI and GL in diets, the risk of metabolic disorder escalates such as type 2 diabetes and cardiovascular disease[56]. Furthermore, diets that high in glycemic load decrease leptin levels and upsurge energy storage plus to the inflammatory markers level such as C-reactive protein [26,57,58].

According to a Canadian study, dietary GI was linked to an increased risk of prostate cancer, while greater dietary GL was linked to an increased risk of colorectal and pancreatic cancer[59]. Furthermore, a high intake of polished white rice appears to have no

negative metabolic consequences among lean and physically active persons such as farmers, despite being a substantial risk factor for diabetes in urbanized Asian populations. In an animal investigation, these findings showed that a carbohydrate-rich diet alters the gut microbiota, which is closely connected with obesity and insulin resistance[60]. Moreover, and according to recent research, insulin resistance can exacerbate the negative metabolic effects of high carbohydrate diets[56].

Nutrition, on the other hand, plays a role in regulating GI and GL response which consequently during pregnancy interfere in the evolution of gestational diabetes along with the risk of clinical outcomes on the mother and her offspring. Therefore, nutrition and some of its compound, such as carbohydrates possibly will be a tool for prevention and management of gestational diabetes[61].

Usually, during pregnancy, the body goes through several metabolic and immunological changes that are meant to keep both the mother and the fetus healthy [62]. One of the most significant metabolic alterations is related to glucose metabolism [61]. Table 2.2 shows the difference between the low-glycemic sources and the high- glycemic sources.

**Table 2.2.** Typical low- and high glycemic-carbohydrates food [61].

| Low-glycaemic sources | High-glycaemic sources |
|---|---|
| Whole grains and unprocessed rice | Processed grains (flour, bread, cereals) |
| Beans and other non-tuberous vegetables | Tuberous vegetables (potato, carrot, parsnip) |
| Pasta (unless overcooked) | Typical desserts (baked goods, confectionery) |
| Most fruits and unsweetened juices | Soft drinks and sweetened juices |
| Unsweetened chocolate | Other snack foods |
| Nuts | Maize |
| Dairy products | Ripe bananas and some tropical fruit |

## 2.4. Fructose:

### 2.4.1. Fructose Metabolism:

Expansion in our understanding of fructose metabolism, beginning with the first step of fructose absorption from the lumen via the gut [63] (Figure 2.7), are important because fructose, were a substitute of sucrose by the food industry and that's because of the development of low-cost corn-derived high-fructose sweeteners [64].

Firstly, the transfer of dietary fructose (F) via the small intestine epithelia across the apical membrane in monosaccharide is attained by a member of the facilitative glucose transporter (GLUT) family, GLUT5.

Most of the fructose (solid arrow) leaves the cytosol and enters the portal vein via basolateral GLUT2, which may also transport glucose and galactose (G) absorbed across the apical membrane by Na+-dependent glucose transporter (SGLT1) via Na+-coupled cotransport. Therefore, deletion of GLUT5 entirely excludes transepithelial fructose transport, whereas total or intestine-specific deletion of GLUT2 only slightly lowers glucose transfer, for reasons that are yet unknown.

Secondly, ketohexokinase (KHK) phosphorylates some fructose, which keeps the lumen-to-cytosol gradient favorable for fructose absorption. The transapical fructose transport rate is reduced when KHK is deleted.

Thirdly, When the concentration of luminal fructose is high, a product(s) of fructose metabolism (M) stimulates the transcription and translation of GLUT5 along with fructolytic enzymes. After that, and on the fourth step, when luminal fructose levels are high, new GLUT5 is supplied to the apical membrane via Ras-related protein-in-brain 11a (Rab11a)-mediated (R) endosomes, increasing transapical fructose transport. Moreover, Rab11a deletion in the intestine also inhibits SGLT1-mediated glucose transport, indicating that this GTPase is involved in the trafficking of transporters bound for the apical membrane. Lastly, with increased fructose consumption, portal fructose concentrations rise dramatically. Fructose rate appearance in the blood depends on the number of GLUT5 transporters and the site of fructose absorption; thus, regulation of GLUT5 levels by its own substrate is crucial physiologically[63].

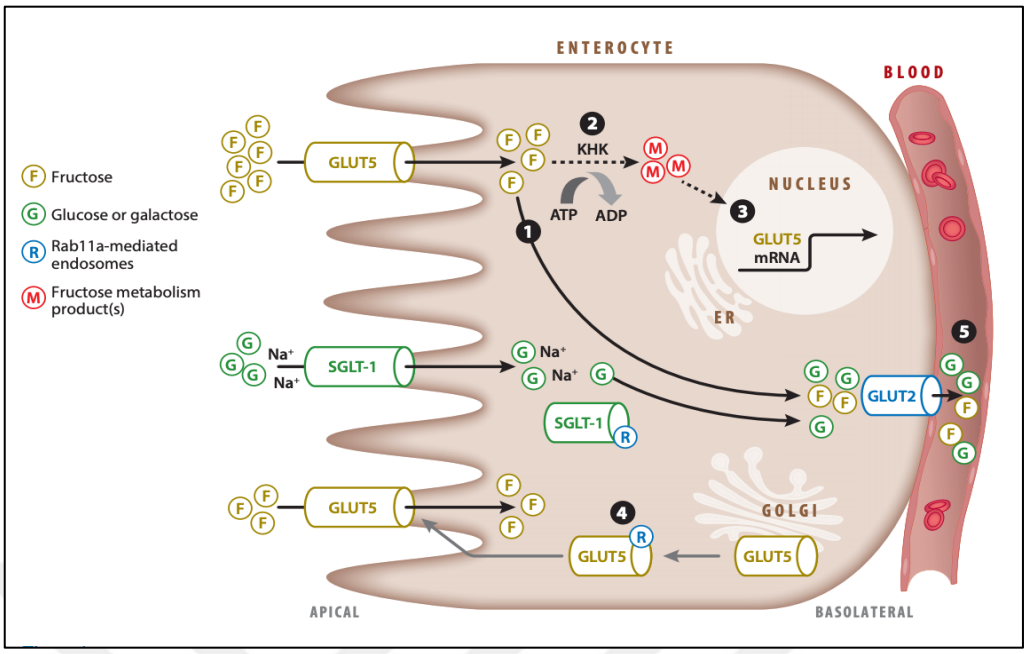



**Figure 2.7.** Intestinal fructose transport across the small intestinal epithelia. (63) modified from reference (65).

### 2.4.2. Obesity:

Obesity has increased during the last 30 years, initially in wealthy countries then, more recently, in emerging ones. Fructose consumption has skyrocketed in recent years. High-fructose corn syrup (HFCS) is widely used in soft drinks, baked goods, sauces, ready-to-eat desserts, and expanding variety of processed goods [61]. Moreover, consumption of high-fructose corn syrup (usually 55% fructose, although it can be as high as 90%) [61] has escalated from 0.5 gram per capita daily in 1970 to 53.9 grams per capita daily in 2003 [66].

For example, soft drink consumption has risen 500% in the last 50 years, and soft drinks are now the major source of added sugar in children's diets [67]. In fact, more recent epidemiological study has confirmed the role of sweetened beverages, most of which include high-fructose corn syrup, in the development of obesity in children aged 9 to 17 [68].

In addition, High fructose consumption has been linked to a variety of diseases in animal studies. Fructose feeding has been demonstrated to gene expression patterns alteration in rats [69], satiety factors alteration in rats' brains [70], and promote inflammation in rats[71], thus increase the amount of reactive oxygen species in rats [72].

On the other hand, exposure to excessive levels of added sugar through maternal nutrition may contribute to an increased risk of grownup obesity[73]. Also, several studies had investigated the effects of extreme carbohydrate consumption through the lactation period on the metabolic state of the offspring. These animal model studies revealed that a high-fructose diet during pregnancy and lactation causes metabolic dysfunction, increased body weight, retroperitoneal adipose tissue, and insulin resistance in both the dams and their neonates[74-76].

Despite significant advances in our understanding of the molecular and physiological pathways that govern the relationship between the early life environment and disease risk, the basic mechanisms underlying the link between maternal and childhood obesity remain unknown.

## 2.5. Short-Chain Fatty Acid (SCFA):

### 2.5.1. SCFA Production:

The colon contains approximately 1011 to 1012 bacteria, primarily Clostridium Type IV and XIV, Bacteroidetes, Bifidobacterium, and Enterobacteriaceae[77]. Consequently, it is obvious that the majority of bacteria reside in the colon and are classified into four major groups: Firmicutes alone account for 64% of the microbiota, followed by Bacteroidetes (23%), Actinobacteria, and Proteobacteria[77].

The microbiota in the gut performs a variety of functions, including the fermentation of amino acids and saccharides, the production of short-chain fatty acids (SCFAs), succinate, ethanol, H2, amines, lactate, phenols, thiols, and indoles, the disposal of hydrogen (as acetate, H2S, and methane), the degradation of undigested proteins and carbohydrates, and the transformation of bile acids [78].The SCFA are essential nutrients for colonocytes, but excessive amounts cause diarrhea and abdominal cramps[79].

### 2.5.2. Butyrate Transportation:

SCFA have a wide range of effects on the host, including metabolism, differentiation, and proliferation, owing to its effect on gene regulation. Several studies have found that butyrate influences the expression of 5-20% of human genes [80-82].

However, SCFA are taken up by cells in their anionic state via H+ or Na+-coupled transporters. As a result, butyrate transport directly contributes to electrolyte absorption

by increasing Na+ and Cl- absorption and releasing bicarbonate (HCO3) in the lumen [83-85].

On the other hand, GPR41 (FFAR3), GPR42, GPR43 (FFAR2), GPR109a (HCAR2), GPR164 (OR51E1), and OR51E2 are all possible G protein-coupled receptors (GPCR) responsive to SCFA encoded by the human genome[86].

Butyrate inhibits Cl secretion by reducing the expression of the Na-K-2Cl cotransporter and promotes the expression of the Na/H transporter NHE3 via histone deacetylase (HDAC) inhibition and a specificity protein dependent mechanism. As it shows in (Figure 2.8. b) [87-90], GPR43 (FFAR2) and GPR41 (FFAR3) recognize acetate, butyrate, and propionate with varying affinities, whereas only butyrate activates GPR109a.

Therefore, GPR41 activation by propionate and butyrate, and GPR109a activation by butyrate, inhibit cAMP accumulation and the activation of protein kinase A and mitogen-activated protein kinases (ERK and p38)[86].

### 2.5.3. Transcriptional Regulation of Butyrate:

Noting that butyrate inhibits lysine and histone deacetylase (K/HDAC) activity more than propionate within the cell. Butyrate is also metabolized into acetyl-CoA, which stimulates histone acetyltransferase by increasing histone acetylation, (Figure. 2.8b)[87-90].

Also, SCFA promote post-translational modification of histones by increasing acetylation through their HDAC inhibitor and histone acetyltransferase stimulatory properties[86]. Peroxisome proliferator-activated receptor gamma (PPAR- γ) normally maintains microbial homeostasis. PPAR- γ is a nuclear receptor found in adipocytes and colonocytes that is activated by butyrate and other ligands. It is responsible for activating genes involved in glucose and lipid metabolism [91].

Butyrate deficiency silences metabolic signaling in the gut. Mitochondrial beta-oxidation in colonocytes is inhibited, resulting in a transfer of oxygen from the blood to the GI lumen via cell membranes. Because of the increased availability of oxygen in the colon, pathogenic facultative anaerobes such as E. coli [92], can outcompete the benign obligate anaerobes that characterize a healthy gut [93,94]. Hence, in the absence of butyrate signaling, nitrate electron acceptors are released into the colon, which facultative anaerobes can also use for cell respiration, breaking down carbs into carbon dioxide rather than digesting them[95].

At last, high butyrate concentrations are linked to the inhibition of stem and proliferative cells in the crypts via HDAC inhibition-dependent binding of Foxo3 to promoters of key genes in the cell cycle [86].

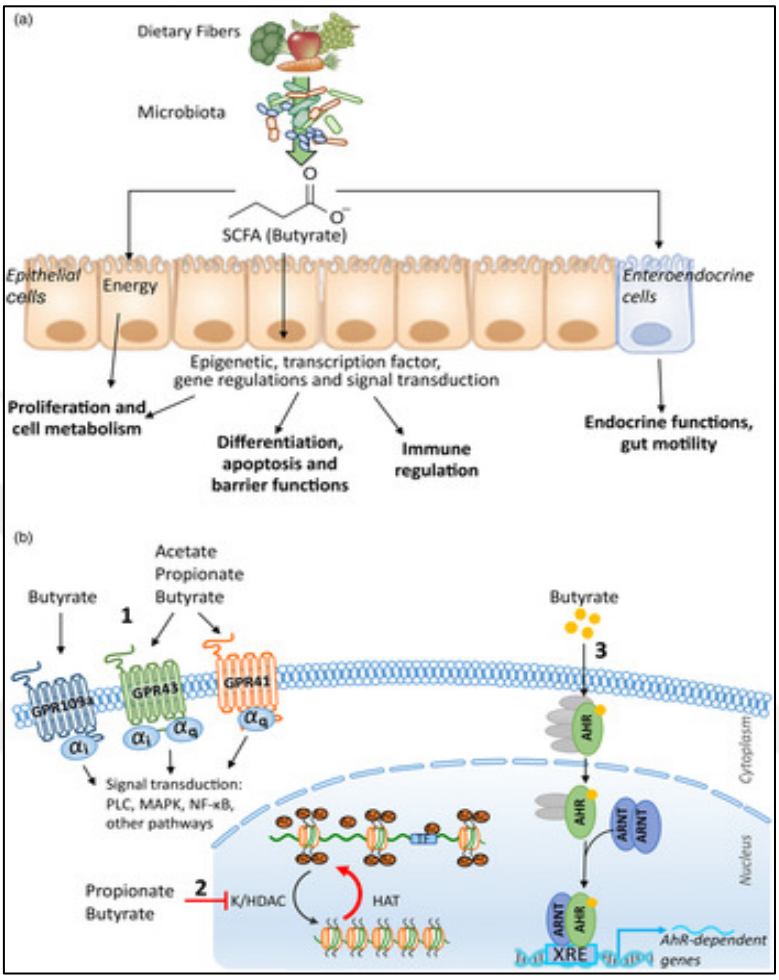



**Figure 2.8.** (a) Functional impact of SCFA on the host. (b) Mechanisms: (1) G protein-coupled receptor (GPCR)-dependent signaling, (2) histone and transcription factor acetylation by SCFA and (3) role of butyrate as a ligand of transcription factors.

## 2.6. Oxidative Stress and Antioxidant:

### 2.6.1. Nutritional Oxidative Stress Consequences:

Oxidative stress is a physiological state characterized by excessive amounts of reactive oxygen species (ROS) and free radicals[96]. Superoxide anion radicals, hydroxyl, alkoxyl, and lipid peroxyl radicals, nitric oxide, and peroxynitrite are examples of reactive oxygenated/nitrogenated species.

Inadequate or excessive nutritional consumption causes oxidative stress, which can disrupt oxidative homeostasis, trigger a cascade of molecular pathways, and change the metabolic status of different tissues. Several diets and consumption habits have been linked to various cancers, and [30–35] percent of cancer diagnoses are linked to malnutrition or overnutrition. However, various contradicting research exist addressing the relationship between food and cancer risk, which has yet to be resolved.

Concurrently, oxidative stress is an important role in cancer growth and therapy. Nutritional oxidative stress can be caused by an imbalance in antioxidant defense and pro-oxidant load because of insufficient or excessive nutrient delivery.

The nutrition-mediated increase in ROS levels, which can accelerate cancer initiation by inducing DNA mutations, damage, and pro-oncogenic signaling, may regulate cancer initiation. In Figure 2.9, Nutrient consumption clearly has a significant oxidative and inflammatory effect on the cellular level, altering tissue metabolism. Nutritional oxidative stress after carbohydrate, protein, and lipid ingestion causes a cascade of metabolic changes in a variety of organs, including the liver, adipose tissue, pancreatic β-cells, and skeletal muscle [97].

Dietary fat (lipids) induces intracellular lipid accumulation in the and results in oxidative stress and insulin resistance-induced liver dysfunction. Moreover, a nutritious diet can promote the inflammatory response and affect FoxO1 expression, adipokine secretions, and antioxidant enzyme activity in the adipose tissue, leading in increased ROS formation and, ultimately, adipose tissue malfunction[97].

However, hyperglycemia can enhance mitochondrial ROS generation in pancreatic β-cells, fostering a native oxidative milieu, which regrettably affects insulin gene expression and activity, increasing oxidative stress. Consequently, a vicious cycle of mitochondrial dysfunctions due to dietary fat overfeeding further intensify the metabolic abnormalities of the skeletal muscle [97].

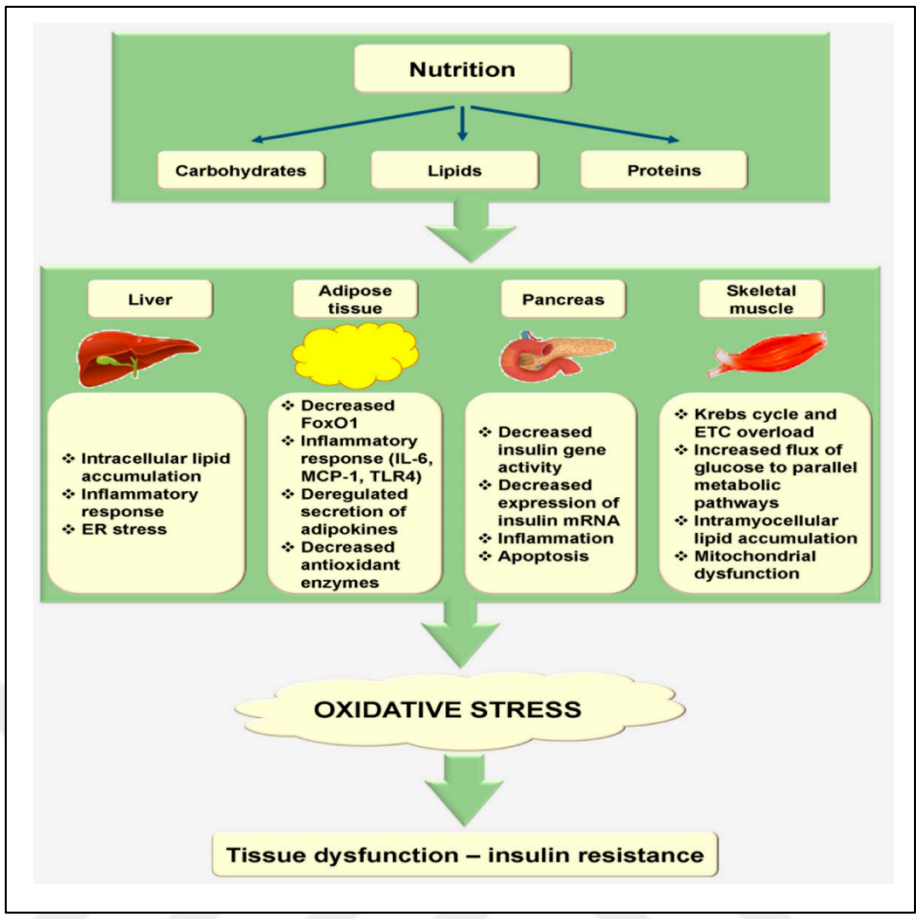



**Figure 2.9**. Nutrition mediates oxidative stress at the metabolic tissue level. Adapted from (98)

Therefore, Maternal nutrition is identified as a potential risk factor for the development of colorectal cancer since it influences both the epigenetic pattern and the gut flora of the individual. This condition has led us to believe that the exposed diet during the maternal period may be helpful at an early stage and modulate the development of colon cancer.

### 2.6.2. Carbohydrate Intake and Oxidative Stress:

Evidence has been shown that the evolution of man has been accompanied by high complex carbohydrate dietary habits, which have a major effect on the colonic microbiota and their ability to create metabolites that maintain colonic health and prevent carcinogenesis[7].

Previous research suggests that oxidative stress may play a significant role in the link between acute hyperglycemia and increased cardiovascular risk [99-101]. Whereas an

acute increase in blood glucose concentrations may increase the generation of free radicals due to an imbalance in the ratio of NADH to NAD and increased non-enzymatic glycation in cells[102,103]. The direct indication from studies presented that enhanced hyperglycemia or

meal consumption and its derived glucose can promote oxidative stress and impair antioxidant defenses [104,105].

Nevertheless, Fructose is processed in the liver by the enzyme fructokinase-c, which has no negative feedback mechanism. Thus, fructose supply causes continuing metabolism, ATP depletion, uric acid formation, mediators of inflammation and oxidative stress, and, ultimately, protein production disruption [106]. In addition, Fructokinase-C is also found in the small intestine, where uncontrolled fructose metabolism leads to ATP depletion and decreased expression of TJ proteins, resulting in increased permeability and endotoxemia. These concepts were validated in fructokinase-C gene knockout mice [106].

Similarly, numerous studies have found a link between elevated oxidative stress and a Western-style diet high in fats and processed sugars. These products, in fact, promote a rise in the inflammatory state with ROS generation and subsequent amplification of the inflammatory cascade when taken regularly and in significant quantities [107-109].

Up to now, the association between carbohydrate supplementation and antioxidant response in the diet has remained unknown. High sugar consumption is widely thought to be associated with a loss of antioxidant potential and an increase in the intensity of oxidative alteration of biomolecules by reactive carbohydrate derivatives[101]. However, evidence from non-vertebrate creatures suggests that carbohydrate restriction causes an increase in oxidative damage/ROS generation [111].

### 2.6.3. The Gut Microbiota and Oxidative Stress:

The gut microbiota (also known as microflora) is made up of a diverse range of bacteria that live in the gastrointestinal system and play a vital role in human health and disease. (Reviewed in 112). In terms of nutrition and mucosal immunity, they are critical to the host's well-being. Numerous studies, however, have implicated some components of the gut microbiota with the development of CRC via various mechanisms, including the production of reactive metabolites (E. faecalis produces hydroxyl radicals, a significant cause of oxidative stress on the intestinal epithelium) [113], and carcinogens,

changes in host carbohydrate expression, and persistent mucosal inflammation [114]. The human gut ecosystem contains 300–500 different types of bacteria, the content of which varies substantially between individuals, potentially implying enormous variation in free radical generation among men (Figure 2.10).

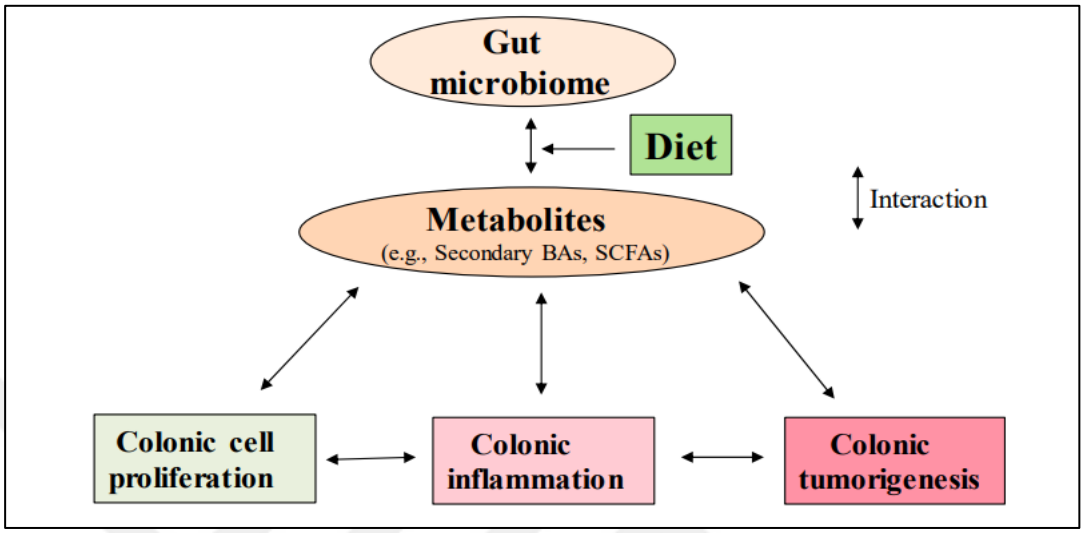



**Figure 2.10.** Interactions between the gut microbiome, metabolites, colonic cell proliferation, inflammation, and cancer[115].

### 2.6.4. Inflammation and Colorectal Cancer:

Colorectal cancer (CRC) is one of the most common cancers worldwide, with the highest incidence rates in western countries[116]. Colon cancer originates from the epithelial cells that line the bowel. These cells divide rapidly and have a high metabolic rate, which has been found as a potential factor that may be responsible for increased oxidation of DNA [117.]

Chronic inflammation appears to be a significant role in the development of carcinogenesis, according to a rapidly rising body of evidence [118,119]. CRC is commonly recognized because of a persistent inflammatory state of the colon. Excessive and unregulated generation of ROS over an extended length of time results in continuous damage of tissue cells and, as a result, prolonged inflammation. In addition to injured cells, inflammatory cells release soluble mediators, which function by recruiting more inflammatory cells to the site of injury and releasing more reactive species [119,120]. In a vicious spiral, the sustained inflammatory/oxidative environment increases the formation of hydroperoxides, which can damage healthy epithelial and stromal cells in the area of injury and, over time, may lead to carcinogenesis[121].

### 2.6.5. Obesity and Colorectal Cancer Risk:

Obesity, particularly abdominal obesity, has been linked to an increased risk of CRC and has been shown to impact oxidative state in obese individuals [122,123]. Adipose tissue is known to produce a variety of adipocytokines (e.g., adiponectin, leptin, and several cytokines such as TNF, IL-6, IL-8, and IL-10) that are involved in the normal functioning of the body. Obesity has been demonstrated to affect adipocytokine levels, increase circulating estrogens, impair insulin sensitivity, and increase the inflammatory response. It induces metabolic syndrome or low-grade chronic inflammation, which may be responsible for the continual rise in free radical production. It is hypothesized that such conditions (oxidative stress) generate a setting favorable to the development of CRC over time [122,123].

When free radicals are created in large and unmanageable quantities, they and their derivative products can react with numerous cellular macromolecules such as lipids, proteins, and DNA, modulating gene expression.

### 2.6.6. Nutrition Effect on Antioxidants:

The antioxidant system is critical in organisms' adaptation to any environmental situations that deviate from physiological optimum[124]. These environmental parameters include macronutrient balance as well [124].

Undoubtedly, Macronutrients such as proteins and carbohydrates, serve many metabolic functions. Thus, overfeeding or a deficiency of one or more macronutrient leads in an unbalanced diet, which affects several important physiological parameters. especially in terms of lifespan and fecundity and ROS generation [124]. However, it is extremely likely that the antioxidant system's response to avoiding ROS-induced effects is linked with protein, carbohydrate, and lipid metabolism, and mitochondria may play a part in this interaction.

Moreover, these organelles are the primary intracellular ROS generators and host critical catabolic events such as the tricarboxylic acid cycle, fatty acid oxidation, urea cycle, glycine cleavage, folate cycle, and others. Similarly, antioxidant response is linked to peroxisomes, which are involved in very long chain fatty acid oxidation and purine catabolism. Since dietary macronutrients regulate catabolic processes in mitochondria and peroxisomes, they can also influence ROS generation and, as a result, antioxidant response.

### 2.6.7. The Mechanisms of Protection Against Oxidative Stress:

As discussed earlier, living organisms are continually subjected to a variety of stressors (both external and internal) that can have a major impact on the redox potential of their cells. As a result, they have evolved a number of defensive mechanisms that endow cells with an extraordinary capacity for redox homeostasis. These antioxidative protective mechanisms are classified into three categories of protection, (Figure 2.11).

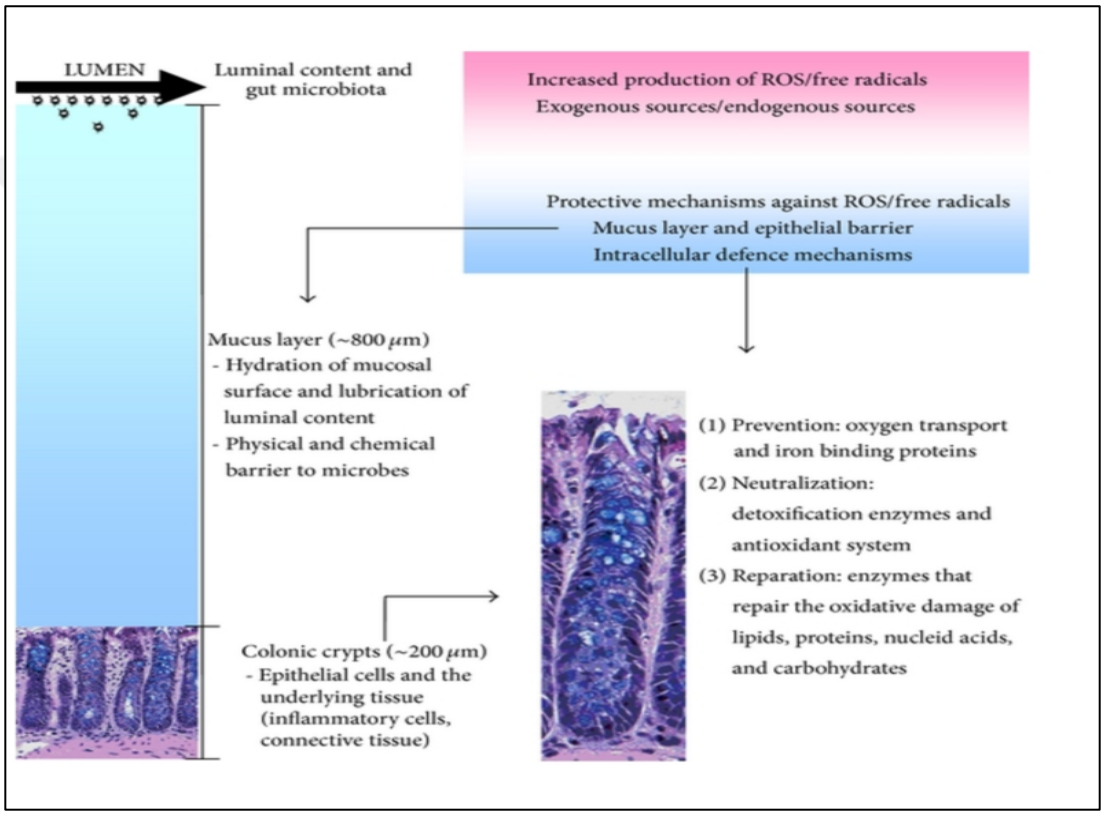



**Figure 2.11**. Sematic representation of the colonic barrier and intracellular protective mechanisms against oxidative stress[125].

The arrangement of oxygen transport or proteins that bind iron and prevent the Fenton's reaction represent the first level of antioxidative defense. Furthermore, the second level includes two basic defense systems: detoxifying enzymes that may be influenced by xenobiotic levels and an antioxidant system that reduces free radical species and maintains the cell's redox state. At last, Enzymes that repair oxidative damage to lipids, proteins, carbohydrates, and nucleic acids are included in the third stage of defense [125].

# 3. MATERIALS and METHODS

## 3.1. Animals

Male and female Sprague Dawley rats were obtained and used from Yeditepe University Experimental Animal Research Center.

## 3.2. Diet

Two different diets were purchased from Ssniff company (Germany). The macronutrient percentages of the experimental diets are available in (Table 3.1).

**Table3.1.** The ingredients of the Control standard fat diet (SFD), high carbohydrate/low fat diet (HCD/LFD).

| Ingredients | Control Diet (SFD) (VRF1-Ssniff) | High Carb/Low Fat Diet (HCD/LFD) (sniff) |
|---|---|---|
| Total Calorie (kcal/gr) | 3,3 | 3,4 |
| Fat (percent kJ) | 13 | 3 |
| Protein (percent kJ) | 23 | 23 |
| Carbohydrate (percent kJ) | 64 | 74 |

## 3.3. Creating Experimental Groups:

This study followed the turkish guidelines for the use and care of laboratory animals plus, the animal research studies in accordance with the Animal Research Ethics Committee of Yeditepe University were applied in the experimental protocol. Animal euthanasia and experiments of Sprague Dawley rats were performed in the Yeditepe University Faculty of Medicine Experimental Research Center. Mating days for male and female rats were approximately 8 days. The mating day was determined by vaginal smears establishment. First day of gestation was assumed by the smear-positive animals.

Approximately the 21st day of gestation the offsprings were born and animals continued feeding with same experimental diet in groups during the lactation period.

In this study, the experimental group was created to apply high carbs diet on 20 rats during gestation-lactation (G + L), in other words maternal period and maturation (M) periods. Each group has five animals.

1. **Control Group (SFD-SFD)**: during gestation-lactation and maturation periods, the animals in this group will be fed with a control diet.
2. **Maturation High Carbohydrate Diets (SFD-HCD):** during gestation-lactation and maturation periods, the animals in this group will be fed with high carbs diet (HCD).
3. **Maternal High Carbohydrate Diets (HCD-SFD):** Animals in this group will be fed with HCD in the maternal period and with a control diet when they switch to the maturation period.
4. **High Carbs Diet Group (HCD-HCD):** Animals in this group will be fed with a control diet in the maternal period and with HCD when they switch to the maturation period.

### 3.4. Determination of Total Antioxidant and Oxidant Capacity:

For the determination of the oxidative stress status of proximal colon tissue a commercial total oxidant status and total antioxidant status kits (Rel Assay) will be used.

In order to determine the oxidant and antioxidant status these steps will be performed. Each one of these steps has their own chemicals, reagents, and procedures.

#### 3.4.1. Homogenization of Samples:

Fifty mg of frozen proximal colon tissue from all experimental groups will be weighed and transferred to tubes. After that, 300 µl of phosphate buffer saline (PBS) was added to the tubes. Homogenizer tissues (Dia X 900 Heidolph, Germany) were disrupted. Then, for 5 minutes, tubes were centrifuged at 3000 rpm. Supernatants were taken into new tubes and used as a sample for assays with TAS and TOS.

#### 3.4.2. TAS Measurements:

Eighty µl of sample, standard, and deionized water placed on a 96-well plate then a 300 µl of on each sample, on standards, and on dH2O was added (all wells). After that, Reactive 1 (R-1) solution is added and mixed with a pipette. Then, With the Microplate

Spectrophotometer, the absorbance is measured at 660nm first measurement (A1). 45 µl of Reactive two solution is added to each well and mixed with the Reactive one solution. The plate is kept at room temperature for 10 minutes (incubation). After incubation, the plate is measured again at 660nm absorbance second measurement (A2). For each well, subtract A1 from A2. ΔAbs is calculated.

**TAS (mmol/L) = [ΔAbs H2O-ΔAbs Sample] / [ΔAbs H2O-ΔAbs Standard]**

### 3.4.3. TOS Measurements:

Forty-Five µl of sample, standard, and deionized water is placed on a 96-well plate, then 300 µl of each sample, on standards, and on dH2O was added (all wells). After that, Reagent 1 (R-1) solution is added and mixed with a pipette. Measurement is made at 530nm absorbance with a Microplate Spectrophotometer first measurement (A1). Add 15 µl of Reagent 2 (R-2) solution to each well and mix with the Reactive one solution. The plate is kept at room temperature for 10 minutes or 37 degrees for 5 minutes (incubation). After incubation, the plate is measured again at 530nm absorbance second measurement (A2). For each well, subtract A1 from A2. ΔAbs is calculated.

**TOS (µmol/L) = [ΔAbs Sample] / [ΔAbs Standard] * 10**

### 3.4.4. Calculation of Oxidative Stress Index OSI:

The oxidative stress index (OSI) was calculated by dividing the TOS level by the TAS level.

**OSI (arbitrary unit) = TOS (µmol H2O2 Eq/L)/TAS (µmol Trolox Eq/L)**

## 3.5. Tissue Tracking and Sectioning of Colon Tissues

Proximal colon tissues taken from all groups will be fixed in 10% formalin solution for 48 in a tracking device while following the tracking protocol (table 3.2). After tissue follow-up, colon tissues will be embedded in paraffin and kept at + 40C until sectioning. Then, 5 µm thick sections will be taken from the paraffin blocks taken into the microtome, and ventilation of the sections taken will be performed on the Ply-L lysine-coated slides for 2 hours at room temperature. It will then be observed under a microscope and used for immunofluorescence staining and Hematoxylin-Eosin (HE).

**Table 3.2.** Tissue Tracking Protocol.

| Reagents | Number of Changes | Duraiton |
|---|---|---|
| Tap Water | 3 | 3x10 min |
| 70% Alcohol | 1 | 3 hours |
| 80% Alcohol | 1 | 3 hours |
| 90% Alcohol | 1 | 3 hours |
| 100% Alcohol | 2 | 2+3 hour |
| Xsilen | 2 | 1 +2 hour |
| Paraffin | 2 | > 10 hours |

### 3.6. Hematoxylin-Eosin Staining:

To determine the change in the proximal colon morphology of a high carbohydrate diet to be applied in different developmental periods, the sections taken from the colon will be placed in the tissue staining device, and hematoxylin-eosin staining will be performed following the protocol (Table 3.3). The coverslip will be added to the device sections; the cover slip will be closed and displayed in the light microscope.

**Table 3.3.** Hematoxylin-Eosin Staining Protocol

| Reagents | Number of Changes | Duration |
|---|---|---|
| Oven | 1 | 3 minutes |
| Xsilen | 3 | 3+3+5 minutes |
| %100 Alcohol | 1 | 2 minutes |
| %96 Alcohol | 2 | 2+2 minutes |
| Tap Water | 1 | 2 minutes |
| $dH_2O$ | 1 | 1 minutes |
| Hematoxilin | 1 | 2,1 minutes |
| Tap Water | 5 | 1x5 minutes |
| %96 Alcohol | 1 | 30 seconds |
| Eosin Y | 1 | 4,1 minutes |
| %96 Alcohol | 3 | 30+30+60 seconds |
| %100 Alcohol | 1 | 1 minutes |
| Xsilen | 3 | 1x2 minutes |

### 3.7. Immunofluorescence Staining

Localization and expression levels of RAD51 and gamma-H2AX protein in the colon are demonstrated by immunofluorescent staining. Sections will be placed in Poly-L-lysine slides and kept in the incubator at 37 ° C for 1 night. Then slides will be incubated at 60 ° C for 1 hour. Then it will be placed in xylene twice for 20 minutes. For the dehydration of the colon, the slides will be passed through an alcohol (ethanol) bath (100%, 90%, 80%, 70%, respectively) for 10 mins. Then it will be kept in dH20 for 2 times, 5 minutes each.

After that, slides will be washed 2 times in phosphate buffer saline (PBS) for 5 minutes, placed in ethylene di-amine-tetraacetic acid (EDTA) buffer, boiled for 4 minutes at the "med-high" setting of the microwave oven, cooled at room temperature for 20 minutes. Then again, slides will be washed with PBS 3 times for 5 minutes and tris buffered saline with tween 20 (TBS-T) 2 times for 5 mins after that the boundaries of the samples will be drawn with a hydrophobic pen. Sections blocked with 5% donkey serum in PBS-T which includes %0.1 Triton-X in PBS at room temperature for 60 min on the wet chamber, primary rabbit Rad51 antibody (1: 500 dilution) to positive groups and PBS to negative groups will be dropped and the sections will be incubated overnight in a refrigerator at +4 C. The sections washed 3 times in PBS-T for 5 minutes the next morning, kept in the secondary anti-rabbit antibody diluted 1: 1000 for 90 minutes, washed 3 times in PBS-T for 5 minutes, and determination of cell nuclei with 4', 6-diamidino-2-phenylindole (DAPI) capping solution will be closed for. The samples whose staining has been completed will be displayed under the confocal microscope and the expression level and localization of Rad51 will be shown. The same procedure will be used to image the Gamma-H2AX protein.

**Table 3.4.** Immunofluorescence Staining Protocol

| Reagents | Number of Changes | Duration |
|---|---|---|
| Section in Poly-L-lysine slides placed in the incubator at 37 ° C for 1 night. Then slides will be incubated at 60 ° C for 1 hour. | | |
| xylene | 2 | 20 minutes |
| %100 Alcohol | 1 | 10 minutes |
| %90 Alcohol | 1 | 10 minutes |
| %80 Alcohol | 1 | 10 minutes |
| %70 Alcohol | 1 | 10 minutes |
| $dH_2O$ | 2 | 5 minutes |
| PBS | 2 | 5 minutes |
| EDTA (Boiled) | 1 | 5 minutes |
| Cool Down | 1 | 20 minutes |
| PBS | 3 | 5 minutes |
| TBS-T | 2 | 5 minutes |
| PBS-T (5% donkey blocking serum) | 1 | 60 minutes |
| Rad51 antibody (1: 500 dilution) to positive groups and PBS to negative groups and incubated overnight in a refrigerator at +4 C. | | |
| PBS-T | 3 | 5 minutes |
| secondary anti-rabbit antibody (1: 1000 dilution), | 1 | 90 minutes |
| PBS-T | 3 | 5 minutes |
| Determination of cell nuclei by adding (DAPI) to close the slides. Then Displayed under the confocal microscope | | |

### 3.8. Statistical Analysis

Using the software statistics program SPSS-18, the results to be obtained at the project end is evaluated. The differences between groups and binary groups will be determined by applying Kruskal Vallis and Mann-Whitney U tests. $P < 0.05$ will be considered statistically significant in assessing the results.

# 4. RESULTS

## 4.1. Total Oxidant Status (TOS) parameter in proximal colon tissue:

**Table 4.1.** TOS measurement of SFD with (0.09± 0.01) mmol/L, SFD-HCD with (0.08± 0.03) mmol/L, HCD-SFD (0.11± 0.03) mmol/L and HCD-HCD (0.09± 0.01) mmol/L during maternal and maturation period.

| **Groups** | **TOS (mean ± standard deviation) mmol/L** |
|---|---|
| SFD | 0.09± 0.01 |
| SFD-HCD | 0.08± 0.03 |
| HCD-SFD | 0.11 ± 0.03 |
| HCD-HCD | 0.09± 0.01 |

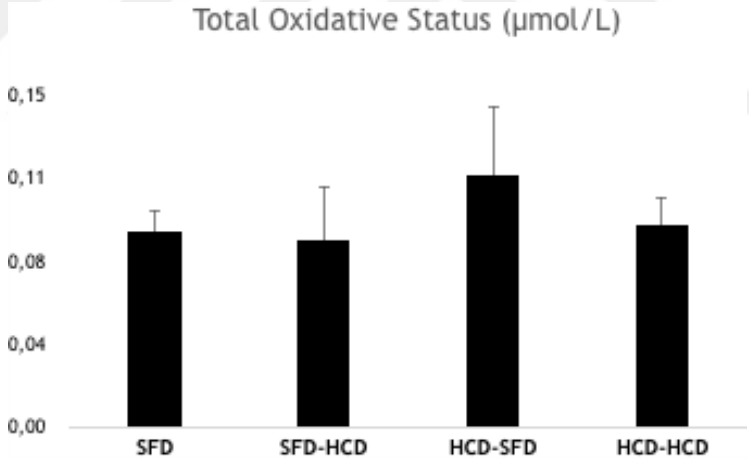



**Figure 4.1**. The distribution of TOS parameter for SFD and HCD during the maternal and maturation period of the proximal colon (mean ± standard deviation) (n=5)

As shown, the distribution of the TOS parameter for each group has no significant difference. Still, we observed an increase tendency of TOS measurement (0.11 ± 0.03) mmol/L in the HCD-SFD group.

### 4.2. Total Antioxidant (TAS) Status parameter in proximal colon tissue:

**Table 4.2.** TOS measurement of SFD with (1.04 ± 0.20) mmol/L, SFD-HCD with (1.09 ± 0.06) mmol/L, HCD-SFD (1.01± 0.12) mmol/L and HCD-HCD (0.53 ± 0.51) mmol/L during maternal and maturation period.

| **Groups** | **TAS (mean ± standard deviation) mmol/L** |
|---|---|
| SFD | 1.04 ± 0.20 |
| SFD-HCD | 1.09 ± 0.06 |
| HCD-SFD | 1.01 ± 0.12 |
| HCD-HCD | 0.53 ± 0.51 |

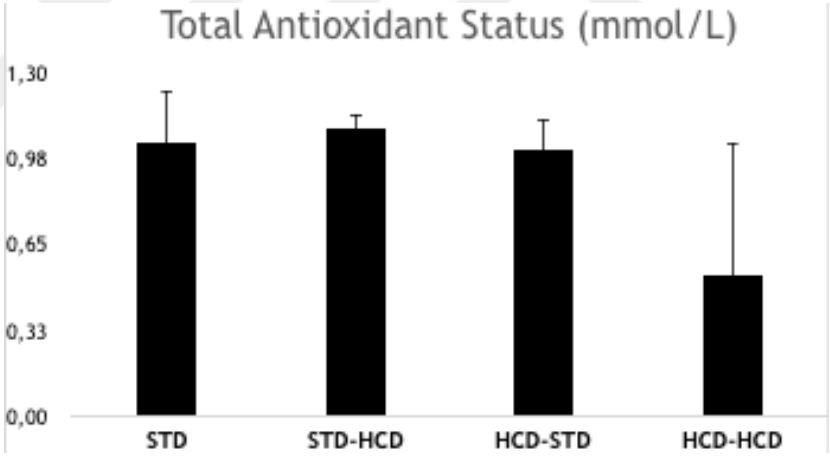



**Figure 4.2**. The distribution of TAS parameter for SFD and HCD during the maternal and maturation period of the proximal colon (mean ± standard deviation) (n=5)

As shown, the distribution of TAS parameter for each group has no significant difference, although we observed a decrease tendency of TAS measurement (0.53 ± 0.51) mmol/L in the HCD-HCD group.

### 4.3. Oxidative Stress Index (OSI) Status parameter in proximal colon tissue:

The oxidative stress index (OSI) values are measured by dividing the TOS level by the TAS level.

**Table 4.3.** OSI measurement of SFD with (0.009 ± 0.002) mmol/L, SFD-HCD with (0.008 ± 0.002) mmol/L, HCD-SFD (0.01± 0.0019) mmol/L and HCD-HCD (0.036 ± 0.0387) mmol/L during maternal and maturation period.

| **Groups** | **TAS (mean ± standard deviation) mmol/L** |
|---|---|
| SFD | 0.009 ± 0.002 |
| SFD-HCD | 0.008 ± 0.002 |
| HCD-SFD | 0.01 ± 0.0019 |
| HCD-HCD | 0.036 ± 0.0387 |

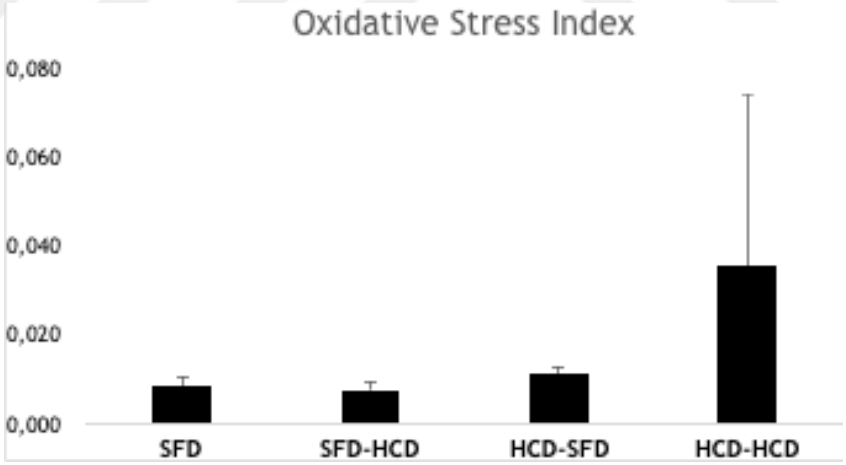
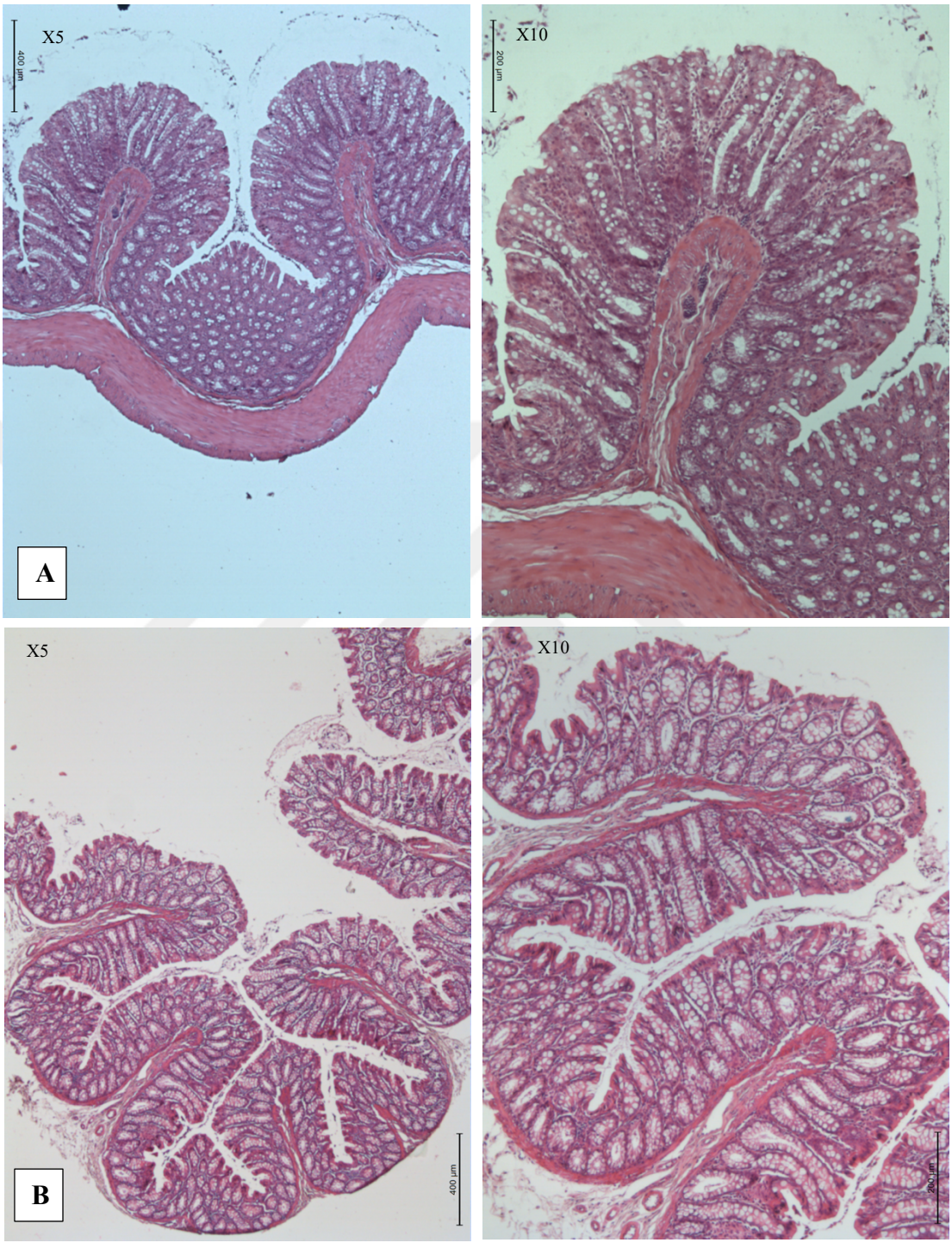



**Figure 4.3**. The distribution of OSI parameter for SFD and HCD during the maternal and maturation period of the proximal colon (mean ± standard deviation) (n=5)

## 4.4. Histomorphological Evaluation of Proximal Colon Tissues with HE Staining



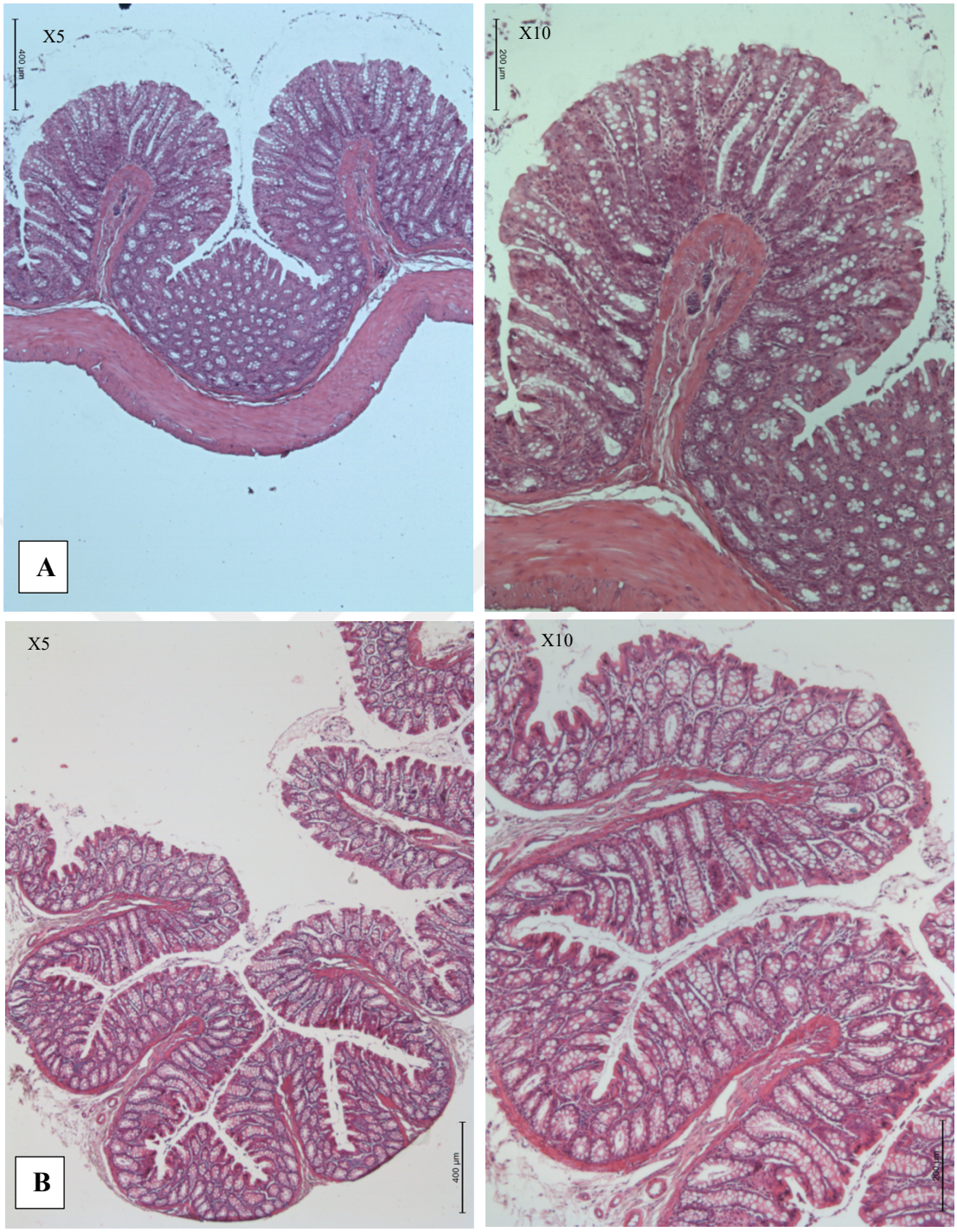



**Figure 4.4**. Representative images of H&E-stained proximal colon sections illustrate the effect of A) SFD on the epithelial crypts of colon (x5, scale bar 400 µm and x10 scale bar 200 µm) B) SFD-HCD on the epithelial crypts of the colon (x5, scale bar 400 µm and x10 scale bar 200 µm) C) HCD-SFD on the epithelial crypts of the colon (x5, scale bar 400 µm and x10 scale bar 200 µm) D) HCD-HCD on the epithelial crypts of the colon (x5, scale bar 400 µm and x10 scale bar 200 µm)

### 4.5. H2AX and Rad51-IF imaging with Confocal Laser Scanning Microscope

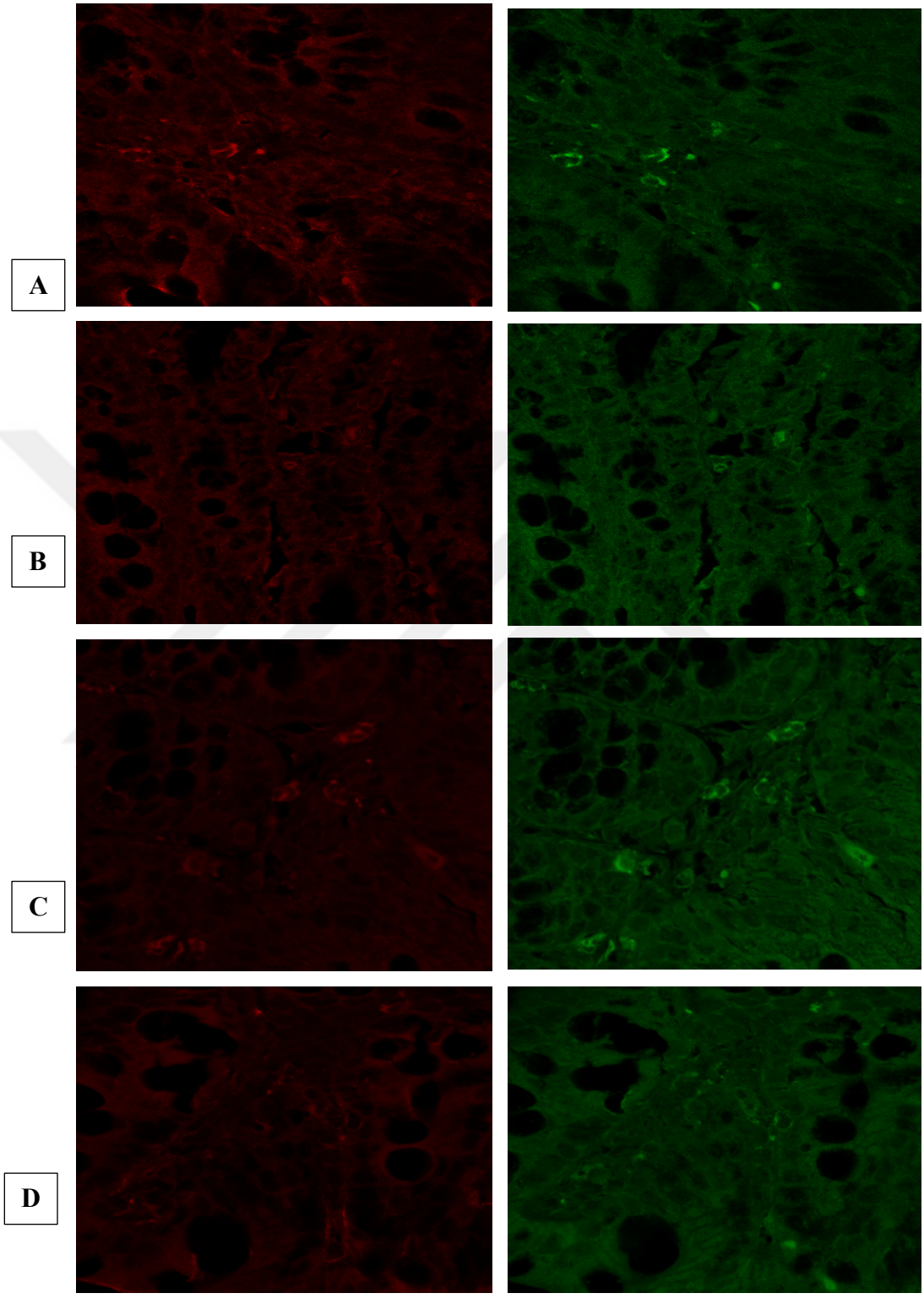



**Figure 4.5**. Results obtained from immunofluorescence staining of the proximal colon. RAD51 imaging showing in red and H2AX imaging showing in the green. A) Confocal imaging of SFD B) Confocal imaging of SFD-HCD C) Confocal imaging of HCD-HCD D) Confocal imaging of HCD-SFD (n=3). For illustrating DNA repair, DNA damage,

and carcinogenesis, we applied important markers RAD51 and H2AX. The slides were closed with DAPI and were visualized by confocal microscopy. Our data show no significant differences were obtained from the experimental group.

### 4.5.1. Expression Level of H2AX and RAD51:

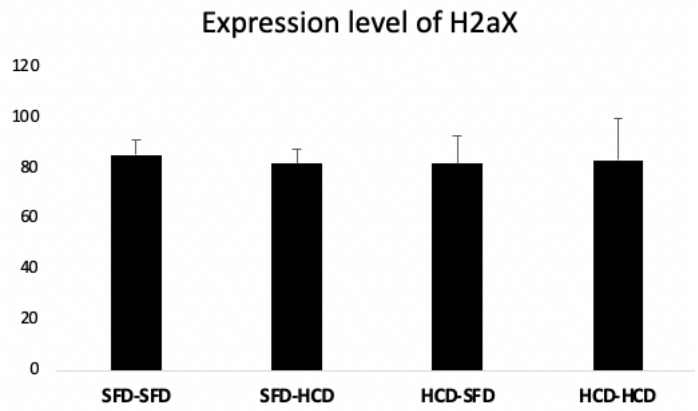



**Figure 4.6**. The distribution of expression level of H2aX on SFD and HCD during the maternal and maturation period of the proximal colon (mean ± standard deviation) (n=5)period of the proximal colon (mean ± standard deviation) (n=5)

As shown, the distribution of expression level of H2AX on each group has no significant difference.

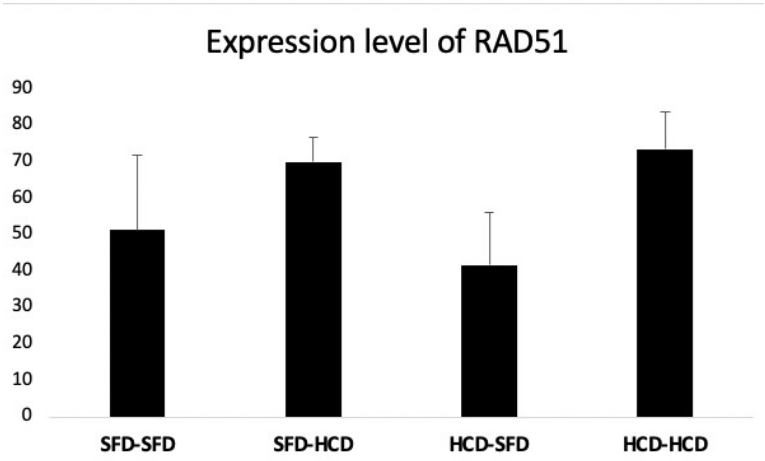



**Figure 4.7**. The distribution of expression level of RAD51 on SFD and HCD during the maternal and maturation period of the proximal colon (mean ± standard deviation) (n=5)

As shown, the distribution of expression level of RAD51 in each group has no significant difference.

## 5. DISCUSSION and CONCLUSION

This study investigated how high carbohydrate diet consumption affects the colon tissue during maternal and maturation periods. In the literature, many studies tested the effect of high carbohydrates on the gut microbiome, glycemic index, glycemic load, and colon distress. However, it's the effect on colon tissue during maternal and maturation still needs attention. The consequences that may appear on the colon tissue inspired us to investigate Gamma-H2AX and RAD51 DNA repair biomarker proteins during gestation-lactation (G+L) and maturation (M) periods. Besides, Total Oxidant Status (TOS) and Total Oxidant Status (TAS) affect the mother's proximal colon tissue. For that purpose, twenty Sprague Dawley rats were divided into four groups of control (SFD) and high carbohydrate diet (HCD) during maternal and maturation periods.

Firstly, we examined the total oxidant status (TOS), antioxidant status (TAS) and Oxidative stress index (OSI) for each group. In determining TOS distribution for SFD and HCD during maternal and maturation periods, we found that statistically no significant difference, but a tendency increases in the maternal HCD-SFD group. Moreover, TAS distribution for the group diets during maternal and maturation periods did not significantly differ. Still, it showed a tendency to decrease the antioxidants status in the maternal and maturation HCD-HCD group. Not only did TAS and TOS measurements not show any significant differences, but OSI, when it was analyzed, presented only a tendency increase of the HCD-HCD group. Even though there were no significant differences in our data result, the tendencies increase in TOS and OSI and the tendency decrease in TAS support the hypothesis that high carbohydrate intake affects colon tissue. It is achieved by the possible elevated production of reactive oxygen species (ROS) and possible reduction of antioxidant reaction.

The second approach examines the effects of a high carbohydrate diet on the morphology and structure of the proximal colon tissue during maternal and maturation periods. This approach was achieved by the hematoxylin-eosin (HE) staining method, and the slides were examined under the microscope. Consequently, we found that the crypts of the control group diet (SFD-SFD) are standard and parallel as we expected in healthy animals. Likewise, in the maturation high carbohydrate diet group (SFD-HCD), we found that the crypts are normal and parallel but with mild disturbance on the epithelial cell surface. However, in the high maternal carb diet group (SFD-HCD), we found that the crypts are normal, healthy, and with no structural changes. This result is

due to the short time of the maternal period. At the same time, the high carbohydrate diet group demonstrated a bulk-like crypt structure. These results will provide us with the information when creating another research study or guidance on how a high carbohydrate diet may shape the colon tissue during pregnancy.

Lastly, we performed immunofluorescence (IF) staining to identify the cells, track protein localization, and provide information about cell function after high carbohydrate consumption. Therefore, we studied and examined H2AX as a DNA damage biomarker and RAD51 DNA repair biomarker protein expression statistically and imaging under confocal microscopy. However, no expression levels of H2AX and RAD51 were found in the IF imaging. Similarly, there were no significant differences in the expression of H2AX on the four groups of diet during maternal and maturation periods. Nevertheless, we spotted a tendency increase in the face of RAD51 on both high maturation diet (SFD-HCD) and high carbohydrate diet (HCD-HCD). At the same time, we noticed a tendency to decrease expression on a high maternal carbohydrate diet (HCD-SFD). However, statistically, there was no significant difference. These data results may benefit us for future high carbohydrate effects in maternal and maturation periods related studies.

In conclusion, our data slightly demonstrated the possibility of reactive oxygen species production, antioxidant status decrease, oxidative index stress increase, morphology mild disturbance, and DNA damage in the proximal colon of different high carbohydrate groups during maternal and maturation periods. By some means, these findings are related to studies mentioned in the literature where the effect of high carbohydrate exposure on colon tissue during pregnancy may cause inflammation by producing free radicals and leads to DNA damage by the interruption of gut microbiota. The limitation of our study hides behind the fact that the standard deviation is very high, which will urge us to use a more significant number of sampling for future studies.

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# 7. APPENDICES

## 7.1. Ethical Approval.

T.C. YEDİTEPE ÜNİVERSİTESİ
Hayvan Deneyleri Yerel Etik Kurulu (HADYEK)

ETİK KURUL KARARI

| Protokol No | Toplantı Tarihi | Toplantı Sayısı | Karar No | Proje Yürütücüsü |
|---|---|---|---|---|
| 2019-811 | 08.11.2019 | 2019/11 | 2019/11-11 | Doç. Dr. Burcu Gemici Başol |

'Sprague Dawley Sıçanlarda Maternal ve Maturasyon Dönemlerinde Farklı Yağ Konsantrasyonlarında Diyete Maruz Kalmanın Kolorektal Kanser ile İlişkisinin İncelenmesi' isimli proje oy birliğiyle etik açıdan uygun görülmüştür.

| Hayvan Türü / Irkı | Toplam Hayvan Sayısı | Hayvanın Cinsiyeti |
|---|---|---|
| Sıçan | 56 | Erkek |

| Görevi | Adı Soyadı | Katılım Durumu |
|---|---|---|
| Başkan | Prof. Dr. Bayram YILMAZ | |
| Başkan Vekili | Prof. Dr. Erdem YEŞİLADA | |
| Üye | Veteriner Hekim Engin SÜMER | |
| Üye | Prof. Dr. M. Ece GENÇ | |
| Üye | Prof. Dr. Rukset ATTAR | |
| Üye | Prof. Dr. Gamze TORUN KÖSE | |
| Üye | Doç. Dr. Ediz DENİZ | |
| Üye | Doç. Dr. Aylin YABA UÇAR | |
| Üye | Hakan GÖKSEL | |
| Üye | Ahmet ŞENKARDEŞLER | |