INTERACTION OF INTESTINAL MICROBIOTA AND INTESTINAL EPITHELIAL HEALTH IN HIGH CARBOHYDRATE DIET

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

MEHMET SEFA ULUTAŞ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

AUGUST 2021

Approval of the thesis:

INTERACTION OF INTESTINAL MICROBIOTA AND INTESTINAL EPITHELIAL HEALTH IN HIGH CARBOHYDRATE DIET

submitted by **MEHMET SEFA ULUTAŞ** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** i**n Biotechnology, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Director, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Güzin Candan Gültekin
Chairperson of the Department, **Biotechnology** _____

Prof. Dr. Güzin Candan Gültekin
Supervisor, **Food Engineering, METU** _____

Assist. Prof. Dr. Aysun Cebeci Aydın
Co-Supervisor, **Nanotechnology Eng., Abdullah Gül Uni.** _____

Examining Committee Members:

Prof. Dr. Sreeparna Banerjee
Biology, METU _____

Prof. Dr. Güzin Candan Gültekin
Food Engineering, METU _____

Prof. Dr. Remziye Yılmaz
Food Engineering, Hacettepe University _____

Assoc. Prof. Dr. Yeşim Soyer
Food Engineering, METU _____

Assoc. Prof. Dr. Ömer H. Yılmaz
Biology, Massachusetts Institute of Technology _____

Date: 27.08.2021

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name Lastname: Mehmet Sefa Ulutaş

Signature:

ABSTRACT

INTERACTION OF INTESTINAL MICROBIOTA AND INTESTINAL EPITHELIAL HEALTH IN HIGH CARBOHYDRATE DIET

Ulutaş, Mehmet Sefa
Doctor of Philosophy, Biotechnology
Supervisor: Prof. Dr. Güzin Candan Gürakan
Co-Supervisor: Assist. Prof. Dr. Aysun Cebeci Aydın

August 2021, 120 pages

Inflammatory bowel diseases (IBDs) are chronic inflammatory disorders that cause prolonged inflammation in the gastrointestinal tract. In western countries high fat and protein consumption is shown as the main cause of inflammatory bowel diseases. However, traditional Turkish diet heavily relies on carbohydrate-based foods, the incidence of IBD in Turkey is very similar to western countries. We hypothesize that this may be due to high carbohydrate consumption. In the first part of the study, the interaction between intestinal microbiota and intestinal epithelial cells were investigated in mice fed with a high carbohydrate diet to mimic high carbohydrate consumption in Turkey. To analyze the changes in the microbiota composition and population dynamics, the stool samples of mice with fed different diets were studied by denaturing gradient gel electrophoresis (DGGE) method. To investigate the effect of different diets on inflammatory markers; pro-inflammatory and anti-inflammatory cytokine expression levels were measured by qRT-PCR in small intestine and colon tissues of mice. Moreover, in order to investigate how tight junction (TJ) proteins changed with different diets, TJ mRNA and protein levels were measured by qRT-PCR and western blotting method respectively. In the second part of the study, microbiota composition of mice fed with either ketogenic diet or fasted for

24-hour were stidied by next generation gequencing (NGS). These dietary interventions are known to increase the rate limiting step of ketogenesis, Hmgcs2 expression in intestinal stem cells. We have also studied the effect of microbial derived short chain fatty acid (SCFA), butyrate, on intestinal epithelial stem cells function and metabolism, using *in vivo* and *in vitro* methods. In order to investigate the effect of microbiota on intestinal stem cells function and Hmgcs2 expression in the proximal and distal colon, antibiotic treatments were used.

After four mounts of feeding, DGGE profiles of stool samples from high carbohydrate with zero fiber (HC-0F), high carbohydrate (HC) and western (WD) groups showed significant reduction of bacterial diversity compared with standard chow diet group (S). Immunoblotting and qRT-PCR results of cytokines and tight junction showed that the inflammation is higher in HC-0F and HC diet groups than S or LC-0F diet groups. There is also a negative correlation between bacterial richness and colonic inflammation in our results. While bacterial diversity was less in HC-0F and HC mice groups than in other groups after diet feeding, it was observed that colonic inflammation is increased.

Next generation sequencing of 24 hours fasting mice showed a significant change in microbiota of colon and small intestine lumen. Microbial derived SCFA, boosted the organoid formation capacity of proximal and distal colon at an optimum 0.5 mM concentration, increased the expression of Hmgcs2, Cpt1a and PPAR-γ. While in antibiotic treated mice, intestinal stem cells proriferation decreased and Hmgcs2 expression decreased.

Keywords: High Carbohydrate Diet, Microbiota, Inflammation, Butyrate, Hmgcs2

ÖZ

YÜKSEK KARBONHİDRATLI DİYETTE BAĞIRSAK MİKROBİYOTASI VE BAĞIRSAK EPİTEL SAĞLIĞININ ETKİLEŞİMİ

Ulutaş, Mehmet Sefa
Doktora, Biyoteknoloji
Tez Yöneticisi: Prof. Dr. Güzin Candan Gürakan
Ortak Tez Yöneticisi: Dr. Öğr. Üyesi Aysun Cebeci Aydın

Ağustos 2021, 120 sayfa

İnflamatuvar bağırsak hastalıkları (İBH) sindirim sistemi yolağında uzun sureli inflamasyonlara neden olan kronik inflamatuvar bağırsak bozukluklarıdır. Gelişmiş ülkelerde yüksek yağ ve protein tüketiminin inflamasyonun ana nedeni olduğu gösterilmiştir. Bununla birlikte, Türkiye'de bu kadar yüksek seviyede protein veya yağ tüketimi olmamasına rağmen, inflamatuvar hastalıkların görülme sıklığı artmaktadır. Bu fark ülkemizdeki yüksek karbonhidrat tüketiminden kaynaklanıyor olabilir. Çalışmanın ilk kısmında, Türkiye'de ana besin kaynağı olan karbonhidrat ile beslenen farelerde bağırsak mikrobiyotası ve bağırsak hücreleri arasındaki etkileşim araştırıldı. Mikrobiyota kompozisyonu ve değişimleri analiz edebilmek için farklı diyetler ile beslenen farelerden alınan dışkı örnekleri denaturant gradient jel elektroforezi (DGGE) metodu ile incelendi. Farklı beslenme tiplerinin inflamatuvar belirteçler üzerindeki etkisini araştırmak amacıyla; farelerin ince bağırsak ve kolon dokularındaki sitokin ekspresyon seviyeleri qRT-PCR yöntemi ile belirlendi. Ayrıca, bağırsak epitel hücreleri arasında bariyer işlevi gören tight junction (TJ) proteinlerinin nasıl değiştiğini araştırmak için TJ mRNA ve protein seviyeleri sırasıyla qRT-PCR ve western blot yöntemleri kullanılarak belirlendi. Çalışmanın ikinci bölümünde ise ketojenik diyetle beslenen veya 24 saat aç bırakılan

farelerin mikrobiyota kompozisyonları yeni nesil dizileme (NGS) ile belirlendi. Bu diyet müdahalelerinin, ketogenezin hız sınırlayıcı adımını olan Hmgcs2'nun ekspresyonunu, bağırsak kök hücrelerinde arttırdığı bilinmektedir. Ayrıca mikrobiyal türetilmiş kısa zincirli yağ asidi (SCFA) olan, bütiratın bağırsak epitel kök hücre fonksiyonu ve metabolizması üzerindeki etkisini görmek için *in vivo* ve *in vitro* çalışmalar yapıldı. Son olarak Mikrobiyotanın proksimal ve distal kolonda bağırsak kök hücresi ve Hmgcs2 expresyonu üzerine olan etkisini araştırmak için farelerde abtibiyotik muamelesi yapılarak incelendi.

Dört aylık beslemeden sonra, yapılan mikrobiyota analizi çalışmaları, lif içermeyen yüksek karbonhidratlı diyet (HC-0F), yüksek karbonhidratlı diyet (HC) ve batı tarzı diyet grubundan (WD) alınan dışkı örneklerinin DGGE profillerinde, standart diyet grubuna (S) kıyasla bakteri çeşitliliğinde önemli azalma olduğu gösterilmiştir. İnce bağırsak ve kolon dokularında sitokin ve sıkı bağlantı (tight junctions) proteinlerinin analizleri, lif içermeyen yüksek karbonhidratlı (HC-0F) ve yüksek karbonhidratlı (HC) diyet gruplarının, standart (S) veya lif içermeyen düşük karbonhidrat (LC-0F) diyet gruplarına kıyasla bağırsak dokusunda artan bir inflamasyon olduğu gösterilmiştir. Sonuçlarımızda ayrıca bakteri zenginliği ile bağırsak inflamasyonu arasında negatif bir bağlantı gözlemlendi. Diyetle beslenme sonrası HC-0F ve HC fare gruplarında diğer gruplara göre bakteri çeşitliliği daha az olurken, bağırsak inflamasyonunun da arttığı gözlemlendi. 24 saat aç tutulan farelerin ince bağırsak ve kolondan alınan örnekleri ile yapılan yeni nesil dizilemesi, kolon ve ince bağırsak içeriğinin mikrobiyotasında önemli değişiklikler olduğunu gösterdi. Mikrobiyal kaynaklı bütirat, optimum 0.5 mM konsantrasyonda proksimal ve distal kolonun organoid oluşum kapasitesini ve Hmgcs2, Cpt1a ve PPAR-γ ekspresyonunu arttırdığı gözlemlendi. Antibiyotikle tedavi edilen farelerde ise, kolon dokusunda bağırsak kök hücre proriferasyonunun ve Hmgcs2 ekspresyonu azaldığı gözlemlendi.

Anahtar Kelimeler: Yüksek Karbonhidratlı Diyet, Mikrobiyota, İnflamasyon, Bütirat, Hmgcs2

To my mother

ACKNOWLEDGMENTS

Firstly, I would like to thank my advisor Prof. Dr. Güzin Candan Gürakan and my Co-Advisor Assist. Prof. Dr. Aysun Cebeci Aydın for their precious guidance and infinite support. It is an honor to study with two valuable scientists. Their big support has continued till the end of my thesis. I would also like to thank Assist. Prof. Dr. Erkin Aydin and Dr. Ayça Lekesizcan for their significant support in this thesis.

Secondly, I would like to thank to Assoc. Prof. Dr. Ömer H. Yılmaz for his support during my research in his lab, in Massachusetts Institute of Technology. I also want to thank Dr. Chia-Wei Cheng and Dr. Gizem Çalıbaşı Koçal for helping me with their knowledge and patience.

In addition, I appreciate to Scientific and Technological Research Council of Turkey for supporting me with the 2214-A International research fellowship program for PhD students.

Finally, I am grateful to my lovely parents, the late Fatma and Ali Ulutaş, for their infinite support during my whole life. I know she is at peace wherever she is.

Last but not least, I would like to thank my friend and wife, Sacide Özlem Ulutaş, for being with me in all circumstances.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

ABBREVIATIONS

FISH : Fluorescence in situ hybridization

DNA : Deoxyribonucleic acid

rRNA : Ribosomal Ribonucleic acid

ATP : Adenosine triphosphate

DGGE : Denaturant Gradient Gel Electrophoresis

DNA : Deoxyribonucleic acid

TAE : Tris-Acetate-EDTA

PCR : Polymerase Chain Reaction

dNTP : Deoxyribonucleotide triphosphate

APS : *Ammonium* peroxosulphate

TEMED : Tetramethylethylenediamine

UPGMA : Unweighted Pair Group Method with Arithmetic Mean

BLAST : Basic Local Alignment Search Tool

RDP : Ribosomal Database Project

OTU : Operational taxonomic unit

CSLM : Confocal scanning laser microscopy

BSA : Bovine serum albumin

DAPI : 4', 6 diamidino-2-phenylindole

FA : Formamide

PBS : Phosphate buffered saline

PFA : Paraformaldehyde

CHAPTER 1

INTRODUCTION

Nutrition, which is essential for the development and survival of individual life, is defined as a critical part of health and development and better nutrition is associated with improved individual health, stronger immune systems and a lower risk of diseases (World Health Organization, WHO). Gastrointestinal tract (GIT) is exposed to various macronutrients that we consume. As the largest interface between human body and environment, the gastrointestinal tract (GIT) enlarges from the mouth to the anal cavity and can be classified as the upper and lower tract. The upper tract comprises oesophagus, stomach and small intestine (duodenum, jejunum and the ileum) and the lower tract contains the large intestine composed of the cecum, colon, rectum and anal cavity, respectively. (Figure 1.1).

The main functions of GIT can be ordered as absorption, secretion, and transport of various nutrients and water and provides physical protection against potentially irritant and antigenic substances (Verhoeckx et al., 2015; Guzman et al., 2013). In the last two decades, a dramatic increase has been observed in the incidence of intestinal diseases, especially in developed countries as well as in developing regions. This increased incidence parallels changes in lifestyle habits, diet and sanitation conditions (Shouval et al., 2017). In addition, there is increasing evidence showing the role of diet in diabetes (Shoelson et al, 2007; Qi et al., 2008; Chen et al., 2019), obesity (Shoelson et al, 2007; Mozaffarian et al., 2011) and various cancers (Divisi et al., 2006; Potentas et al., 2015) specifically in colon cancer (Divisi et al., 2006; Spyridopoulou et al., 2017).

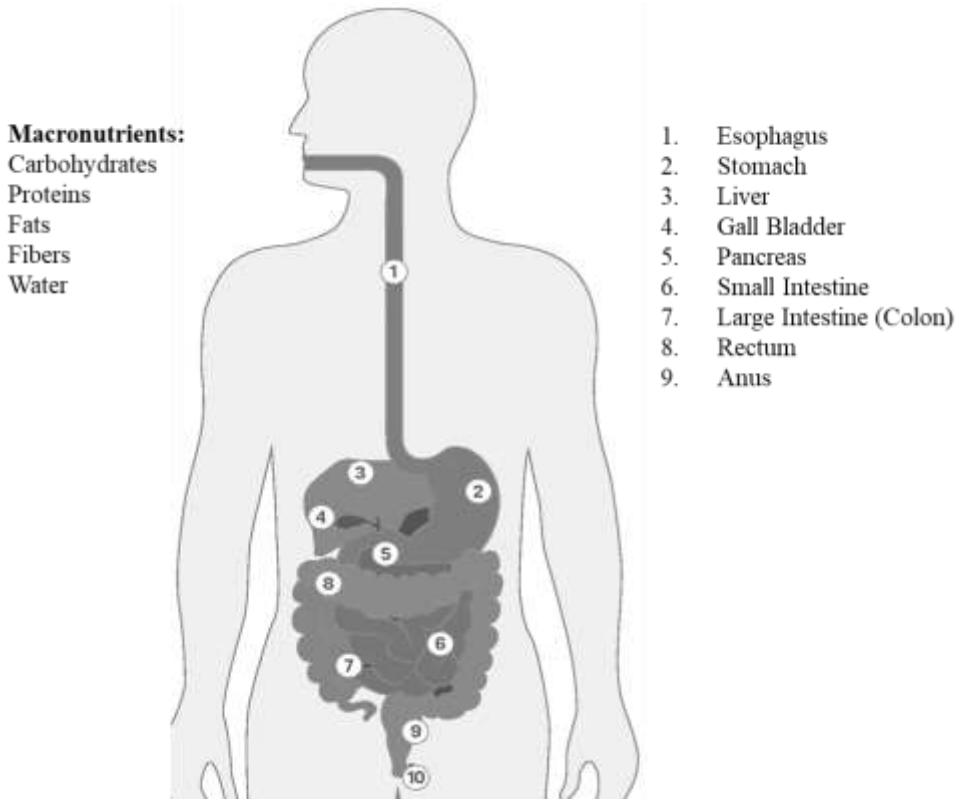


Figure 1.1: The diagram shows the macronutrients and main parts of the digestive system. (Figure was modifed from Verhoeckx et al., 2015).

It is known that diet affects intestinal microbiota and the physiology of intestinal epithelium (Guzman et al., 2013; Conlon et al., 2014). The worldwide increase of obesity and malnutrition which is defined as excesses, deficiencies or imbalances in energy or nutrient intake (WHO) has shifted the attention of researchers to the effect of diet on the intestine (Cangelosi et al. 2016).

It has been observed that different diet types cause rapid changes in the intestinal microbiota and intestinal epithelium (Lepage et al., 2011; Machiels et al., 2014). In addition to its effect on intestinal microbiota, the effect of diet on diseases has been investigated in recent years. These include obesity, cancer and inflammatory bowel diseases (IBD). Inflammatory bowel diseases (IBD) have a high incidence rate among gastrointestinal diseases. Although the effect of different diets on inflammation is not exactly known, several studies have shown that various dietary factors and microbial changes as well as changes in the immune system trigger the

onset of IBD (Brown et al, 2012; David et al. 2014; Tomasello et al 2016; De Filipo et al 2010). Modern diet style; fast food (high fat, protein and carbohydrate) and low vegetable-fruit consumption contribute to the progression of diseases such as Ulcerative colitis (UC) and Crohn's disease (CD), two main inflammatory bowel diseases (IBD) (Hou et al. 2011; Magro et al. 2013; Roushan et al., 2019). However, high fiber diets and greater consumption of fruit and vegetable based foods are typically associated with a lower incidence of these diseases (De Filipo et al. 2010; Lewis et al 2016).

While western diet (high fat, high protein, simple sugar and low fiber-based foods) is preferred over the world, the western diet is assumed to be the most important contributor to the progression of IBD (Rizzello et al., 2019; Knight-Sepulveda et al., 2015). Traditional Turkish diet heavily relies on carbohydrate-based foods (52% caloric intake from carbohydrates, TBSA, 2019). However, the increase in ulcerative colitis and Crohn's disease incidence in Turkey is very similar to western countries (Ozin et al. 2009). In a study of Turkish IBD patients, 4-fold increase of ulcerative colitis and approximately 2-fold increase of Crohn's disease is observed in the years between 2005 and 2013 (Can et al., 2019). This suggests that the relationship of inflammatory bowel diseases with dietary patterns may be different in different geographies.

Microbiota is a common term used for microorganisms living in or on the human body. Microbiota clusters are found in the gastrointestinal tract, mouth, skin, eyes or vagina (Kodukula et al., 2017). The human and other animal intestinal tract is the host to trillions of microorganisms and they live in a symbiotic relationship with their hosts. The most important regulator of intestinal microbiota is diet (David et al. 2014) and the gut microbiota has emerged as a key player in individual health and disease (Trebicka et al., 2020). Digestion and fermentation of non-digestible components by microbiota is crucial for nutrition and intestinal health. In addition, commensal microbiota has functional roles in the development and regulation of the immune system of host organisms. In this study, the effect of high-fat diet and calorie-restricted diet on inflammatory bowel diseases and intestinal microbiota is investigated.

Studies on the composition and role of intestinal microbiota have shown that the effect of intestinal microbiota on human health is more important than anticipated (Costello et al., 2012). Complex intestinal microbiota consisting of a large number of microorganisms is vital for intestinal epithelial tissue growth and intestinal cell differentiation (Soderholm et al, 2011). Changes in intestinal microbiota therefore directly affect intestinal epithelial cells. In a healthy gastrointestinal tract (GI), the mucosa with intact epithelial cells maintains its integrity and produces immunological responses to only a few number organisms. However in IBD, epithelial and mucosal integrity is deteriorated, total bacterial count is increased and bacterial diversity is decreased (Guo et al, 2017).

The effect of diet on gut health and microbiota is a hot topic for two decades. Unfortunately, studies in Turkey remains limited. Figure 1.2 and Figure 1.3 show the number of publications in USA, Turkey and worldwide according to the web of science ‘’Diet and Intestine and Health’’ and ‘’Diet and Intestine and Microbiota’’ searches criteria respectively.

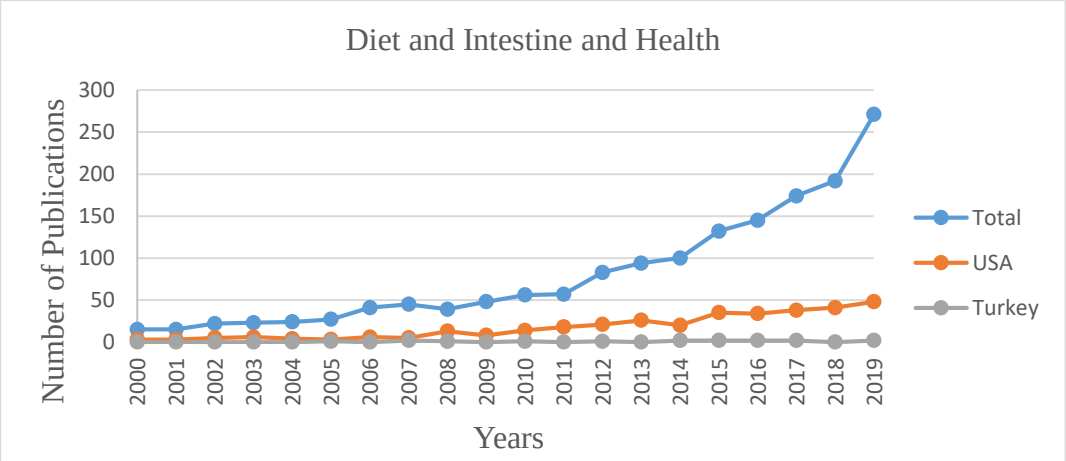


Figure 1.2: Number of publication in USA, Turkey and worldwide according to web of science ‘’Diet and Intestine and Health’’ search criteria.

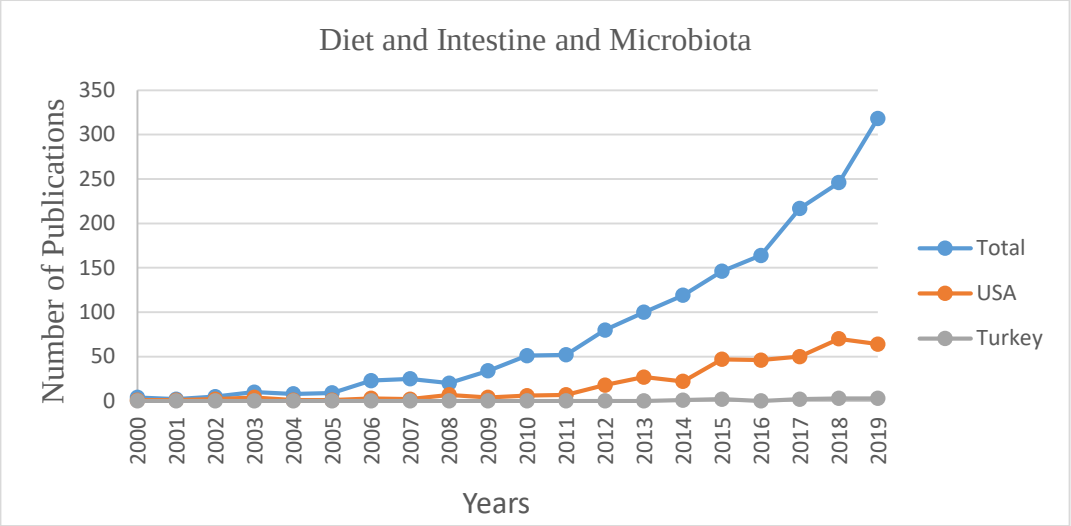


Figure 1.3: Number of publication in USA, Turkey and worldwide according to web of science ‘’Diet and Intestine and Microbiota’’ search criteria.

Diet is also one of the most important parameters affectinf the intestinal development, homeostasis and intestinal metabolism. In the mammalian intestine, unhealthy and excessive eating habits such as Western-diet or high-fat diet (HFD), which is a diet rich in simple carbohydrates and fat, trigger changes in the enterocyte subcellular structure (Zeituni et al., 2016) and disruption of the entire crypt-villus organization (Fu et al., 2019). Specifically, intestinal stem cells (ISCs) produce with specific adaptive responses to HFD or to excessive nutrition, which increases the proliferation of ISCs via increased ß-catenin signalling (Mao et al., 2013). HFD induced ISCs activation is directly related to the high lipid contents that strongly activate Peroxisome Proliferator-Activated Receptor (PPAR-δ) in ISCs, following activation of ISCs (Haller et al., 2016; Beyaz et al., 2016a; Beyaz et al., 2016b). In addition to the adaptive responses of ISCs, HFD also induces tumorigenicity of intestinal progenitor cells. Overall, these studies show that different diets can change the risk of developing IBD and bowel cancer (Alonso et al., 2018; Beyaz et al., 2016a; Nystrom et al., 2009).

1.1 Diet and Intestinal Health

Human nutrition has profound effects on health, both individually and in the community. Therefore, nutrition researches are always at the center of economic, medical, cultural and social focus. Moreover, diet is one of the important parameters of a healthy body. The food components can have various direct and indirect effects on intestinal health, and can result in either positive or negative outcomes.

The gastrointestinal epithelium provides an immune response and a protective barrier against complex luminal environment through the semi permeable and selective transformation of nutrients. It has an enormous surface area to increase the absorption of processed and digested dietary nutrients and consists of pocket-like crypts including intestinal stem cells which produce all the other cells necessary for the gut epithelium. Intestinal stem cells (ISCs) are located at the bottom of the crypts and divide every 24 hours to generate rapidly proliferative progenitors that migrate upward while they differentiate into nutrient absorbing-enterocytes, secretory cells consisting mucin producing goblet cells or hormone producing enteroendocrine cells. Another type of secretory cells are Paneth cells but they migrate downward to reside at the bottom of the small intestinal crypt. They provide the niche for ISCs maintenance by secreting signals such as EGF, NOTCH, and WNT ligands and produce antibacterial peptides which control the microbial growth (Ayabe et al., 2000; Sato et al., 2011; Koo et al., 2014; Fellow et al., 2020). The two most recently discovered cell types, M cells and Tuft cells are rarely forming from ISCs (Gerbe et al., 2011; de Lau et al., 2012). Tuft cells produce signals to regulate immune response to their underlying tissues (Ting et al., 2019) and M cells transport antigens from the intestinal lumen to cells of the immune system and initiate the immune response (Gebert et al., 1996).

The small intestine is composed of one scattered mucus layer not connected to the epithelium and includes a few bacteria, however, the colon is composed of two mucus layer, an outer and an inner layer. The inner mucus layer is tight and connected with the epithelium and is a bacteria-free environment. But the outer

mucus layer of the colon is more loose without defined lines and hosts intestinal microbiota. The colon hosts larger numbers of bacteria and a more diverse microbial population than the small intestine (Hansson et al., 2012, and Figure 1.4).

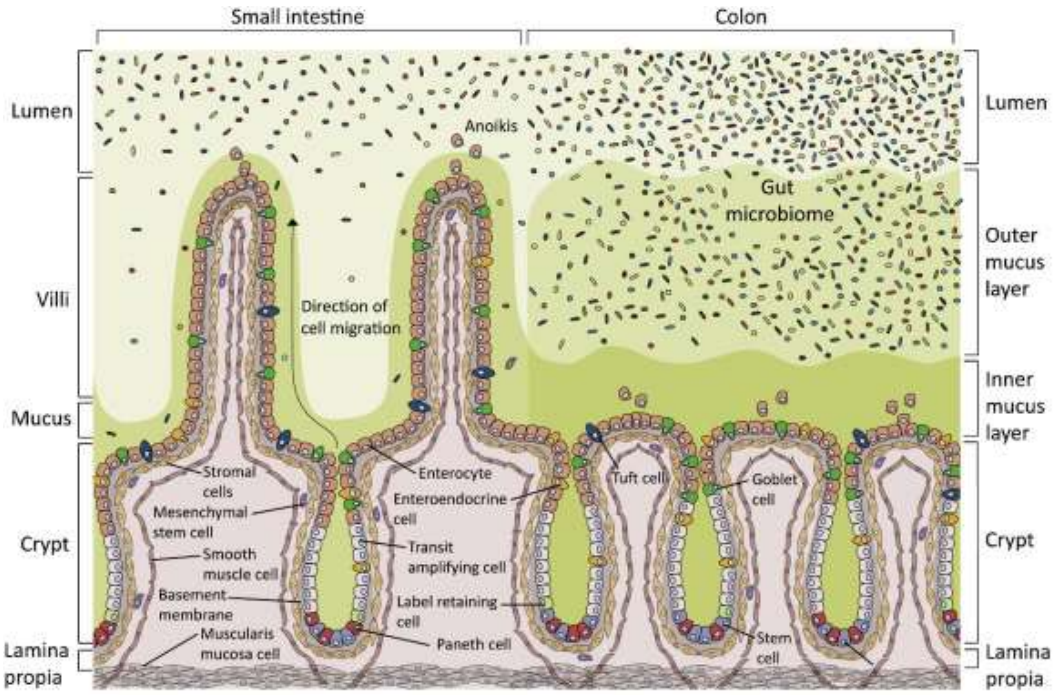


Figure 1.4: The structure of the small intestine and colon epithelium (Fellow et al., 2020).

Intestinal stem cells are located at the bottom of the crypt next to the paneth cells in small intestine and next to deep secretory cells in the colon. ISCs express markers such as LGR5 (leucine-rich-repeat-containing G-protein coupled receptor) and drive the rapid renewal of the intestinal epithelium (Barker et al 2007 and Clevers et al, 2013). Being the neighbor of ISCs, Paneth cells shape the crypt microenvironment, produce antimicrobial compounds for regulation of microbial interactions and secrete niche factors for intestinal stem cells (Clevers et al, 2013 and Beumer et al, 2021). In the mammalian intestine, $LGR5^+$ intestinal stem cells ($LGR5^+$ ISCs) are involved in the rapid regeneration of the intestinal epithelium and reshape its composition in response to dietary signals (Barker et al, 2007).

1.1.1 Inflammatory Bowel Diseases

Inflammatory bowel diseases (IBD) are intestinal diseases characterized by chronic inflammation of the gastrointestinal tract. Ulcerative colitis (UC) and Crohn's disease (CD) are two main chronic relapsing inflammatory bowel diseases (IBD). But there is also one more type of disease; IBD unspecified (IBDU) which is formerly named as indeterminate colitis. Endoscopic, histological, clinical and radiologic characteristics are used to diagnose CD and UC (Sairenji et al, 2017). When the differential diagnosis between UC and CD cannot be achieved, the term IBDU is applied (Magro et al. 2013).

Being a multifactorial complex disease, IBD is mainly affected by diet, genetic susceptibility, environmental factors, microbiome, immune dysregulation and infectious agents. (Knight-Sepulveda et al, 2015). In a healthy GI, the epithelial barrier and upper mucus layer provide a semi-permeable barrier to exclude the host from the intestinal lumen content, IBD patients show increased intestinal paracellular permeability reflecting reduced epithelial barrier function (Hollander et al 1986; May et al 1993).

During the intestinal barrier damaged commensal bacteria, food antigens, and their products leak through the intestinal wall and activate the underlying intestinal immune cells, causing tissue damage and chronic inflammation. But it is not yet clear whether barrier dysfunction causes the disease or active inflammation increases intestinal permeability (Katz et al., 1989; Buhner et al., 2006). Despite similar features, Crohn's disease and ulcerative colitis differ in genetic predispositions, risk factors and in endoscopic, clinical, and histological images. In ulcerative colitis, the inflammation process contains only the mucosa and starting from the rectum and extending continuously to the proximal segment of the colon. A characteristic symptom of ulcerative colitis is bloody diarrhea, which may be accompanied by abdominal pain or fever (Ordas et al., 2012; de Britto et al., 2013). In addition, patients with inflammation in the rectum may only develop constipation during the

inflammation period of the IBD. In Crohn's disease, the inflammation process contains the whole wall of the intestine (from the oral cavity to the rectum) and it is prograssed segmentally (Baumgart et al., 2012). Characteristic symptoms of Crohn's disease are abdominal pain, fever, loss of body mass, diarrhea and anemization (Feuerstein et al., 1998).

Lymphoid tissues are a well characterized part of the underlying intestinal immune system. Although the number and variety of immune cells vary across the gut. These cells are predominantly found in the lamina propria or in the intestinal mucosa (Mowat et al., 2014). Intestinal epithelium mainly hosts when lamina propria hosts both adaptive and innate immune system cells, which are B cells, T cells, dendritic cells, innate lymphoid cells (ILCs), macrophages, eosinophils and mast cells (Mowat et al., 2014).

Although cytokines and chemokines are soluble protein mediators that promote intestinal mucosal homeostasis, they may also be major agents of tissue damage or intestinal inflammation (Peterson et al., 2014; Neurath et al., 2014). Cytokines can affect intestinal epithelial barrier integrity both positively or negatively and may be produced from adaptive immune or resident innate cells or epithelial cells themselves but mainly by T cells (Th) and macrophages (Zhang et al., 2007; Andrews et al., 2018). A representative image of intestinal epithelium and associated immune system cells is shown in Figure 1.5 (Figure 1.5 was taken from Andrews et al., 2018).

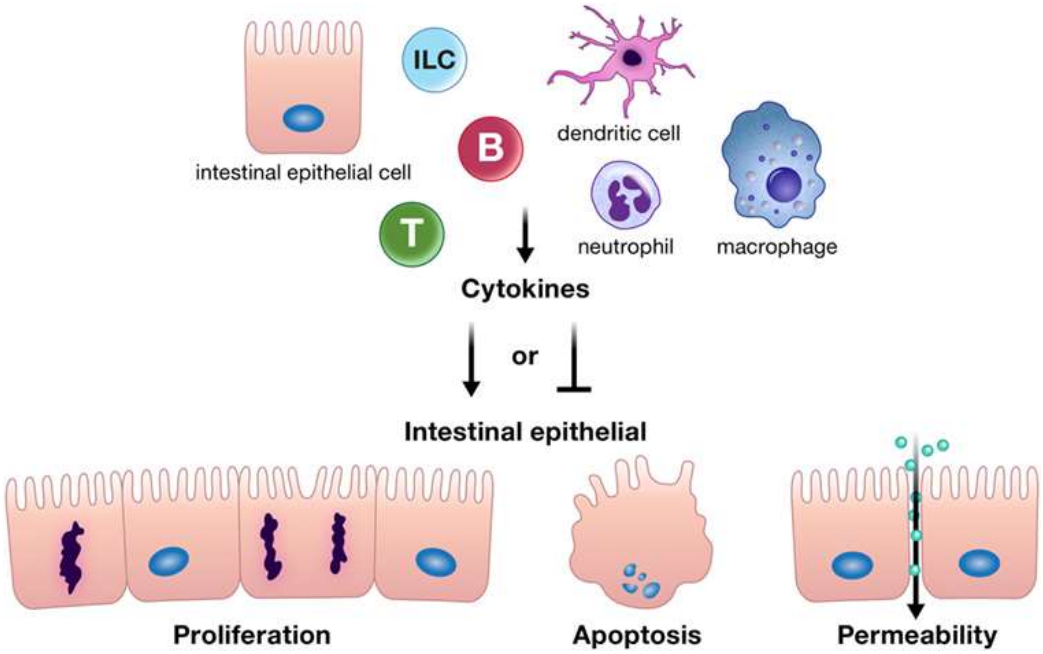


Figure 1.5: A representative image of intestinal epithelium and assocated immune system cells (Andrews et al., 2018).

For example, the interleukin 10 ablation in mice, induced inflammation, suggesting that IL-10 is essential for colon homeostasis (Andrews et al., 2018). But expression level of some other cytokines such as tumor necrosis factor (TNF), IL-6 and IL-1β are increased in inflamed intestinal tissues, suggesting that these cytokines are potential agents to trigger inflammation or damage (Neurath, 2014). In addition, studies contain conflicting results for both pro-inflammatory and anti-inflammatory markers of specific cytokines in the intestine (Neurath et al., 2014, Andrews et al., 2016). For instance, IL-10 is an anti-inflammatory cytokine but, in one study it was shown that IL-10 expression levels either remained same or were higher than in the control group (Schreiber et al., 1995). There may be a few explanations for such contradictory results in the studies; such as the action time of cytokines, the animal model and method used (Schreiber et al., 1995 and Waddell et al., 2015).

Pro-inflammatory and anti-inflammatory cytokines have diverse functions including the initiation and moderation of inflammation, and are produced by variable immune cells (Lee et al., 2018; Neurath, 2014). For example, tumor necrosis factor (TNF) pro-inflammatory cytokine is mainly produced by macrophages, dendritic cells

(DCs) and T cells and it stimulates pro-inflammatory cytokines and activates fibroblasts. Interleukin-6 (IL-6) pro-inflammatory cytokine is produced by macrophages, fibroblasts and T cells and triggers proliferation of epithelial cells and facilitates tumor growth. Another pro-inflammatory cytokine, Interleukin-1 (IL-1) is predominantly produced by macrophages and neutrophils and stimulates production of IL-6 by macrophages. One of the important anti- inflammatory cytokine, Interleukin-10 (IL10) is produced by T cells and suppresses production of pro-inflammatory cytokines by antigen-presenting cells and T cells (Neurath et al., 2014).

In addition to responding to immune cells, the intestinal epithelium is also a rich source of cytokines (Andrews et al., 2018). Anti-inflammatory IL-10 is also produced by intestinal epithelium and contributes to tolerance to commensal bacteria (Hyun et al., 2015) and pro-inflammatory cytokine IL-6 is also produced by intestinal epithelium and regulate crypt homeostasis (Jeffery et al., 2017). Furthermore, intestinal epithelial cells (IECs) can also produce many cytokines, specifically IL-18, IL-33 and IL-37. During the inflammation, expression of IL-37 in IECs are increased and they suppress innate immune responses in epithelial mucosa (Imaeda et al., 2013; Neurath, 2014).

Intestinal epithelium creates a tight but semipermeable barrier that prevents the passage of molecules such as toxins, pathogens, and antigens from the lumen to lamina propria and to the circulatory system (Turner et al., 2009). Maintenance of the gut barrier and the permeability of ions, nutrients and water is carried out by epithelial tight junctions (TJs), a multi-protein complex that creates a selectively permeable anchor between intestinal epithelial cells (Turner et al., 2006; Lee et al.,

2015).

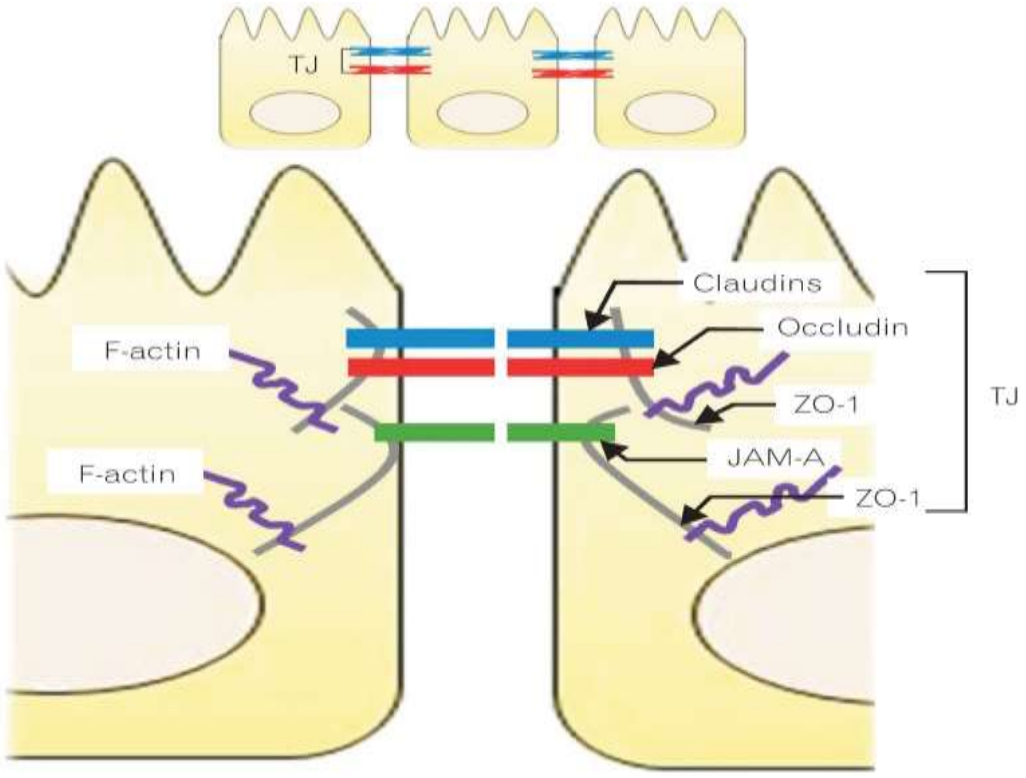


Figure 1.6: Molecular structure of the intracellular junction of intestinal epithelial cells (Lee et al., 2015).

Claudins, occludin, tricellulin and junctional adhesion molecule (JAM) are TJs transmembrane proteins and interact with zonula occludens (ZO) proteins that is a cytosolic scaffold proteins (Figure 1.6, Lee et al., 2015). After the permeation of pro-inflammatory molecules such as pathogens, toxins, and antigens, increased intracellular permeability and TJ barrier disruption results in immune system activation and finally tissue damage and inflammation occurs (Lee et al., 2015).

Although IBD is more prevalent in developed countries, it has been increasing in developing countries in recent years, and it clearly reveals the important role of the environmental factors in disease development. But it has not been established that a single environmental factor has a definite causal function (Hou et al, 2011). Especially high utilization of western diet fat and protein, simple carbohydrate

prepared foods and low vegetables – fruit has been shown as the main reason for the increase of IBD incidence (Amre et al, 2007).

1.1.2 Microbiota

The human and other animal intestinal tract is the host to trillions of microorganisms. Using advanced molecular analysis techniques such as 16S ribosomal RNA gene sequencing over the traditional culture dependent techniques have increased the number of defined colonic microbiota from 200–300 species to between 15,000 and 36,000 species (Frank et al, 2007). The human intestine hosts about 1.3 times more bacteria than human cells. But this number varies individually and sexually (Sender et al., 2016).

The composition and quantity of the intestinal microbiota varies depending on the specific microenvironment, the intestinal site and also differs between individuals (Costello et al, 2009). Both the concentration and complexity of these bacteria and other microorganisms increase throughout the intestine from gastric to colon. While the proximal gastric and duodenal population harbor mostly aerobic microorganisms to the number of 10^2–10^3 per gram of luminal contents, the cecum and colon harbor predominantly anaerobic bacteria to the number of 10^{11}–10^{12} per gram of luminal content. (Figure1.7, Sartor et al, 2008).

The Firmicutes (64% of attached colonic species) and Bacteroidetes (23 %) are the two dominant phyla and Proteobacteria (8 %), Actinobacteria, Tenericutes, Deferribacteres, Verrucomicrobia and Fusobacterial are the lower abundance phyla in the healthy adult intestinal microbiota (Sartor et al, 2008). The Firmicutes phylum includes more than 200 different genera such as, *Clostridium, Lactobacillus, Enterococcus, Bacillus,* and *Ruminicoccus*. *Clostridium* is most aboundant genus in the Firmicutes and accounts for about 95% of phylum. Bacteroidetes phylum includes *Prevotella* and *Bacteroides* as predominant genera. The Actinobacteria

phylum mainly includes *Bifidobacterium* genus (Arumugam et al., 2011 and Rinninella et al., 2019).

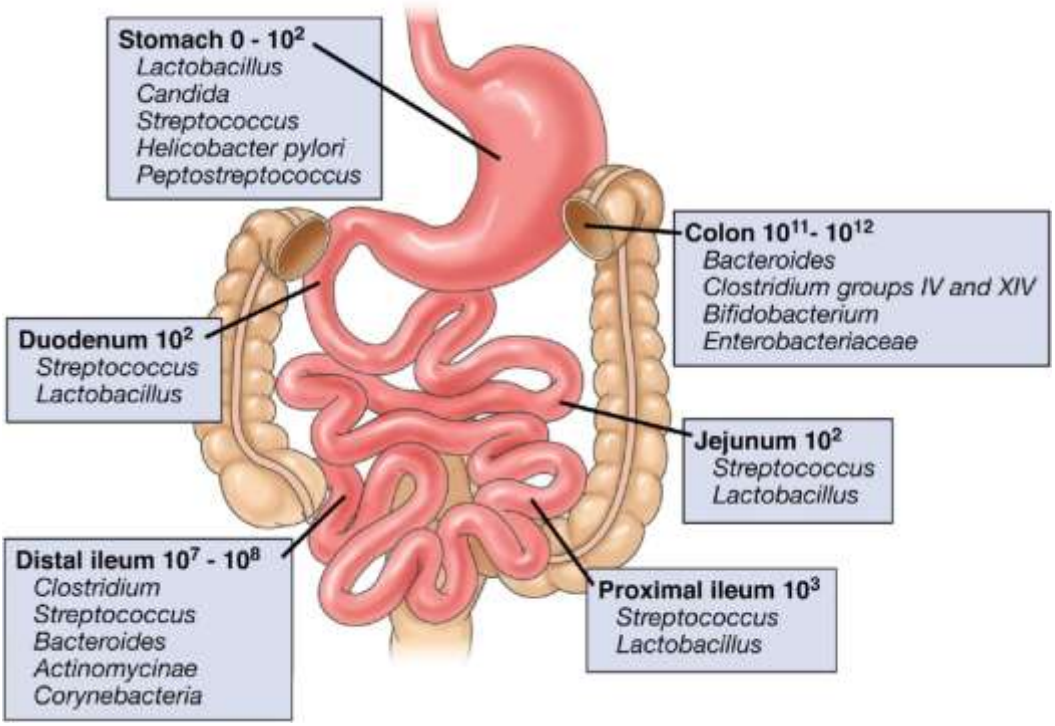


Figure 1.7: Composition and luminal concentrations of dominant microbial species in various regions of the gastrointestinal tract (Sartor et al, 2008).

The intestinal microbiota has broad effects on host physiology. The beneficial effects can be classified in three groups; nutritional effect, helping in host defense and immune development. Gut bacteria play a key modulator effect on digestion of various nutrients throughout the GIT. Synthesis and absorption of different nutrients such as lipids, bile acids, amino acids, short chain fatty acids (SCFA) and vitamins by microbiota is crucial for epithelial growth and differentiation. Also, beneficial bacteria play a crucial role in meeting the energy needs of the intestinal epithelium and in the development and differentiation of epithelial cells by digesting and fermenting some non-degradable compounds such as some oligosaccharides. (Rinninella et al., 2019). In addition, microbial flora have an important immune function by preventing infections and inhibitions of colonization by pathogenic microorganisms. The gut microbiota inhibits bacterial invasion by preserving the

integrity and maintenance of the intestinal epithelium (Khosravi et al., 2013). Lastly, this commensal microbiota has functional roles in the development and regulation of the immune system of host organisms. (Lazar et al, 2011). Metagenome analysis of fecal samples from healthy individuals revealed that the gut microbiota mainly bears the enzymes involved in metabolism of starch, sucrose, xylose, mannose, arabinose and xenobiotic compounds and production of isoprenoids, vitamins, methane and short chain fatty acids (SCFA) such as propionate, acetate and butyrate (Gill et al, 2006). These bacterial enzymes are required for the utilization of non-digestible components such as pectin xylan and arabinose. Especially *Bifidobacterium* and *Clostridium* genus digest dietary fiber to SCFA which in turn, used as an energy source mainly in the colon (Figure 1.8, Sartor et al, 2008).

Maintenance of homeostatic state of intestinal epithelium is associated with increased extracellular and secreted molecules that decrease adaptive and innate immune response, inducing protective molecules that drive mucosal barrier function and down regulating of bacterial receptors. The homeostatic gut has identified and controlled microbial antigen uptake, exclusion of living organisms, a nonresponsive level of adaptive and innate immune cells, and limited TGF-β and IL-10 secretion. In IBD, aberrant bacteria bind and surround epithelial cells in the absence or shortage of antibacterial peptides (defensins) and remain within phagocytic and epithelial cells when cellular killing mechanism is defective (Sartor et al, 2008 and Zheng et al., 2020).

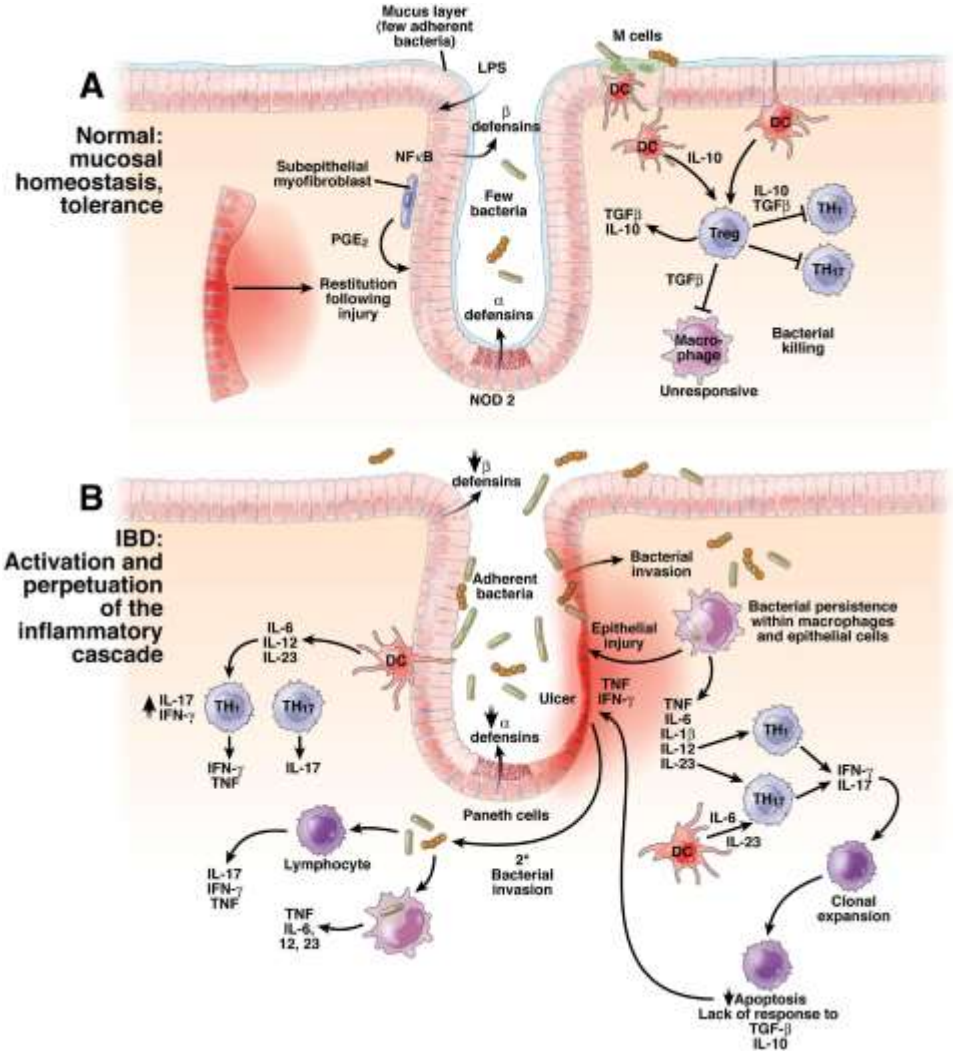


Figure 1.8: State of epithelial/bacterial and epithelial/immune interaction in intestinal homeostasis and IBD (Sartor et al, 2008).

It is known that nutrition affects the structure and activity of intestinal microbiota in the long term. In the previous studies (David et al., 2014) microbiota changes were observed in mice fed with different diets. As a result of studies, it was found that different feeding types cause rapid changes on the microbiota, and diet has been found to be the most effective mechanism on the microbiota (Lepage et al., 2011; Machiels et al., 2014). In addition to the importance of microbiota on intestinal health, the effect of intestinal microbiota in intestinal diseases has been demonstrated in recent years (Frank et al., 2007).

1.1.2.1 Methods for the Analysis of Intestinal Microbiota

Molecular methods give a new sight as a strong alternative to the traditional culture based methods for identification and analysis of microbial community. Since culture conditions are not determined for the majority of bacteria that colonize in the intestine, culture-dependent techniques lead to a biased and an unsufficient prifile of the biodiversity of the gut microbiota. Therefore, 16S rRNA gene based culture independent molecular methods such as targeted qPCR with primers, dot blot hybridization technique by using oligonucleotide probes targeting rRNA, fluorescent in situ hybridization (FISH), sequence based or traditional community fingerprinting: denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and temperature gradient gel electrophoresis (TGGE), 16S rDNA based clone library sequencing and next generation sequencing (NGS) have been used to characterize and analyze diversity of intestinal microbiota (Sheh et al, 2013). Although, new technological approaches have helped development of characterization of cultured organisms, culture conditions remain limited not only because of the inability of culturing all microorganisms, but also by technical challenges and being labor intensive. The quality of DNA is critical in the process of the 16S rRNA gene hybridization, amplification, sequencing or identification. It may lead to some bias results because of the different level of bead beating processes, chaotropic agents and microbial resistance to processing by enzymes during the DNA isolation. (Yang et al, 2013). 16s rRNA gene based techniques including Sanger sequencing of the 16S rRNA gene clone libraries, 16S rRNA sequence aided community fingerprinting (DGGE, T-RFLP and TGGE), and 16S rRNA genes analysis by next generation sequencing (NGS) provide an unbiased monitoring of the whole microbial community without prior information about the microbial diversity (Sheh et al, 2013). Being a homologous gene in all bacteria, 16S rRNA gene has highly conserved general structure and includes 9 variable regions which allow assessment of operative taxonomic units (OTUs) or phylogenetic identification of species (Gevers et al,

2005). Although, being not free of biases in PCR amplification, increased parallel sequencing by NGS exclude biases that could happen in 16S rDNA clone libraries, increase sequencing coverage, provide simple processing and high quality than other methods. Techniques like 16S rRNA gene profiling become a quite standard. Basically, NGS generated large amount of sequence data by the program that eliminate low resolution sequences and forward processing like trimming adaptor sequences and barcodes and preparing sequences for comparison. On the other hand, NGS become mostly used method for 16S rRNA profiling because of being accessible and provide lower cost for microbiota studies (Sheh et al, 2013). Next generation sequencing (NGS), being a massively parallel or deep sequencing technique, describes a DNA sequencing technology that has redirected the genomic studies. In contrast to the previous sequencing techniques, such as Sanger sequencing technology, NGS is fast, less labor and enables high throughput research (Behjati et al., 2013).

Denaturing gradient gel electrophoresis (DGGE) is a traditional DNA sequence based molecular identification technique which is used to identify and analyze bacterial composition by 16S rDNA gene. In DGGE technique, DNA molecules are separated based on melting behavior of double strand DNA. The lower melting point DNA region is opened first, while the higher melting point DNA region is then opened. The partially opened DNA molecule suddenly reduces ıts travel throughout the acrylamide gel. In conventional agarose gel electrophoresis, DNA molecules of similar size stay on the same line in the gel, but this can be overcome by using DGGE including acrylamode and urea (Lerman et al., 1984).

1.1.3 Ketogenic Pathway

Ketogenesis is a catabolic pathway involving breakdown of fatty acids and production of ketone bodies (acetoacetate, β-hydroxybutyrate, and acetone) to obtain energy by alternative ways. Mitochondrial HMGCS2 (3-hydroxy-3-methylglutaryl-CoA synthetase 2) is the the rate-limiting step of ketogenesis and catalyzes the

condensation of acetyl-CoA which is eventually converted into ketone bodies (Figure 1.9; Adijanto et al., 2014). The ketogenic pathway is preferred to meet energy needs in specific situations such as hunger, when carbohydrates are not available in sufficient amounts in the diet (Hegardt, 1999). Ketogenesis is a mitochondrial metabolic pathway mainly occuring in hepatocytes but also in colonocytes of human and other mammals (Serra et al., 1996) and takes roles in epithelial homeostasis, cell differentiation and cell signalling. (Wang et al, 2017). A recent work has demonstrated that $LGR5^{+}$ ISCs in the mouse small intestine strongly express Hmgcs2 compared to non-stem cell populations and that Hmgcs2 takes a crucial role in intestinal stem cell differentiation fate in small intestine (Cheng et al 2019).

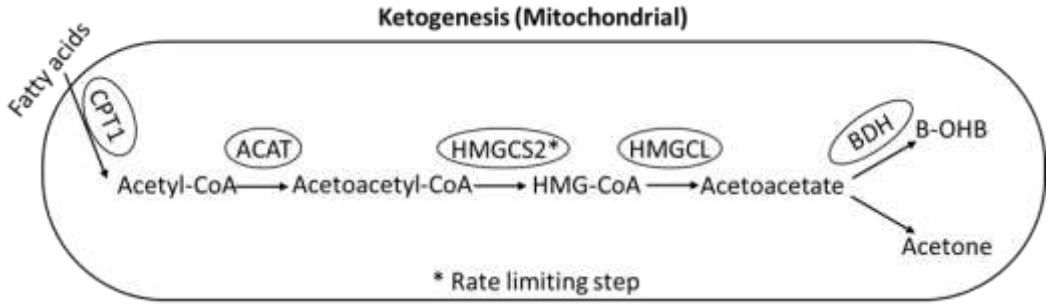


Figure 1.9: Representative drawing for ketogenic pathway. Carnitine palmitoyltransferase I (CPT1); Acetyl-CoA acetyltransferase (ACAT); 3-hydroxy-3-methylglutaryl-CoA synthetase 2 (HMGCS2); 3-hydroxybutyrate dehydrogenase (BDH); HMG-CoA lyase (HMGL). Image was modified from Adijanto et al., 2014.

1.1.3.1 Ketogenic Diet

A Ketogenic diet is composed of a high amount of fat (approximately 55% to 60%), moderate protein (30% to 35%) and small amounts of carbohydrates (5% to 10%). It was shown that ketogenic diet has a broad effect on intestinal stem cell proliferation and activity (Kappagoda et al., 2004). Although this diet was first used for the treatment of epilepsy and diabetic patients, nowadays it has become a very widely consumed diet type for many different purposes (Ludwig, 2020). It is proposed to cure Alzheimer, inflammation, neurodegeneration and cancer and many other

diseases (Pinto et al., 2018). It has been shown that ketogenic diets contribute to intestinal cell differentiation via Hmgcs2/ β-OHB (Wang et al., 2017). Ketogenic diet increases the expression of Hmgcs2 and β-OHB level in intestinal stem cells of small intestine as compared with chow diet fed mice (Cheng et al 2019). Moreover, ketogenic diet improved crypt survival/regeneration *in vivo* after damage caused by ionizing radiation (Cheng et al 2019).

1.1.3.2 Fasting

The most rapid response to starvation is an increase in serum ketone body in mammals. 4-8 hours fasting has elevated the ketone body concentration in serum and reach to millimolar concentrations within 24 hours (Foster et al., 1967; Geisler et al., 2016; Longo et al., 2021). This metabolic change occurs more slowly in humans; After 8-12 hours of fasting, ketone bodies reach 0.2-0.5 mM. With 48 hours of fasting, the concentration level rises to 1 and 2 mM (Cahill et al., 1970; Browning et al., 2012). However, the response of intestinal stem cells to starvation takes longer time in mice and humans. Studies have used 24 hours fasted mice to see the effect of fasting on hematopoietic stem cells (Cheng et al 2014) and intestinal stem cells (Mihaylova et al, 2018). For fasting research on human stem cell function, intermittent fasting (IF) or periodic fasting (PF) has been used (Longo et al., 2014; Longo et al., 2021).

Fasting improves adult stem cell function in diverse tissues (Mihaylova et al, 2018, Longo et al, 2014 and Cheng et al 2014). It has also been shown that 24 hours fasting improves LGR5+ ISCs function in the mouse intestine (Mihaylova et al 2018). They have shown increased ISCs activity in 24 hours fasted mice using organoid cultures. Organoid-forming capacity of 24 hours fasted mice significantly increases as compared with mice fed standard chow *ad libitum* (AL). Fasting also improves crypt survival/regeneration *in vivo* after damage caused by ionizing radiation. These *in vivo* and *in vitro* findings suggest that a 24 hour fasting has measurable pro-regenerative effects on ISCs. Overall, it was demonstrated that fasting or ketogenic

diet induced ketone bodies, especially β-OHB to improve LGR5+ ISCs function and affect the intestinal stem cell renewal and differentiation. Conversely, pharmacological or genetic inhibition of β-OHB in the small intestine abrogates regeneration of intestinal stem cells and stem cell differentiation fate is cahnged (Mihaylova et al 2018, Cheng et al 2019).

1.1.4 Microbial Derived Butyrate

The intestinal microbiome has long been suggested to be involved in both homeostasis and diseases in the gastrointestinal tract and as a key contributor to host physiology and metabolism. Perturbation of the intestinal microbial composition by diet or fasting regimes may underlie many human illnesses, however the mechanisms that maintain homeostasis are poorly understood (Byndloss et al, 2017). One of the important microbial products, short chain fatty acids (SCFA) have many intrinsic roles in the homeostatic state of the intestine. Resistant starch or wheat bran which include high amounts of dietary fiber are metabolized by the small intestine and large intestine microbiota and converted into short-chain fatty acids (SCFA) such as butyrate (Tremaroli et al., 2012). Recently a study has shown that a high fat diet decreased the number of butyrate producing bacteria (*Faecalibacterium* and *Blautia* bacteria) compared with a low fat diet in humans (Wan et al., 2019). Reduction of certain carbohydrates in diet may also have reduced the butyrate producing bacteria directly (Lindefeldt et al., 2019).

Based on the recent studies, PPAR-γ stands out as a strong candidate to examine the communication between microbiota and ketogenesis. Microbial derived butyrate is a well-known short chain fatty acid (SCFA) by upregulating the PPAR-γ in the intestine (Alex et al, 2012 and Byndloss et al 2017). The mechanism of how PPAR-γ senses butyrate remains poorly understood. A study has shown that PPAR-γ stimulation may occur after the inhibition of NFκB activation by butyrate (Schwab et al., 2007). Butyrate has a dose dependent responsive effect on activity of PPAR-γ and its target genes. Increased butyrate until a certain concentration strongly

activated PPAR-γ but not activated other PPARs. Treatment of colon cell line (T84) with rosiglitazone (a PPAR-γ agonist) and 1 mM butyrate, increased the expression of two important proteins in the ketogenic pathway cpt1a (Carnitine Palmitoyltransferase 1A) and Fabp3 (Fatty acid binding protein 3), however, when butyrate concentration is increased to 8mM, the expression levels of these proteins decreased compared with non-treated group. (Alex et al, 2012). Microbiota-induced PPAR-γ drives the energy metabolism of colonic epithelial cells toward beta-oxidation and limits the usable oxygen for *Enterobacteriaceae* in the intestinal lumen. So, the microbiota induced PPAR-γ signaling is a homeostatic state and prevents the expansion of pathogenic *Salmonella* and *Escherichia* (Byndloss et al 2017). In addition, fecal microbiota transplantation from high fat diet induces PPAR-γ driven programing of the liver circadian clock and this effect is neutralized by antibiotic treatment (Murakami et al, 2016). LGR5 positive intestinal stem cells (Lgr5+ISCs), compared to non-stem cell populations, strongly express enzymes of the ketogenic pathway, including the rate-limiting enzyme Hmgcs2 in the small intestine. Moreover, after feeding with ketogenic diet or 24 hours fasting, the expression of Hmgcs2 in $LGR5^{+}$ ISCs significantly increases compared to chow diet or ad libitum cohort respectively (Cheng et al, 2019). In a study that used human intestinal cell lines and mouse intestinal organoids it was shown that Hmgcs2 and beta hydroxybutyrate (βOHB) concentration are regulated by Wnt/B-Catenin/ PPAR-γ pathway and Hmgcs2 regulates epithelial cell differentiation through inhibition of glycolysis (Kim et al, 2019). Rosiglitazone treatment of wild type mice induced Hmgcs2 in the colonic epithelial cells. The deletion of the PPAR-γ gene in the small intestine results in decreasing Hmgcs2 levels by half. In addition, induction of Hmgcs2 was not observed with the rosiglitazone treatment in the PPAR-γ-KO mice. (Harmon et al, 2010). As a result it has been shown that microbial derived butyrate induces fatty acid oxidation by activating peroxisome proliferator-activated receptor-γ (PPAR-γ) (Byndloss et al, 2017 and Harmon et al, 2010). Yet, the mechanisms of how microbial derived butyrate modulate PPAR-γ and cell signaling

in the intestine and how this affects intestinal homeostasis and tumorigenesis are poorly understood.

1.2 Aim of the study

Today, modern eating habits in developed countries caused significant increases in inflammatory diseases. In particular, high in fat, protein and simple carbohydrates and consuming low amount of vegetables and fruits carry the risk of inflammatory diseases in the long term. In addition, diabetes, obesity, Crohn's diseases, ulcerative colitis and various cancers can be seen in advanced cases. The aim of this study is to examine how dietary differences lead to inflammation and change the intestinal microbiota. In order to see the effects of nutritional differences on gut epithelial health and gut microbiota, mice were fed with different diet types. Mice stool were analyzed for changes in microbiota, and colon and small intestinal tissues were examined for intestinal inflammation markers and expression of TJ proteins. To see the effect of microbial derived short chain fatty acid (SCFA), butyrate, on intestinal epithelial stem cells function and metabolism, *in vivo* and *in vitro* experiments were performed.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animal Care and Experimental Design

2.1.1 Animal Care and Diet

In the first part of the study, 5 weeks old Balb/c male mice were purchased from Erciyes University, Experimental Research and Application Center (Deneysel Araştırmalar Uygulama ve Araştırma Merkezi, DEKAM, Kayseri, Turkey). Three mice were housed per cages and mice were maintained at controlled air humidity (set point 50%), temperature (set point 22-25 ^{o}C) and under regular light-dark cycle (12h:12h). Animal procedures were evaluated and approved by Erciyes University, Central Ethics Committee and Animal Experimentation (CECAE). Diet groups were fed with different diet types and water *ad libitum.* While the standard chow diet provided from DEKAM (Kayseri, Turkey) the other diet types were purchased from TestDiet (USA). (Table 2.1).

Table 2.1: The composition of diets in this study.

		Macronutrients (Calorie %)				
Diet type	**Short Name**	**Protein**	**Fat**	**Carbohydrate**	**Fiber**	**Origin**
Standard chow diet	S	29	13	58	5.2	Optima (Bolu Turkey)
High carbohydrate diet , 0 fiber	HC-0F	15	11	74	0	TestDiet (USA)
High carbohydrate diet	HC	16	12	72	5.8	TestDiet (USA)
Low carbohydrate diet, 0 fiber	LC-0F	15	59	26	0	TestDiet (USA)
Ketogenic diet	K	33	65	2	7.8	TestDiet (USA)
Western diet	WD	16	39	45	6.7	TestDiet (USA)
High protein diet	HP	38	40	22	6.8	TestDiet (USA)

The experiment regarding effect of ketogenic diet and 24 hour fasting on intestinal stem cell function and microbiota performed at Koch Institute Integrative Cancer

Research Center, Massachusetts Institute of Technology (MIT), Massachusetts USA. C57BL/6 mice at the age of 5-6 weeks were housed in groups of 2-3 mice per cage and were maintained at controlled air humidity (set point 50%), temperature (set point 22-25 ^{o}C) and under regular light-dark cycle (12h:12h). Each group was concist of 6 mice. Diet groups were fed with a ketogenic diet (KTD, 15% protein, 5% carbohydrate and %80 fat, Research diet inc., D0604601) and water *ad libitum.*

2.1.2 Experimental Design and Sample Collection

Animal studies were carried out with 7 groups with each group consisting of 6 mice. 5 weeks old mice were first fed with a mixture of standard chow diet and target diet for one week. Then animals were fed with different diets for 4 months (16 weeks). During the feeding, mice stool was collected and mice body weight were measured at time 0 and weeks 1, 2, 3, 5, 9, 13, 17. Stool samples were collected in sterile Eppendorf tubes and stored at -80 °C until analysis.

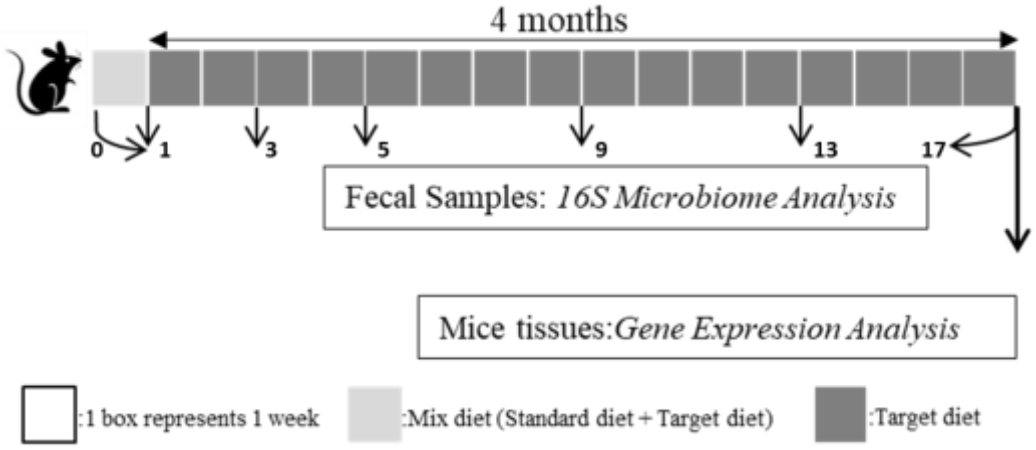


Figure 2.1: Schematic representation of experimental design: 5 weeks old, Balb/c male mice were fed 17 weeks with 7 different diets. The first week, mice were fed with a mixture diet (standard chow diet + target diet), then mice were fed 16 weeks only with target diet. Numbers (0, 1, 3, 5, 9, 13, 17) indicate the time (weeks) that stool samples were collected. One box (□) represents one week.

After 4 months of feeding with different diets, mice were euthanized with ketamine/ xylazine cocktail injection. For protein analysis, small intestine and colon tissue samples were placed in Eppendorf tubes. For real time PCR analysis, small intestine

and colon tissue were placed in Eppendorf tubes with RNanse later. All tissue samples were snap frozen in liquid nitrogen and then were taken to -80 C until study. Schematic representation of experimental setup is shown in Figure 2.1.

Fasting and ketogenic diet experiments were performed at Massachusetts Institute of Technology (MIT), Massachusetts, at the Koch Institute Integrative Cancer Research Center with 6 C57BL/6 mice in each group. Control mice group was fed with a balanced standard chow diet and water *ad libitium*. Diet group was fed with a ketogenic diet (KTD) (Research diet inc., D0604601) and water *ad libitium.* The first experimental diet group (control and ketogenic) was fed for 4 months to see the changes in microbiota and mice intestinal health. For the fasting experiment chow (standard) food was taken out from the fasted mice group. Control group was fed as *ad libitum* (Figure2.2: Schematic representation of experimental setup). Stool samples were collected and mice weights were measured for each experiment set.

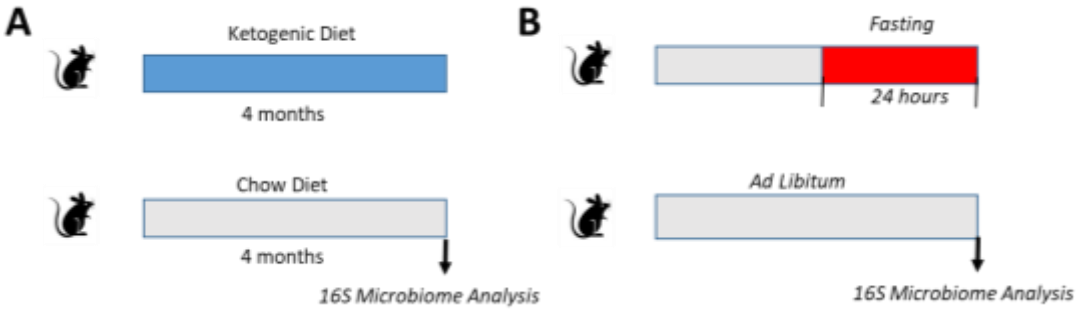


Figure 2.2: Schematic representation of experimental design of ketogenic diet and fasting experiment: (A) Ketogenic diet experimental setup; 5-6 weeks old C57BL/6 mice were fed with ketogenic diet and chow diet for 4 months. (B) Fasting experiment setup; 5-6 weeks old C57BL/6 mice were fasted for 24 and control group were fed ad libitum. After the diet intervention mice were sacrificed for 16S microbial analysis.

2.2 DNA Extraction and PCR amplification

2.2.1 DNA Extraction from Stool Samples

Bacterial DNA was extracted from mice stool samples that were collected at weeks 1, 2, 3, 5, 9, 13, 17 by using the AxyPrep Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences) according to the manufacturer's protocol with minormodifications. Firstly, stool samples were weighted between 300-400 mg then, suspended in 1 ml PBS (Phosphate buffer saline) solution and centrifuged at 12000 g for 10 minute. Then the isolation kit protocol was followed. After DNA isolations, samples were run with agarose gel electrophoresis and visualized with Gel DoC XR (Bio-Rad, Hercules, CA, USA), then DNA concentrations were measured with Nanodrop 2000 (Thermo Scientific, MA, USA). Extracted DNAs were stored at -20°C until use.

During the Ketogenic diet or Fasting experiments, mice stool samples were collected in sterile Eppendorf tubes. After the diet or fasting experiments, mice were euthanized with cervical dislocation. Intestinal lumen content was collected for microbiota analysis. DNA isolation of stool samples and intestinal lumen content samples were isolated by Quick-DNA Fecal/Soil Microbe Microprep Kit (Zymo Research, D6012). All stool and intestinal content samples and isolated DNA samples were stored at -80 °C until microbiota analysis.

2.2.2 Polymerase Chain Reaction (PCR)

The hyper variable 16S ribosomal DNA gene, V3 region was amplified using by using GC357F (5'CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGC ACGGGGGGATTACCGCGGCTGCTG3') and 518R (5'CCTACGGGAGGCAGCAG3') primers. The Polymerase chain reaction (PCR) mixture including of 3 µl of isolated bacterial DNA, 20 µl of 2x AmpMasterTM Taq

(GeneAll Biotechnology, South Korea), 1,5 µl of each primer (10 pM), and distilled sterile water to final volume of 40 µl.

PCR was performed on a C1000Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA). Modified touchdown PCR program was conducted with pre denaturation step at 94 ^{o}C for 5 min, followed by 20 cycles consisting of 30 second at 94 ^{o}C, 30 seconds at initially set at 65 ^{o}C and was then decreased by 0.5 ^{o}C every cycle until it reached 55 ^{o}C and 30 seconds at 68 ^{o}C. Then, 15 additional cycles were carried 30 second at 94 ^{o}C, 30 second at 55 ^{o}C, 30 second at 68 ^{o}C, and finally 7 min of extension at 68 ^{o}C. Quality of PCR products was analyzed by agarose gel electrophoresis and visualized with Gel DoC. All PCR products were stored at 4 ^{o}C until DGGE analysis.

2.3 Denaturing gradient gel electrophoresis (DGGE)

2.3.1 Microbial analysis by DGGE

The DGGE analysis was used to characterize the microbial population in the gastrointestinal trackt (GIT) and to analyze the changes between the different groups of mouse and the effect of diet type to the microbial community. DCode Universal Mutation Detection System (BioRad, Hercules, CA, USA) was used to analyze amplified PCR product from stool samples of different diet groups. 8% polyacrylamide (37.5:1 acrylamide-bisacrylamide) gel conteining urea and formamide gradient from 35% to 54% were used. Gels were firstly run 20 minutes at 100 V and then 5 hours at 200V in 1×TAE (Tris (20 mM), acetate (10 mM), EDTA (0.5 M), pH 7.4). After running, gels were stained with Ethidium Bromide solution (Roth) for 25 minutes and destained for 15 minutes and imaged. Gels were normalized with a lab-made ladder.

2.3.2 DGGE Profile Analysis

DGGE Gel images were aligned and analyzed using GelComparII (Applied Maths) by running the same ladder on both sides of the gels. Dice similarity coefficient was used to compare gel profile as 0.5 optimization and 0.5 tolerance level. The UPGMA (Unweighted pair group method with arithmetic mean) was performed to compare the relations of smaples in dendograms. Firstly, dominant bands displaying different electrophoretic mobility in the DGGE gels were sequenced and the microorganisms represent each dominant bands were identified. Then PCR products of these microorganisms were used as standard (lab made standard, reference strains) for further DGGE in this study. DNA fragments, showed same gel mobility represent the same species and the lab made standard were able to identify the bacterial strains from the stool samples of different feeding groups (Donskey et al., 2003).

2.3.3 Sequencing of DGGE bands.

Strong DGGE bands that frequently appeared in stool samples were excised with a sterile scalpel under the UV light. Excised gel slices were eluted into 20 ml of sterile water overnight at 4 ^{o}C. The resulting DNA solution was then amplified again using the V3 16S rRNA forward primer (without GC clamp) (357F 5' ATTACCGCGGCTGCTGG 3') and the reverse primer (518r 5' CCTACGGGAGGCAGCAG 3'). The 40 µl PCR reaction mixture and PCR conditions were same as mentioned above. PCR products (194 bp) were sequenced on an ABI 3730 automated sequencing system. (Medsantek, Istanbul, Turkey). The retrieved sequences were compared with NCBI GenBank databases using the BLAST tool. All stool samples were processed with Next Generation Sequencing (NGS) for analysis of microbial composition.

2.3.4 Next Generation Sequencing

Isolated stool DNA samples were send to the DCM Microbiome services at Massachusetts Institute of Technology for 16S microbial NGS and analysis. Forward (515F) (GTGCCAGCMGCCGCGGTAA) and reverse (806R) (GGACTACHVGGGTWTCTAAT) primers were used to amplify variable V4 region of the 16S rRNA gene. Paired-end Illumina libraries were **sequenced using** the **Illumina** MiSeq platform at the Biomicro Center (MIT, Cambridge, M A) (Perrotta et al., 2020).

2.4 Quantitative Real-Time PCR

2.4.1 RNA Isolation

100 mg small intestine and colon tissue were homogenized in RiboX^{TM} (GeneAll Biotechnology, South Korea) and total RNA was isolated according to GeneAll Hybrid-R RNA purification kit (GeneAll Biotechnology, South Korea). RNA concentrations of samples were measured with Nanodrop 2000.Extracted RNAs were stored at -80°C until use.

2.4.2 cDNA synthesis

Isolated RNA was converted to cDNA with High Capacity cDNA Reverse Transcription kit (Applied biosystem, Thermo Fisher Scientific, CA, USA). Reverse transcription PCR were performed according to manufacturer protocol and 2 µg of total RNA were used as template. cDNA synthesis was conducted by using CFX96 Real Time PCR Detection System (Bio-Rad, Herculas, CA, USA). The cycling conditions were: first step at 25 ^{o}C for 10 min, second step at 37 ^{o}C for 120 min and last step at 85 ^{o}C for 5 min then samples were kept at 4 ^{o}C until further analysis.

2.4.3 Quantitative PCR

Quantitative real time PCR (qRT-PCR) analyses were performed using CFX96 Real Time PCR Detection System. The reaction mixture consisted of Maxima SYBR green qPCR master mix (Thermo Scientific, MA, USA), one µmol/L of each primer and standardized cDNA as template. qRT-PCR was performed in triplicate for each sample for 40 cycles with a two-step program (5 s of denaturation at 96°C and 10 s of annealing at 60°C). Target gene copy numbers were normalized against the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Cytokine genes were studied with pro-inflammatory cytokines (interleukin -1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNFα)) and anti-inflammatory cytokines (interleukin-10 (IL-10), transforming growth factor beta (TGF-β)). Tight junction protein genes were evaluated with Occludin, Zona Ocludens (ZO-1) and Claudin-1. The primers that are used in qRT-PCR studies are shown in Table2.2.

Table 2.2: The primers that are used in this study

Primer Names	Primer sequence	An. Temp.	References
IL-10	5'-TCAAGGATGCACATCAAAAGGC-3' 5'-AGGCAGCAACTTCCTCCCT-3'	60 ºC	Harvard University primer databank (https://pga.mgh.harvard.edu/primerbank/)
IL-1 β	5'-GCAACTGTTCCTGAACTCAACT-3' 5'-ATCTTTTGGGGTCCGTCAACT-3'	61 ºC	Harvard University primer databank (https://pga.mgh.harvard.edu/primerbank/)
TGF-β1	5'- CAGAGCTGCGCTTGCAGAG -3' 5'- GTCAGCAGCCGGTTACCAAG -3'	60 ºC	(Eijkelkamp et al, 2016)
IL-6	5'-AGTTGCCTTCTTGGGACTGA-3' 5'-CAGAATTGCCATTGCACAAC-3'	60 ºC	(Takuda et al., 2012)
TNFα	5'-GAACTGGCAGAAGAGGCACT-3' 5'-AGGGTCTGGGCCATAGAACT-3'	60 ºC	(Wang et al., 2012)
Occludin	5'-TTGAAAGTCCACCTCCTTACAGA-3' 5'-CCGGATAAAAAGAGTACGCTGG-3'	61 ºC	Harvard University primer databank (https://pga.mgh.harvard.edu/primerbank/)
ZO-1	5'-CCAGTCCCTTACCTTTC-3' 5'-CTCCTCCAGTCTGACATTAG-3'	52 ºC	(Wang et al., 2012)
Claudin-1	5'- CTGGGTTTCATCCTGGCTTC -3' 5'- TTGATGGGGGTCAAGGGGTC -3'	55 ºC	(Van den Bossche et al., 2012)

2.5 Western Blotting

Western blotting analyses were performed from small intestine and colon samples, crypts and organoid. Total protein was extracted from large and small intestine tissue using modified RİPA lysis buffer (Thermo Fisher Scientific) including Pierce Protease and Phosphatase Inhibitor Mini Tablets (Thermo Fisher Scientific). The tissue was homogenized by ultrasonic homogenizer (Sonopuls, Bandelin, Berlin, Germany) for 2-3 minutes, followed by the incubation for 3h on ice. The resulting solutions were centrifuged at 13000 rpm for 15 min at 4°C and supernatant part was taken to a new eppendorf tube. Protein concentration was measured with Pierce™ BCA Protein Assay (Bio Rad) according to bovine serum albumin (BSA) protein standard and the protein amounts were adjusted to the minimum of the sample by adding RIPA lysis buffer to normalize samples. Then, equal amounts (20 µg/sample) of tissue lysates were solubilized in gel loading sample buffer and boiled 95 °C for 5 min. Protein samples were subjected to SDS-PAGE, using precast gels (on 4%-15% Mini-Protean Precast Gels, Bio-Rad Laboratories, CA, USA), for 90 minutes at 150 Volts. Proteins were then transferred to a PVDF membrane (Bio-Rad Laboratories, CA, USA) at constant 350 mA for 1 hour using a wet system (Bio-Rad Laboratories, CA, USA). Membranes were incubated with primary antibodies overnight at 4°C. It was then incubated with horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody (1:10000 Invitrogen 31460) or anti-mouse secondary antibody (Cell Signalling 7076) for 1 hour at 4°C. Antibody-labeled proteins on the membrane were detected using SuperSignal Chemiluminescent Substrates (Thermo Fisher Scientific, MA, USA), which upon exposure to HRP luminesce. The light from this reaction was captured by a ChemiDoc MP Imaging System (Bio-Rad Laboratories, CA, USA) and analyzed using Image Lab software (Bio Rad). The primary antibodies that used in this stusy was listed in Table2.3.

Table 2.3:The list of primary antibadies that used in this study.

Antigen	Host	Company (Cat)	Application	
			Western B.	IHC
ZO-1	Rabbit	Invitrogen 40-2200	1;1000	-
Occludin	Rabbit	Invitrogen 40-4700	1;1000	-
Claudin 1	Rabbit	Invitrogen 717800	1;1000	-
TNF	Rabbit	AFG Bioscience A15635	1;1000	-
IL-1β	Rabbit	AFG Bioscience A14428	1;1000	-
Hmgcs2	Rabbit	Abcam Ab137043	1;2000	1;200
Cpt1	Mouse	Abcam ab128568	1;1000	1;500
Gapdh	Mouse	Invitrogen MA5-15738	1;1000	-

2.6 Organoid experiments

After sacrificing mice, small intestine or colon tissues were washed with cold PBS and incubated in 5 µM EDTA in PBS for 45 minutes in cold room. Dissociated crypts were filtrated with 70 ul mesh and filtrate was centrifuged at 300 g for 5 minutes. Isolated crypts were counted under microscope and embedded in Matrigel™ (Corning 356231 Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix) at 5–10 crypts per µl. Twenty-five µl droplets of Matrigel™ with crypts were plated onto a flat bottom 48-well plate (Corning 3548) and allowed to solidify for 10-15 minutes in a 37°C incubator. Then 300 µl modified Advanced DMEM (Gibco) were added on solidified Matrigel™ with crypts. Advanced DMEM (Gibco) are supplemented by EGF 40 ng ml−1 (R&D), R-spondin 500 ng ml−1 (R&D or Sino Biological), Noggin 200 ng ml−1 (Peprotech), N-acetyl-L-cysteine 1 µM (Sigma-Aldrich), N2 1X (Life Technologies), Chiron 10 µM (Stemgent), B27 1X (Life Technologies), Y-27632 dihydrochloride monohydrate 20 ng ml−1 (Sigma-Aldrich). Plates were incubated at 37°C in fully humidified chambers containing 5% CO2 and medium were changed every three days. Clonogenicity (colony-forming efficiency) was calculated by plating 100–300 crypts and assessing organoid formation 3–7 days or as specified after initiation of cultures (Beyaz et al, 2016). Isolated proximal and distal colon crypts were cultured. Also, to see the effect of

microbial derived butyrate, crypts were treated with 0, 0.25, 0.5 and 1 mM butyrate and organoid frequency of proximal and distal colon crypts were tested.

2.7 Antibiotic Treatment

Antibiotic treatment was performed to the C57B/6 mice for 24 days. Each group; control and antibiotic (Abx) treated group had 4 mice (2 males, 2 females). Antibiotic cocktail was composed of 1 g/L streptomycin (Sigma S6501-25G), 1 g/L gentamycin (Sigma G3632-10G), 1 g/L neomycin (Sigma N1876-25G), 1g/L metronidazole (Sigma M3661-25G) and 0.5 g/L vancomycin (Sigma 94747-1G) (Zarrinpar et al, 2018). The water of the Abx group was changed with antibiotic cocktail water. Stool samples were collected for the validation of microbial depletion and body weight measured at defined intervals.

2.8 Crypt Depth Measurement and Immunohistochemistry Analysis

After the experimental feeding or antibiotic treatment, mice were sacrificed. Small intestine and colon tissues were cleaned with cold PBS and were incubated in 4% formaldehyde solution for 3 days. Paraffin embedding and sectioning of tissues were performed according to the previously described protocol (Gul et al., 2019). Then, samples were deparaffinized. Deparaffinization process consists of 3 times 15 minutes in xylene, 5 minutes in 100% ethanol, 5 minutes in 95% ethanol, 5 minutes in 80% ethanol and 5 minutes in tap water. After that, samples were stained in hematoxylin and eosin solutions and transferred to 80%, 95% and 100% ethanol solutions respectively. Lastly samples were transferred to the xylene and mounted with Vectashield. Small intestine and colon samples were visualized and photographed under the Leica (DMRB, Bx15) microscope with 20X and 40X magnifications. Crypt depth was measured by using the Image J program.

For immunohistochemical analysis, after the deparaffinization process, antigen retrieval was carried out with Borg Decloaker RTU solution (Biocare Medical) in a

pressurized Decloaking Chamber (Decloaking Chamber NxGen, Biocare Medical). The Vecta stain Elite ABC immunoperoxidase detection kit (Vector Labs PK-6101) followed by Dako Liquid DAB+ Substrate (Dako) was used for visualization. The Vecta stain Elite ABC immunoperoxidase detection kit (Vector Labs PK-6101) followed by Dako Liquid DAB+ Substrate (Dako) was used for visualization. Antibodies used in this study were: Hmgcs2 (1:2000, Abcam Ab137043), BrdU (1:2000), Biotin-conjugated secondary donkey anti-rabbit (1:500, Jackson Immuno Research) were used (Table 2.3).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Sample Collection and Body Weight

During feeding, mice body weights were measured at time zero (0) and weeks 1, 2, 3, 5, 9, 13, 17. Figure 3.1 shows mice body weights normalized to the average starting weight of each group on day zero. After starting the diets, it was observed that, mice body weight decreased except standard chow diet group and western diet group. Changes in diet frequently lead to short-term weight loss (Matikainen-Ankney et al., 2020). However, similar to the standard chow diet group whose diet remained the same, western diet group did not show any weight loss in the beginning time. The calories percentage of the macronutrients in the Western diet is similar to the Standard chow diet this may explain why Western diet fed mice did not need any adaptation time after the diet changed. After adaptation to the new diets, all groups started to gain weight and there were not any significant differences in weight gain between diet groups.

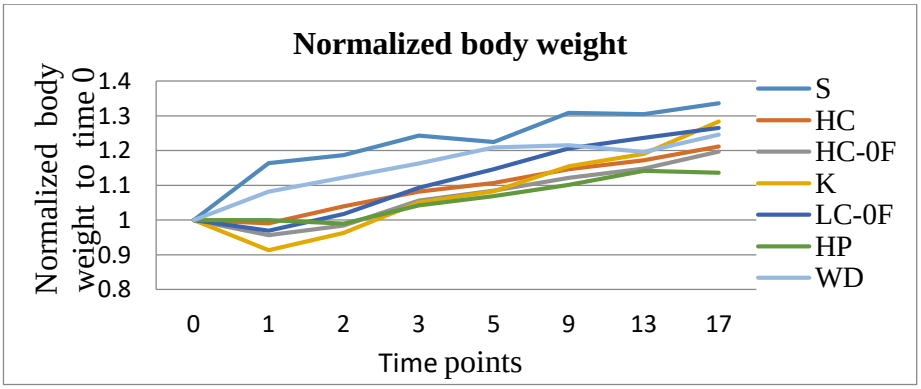


Figure 3.1: Normalized body weight: Mice body weights which were normalized to the average starting weights (time zero) of each group. (S: Standard chow diet, HC-0F: High carbohydrate with zero fiber, HC: High Carbohydrate diet, LC-0F: Low Carbohydrate diet with zero fiber, K: Ketogenic diet, WD: Western diet, HP: High Protein diet).

High fat diet feeding induces body weight increase not only in humans but also in different animal models (Buettner et al., 2007; Hariri et al., 2010 and Beyaz et al., 2016). The low carbohydrate diet with zero fiber (LC-0F) and ketogenic diet (K) contain 59% and 65% percentages of fat respectively in this study. But neither of these two groups showed a higher body weight increase than the standard chow diet group. The reason for the lower body weight in ketogenic diet than standard chow diet in this study may be due to increase of some metabolic pathways such as fatty acid oxidation and ketogenic pathways and decrease of lipid synthesis pathway. Animal studies with the ketogenic diet have shown that these metabolic changes are thought to be the main reason for the weight stability or decrease which is consistent with our ketogenic diet results (Kennedy et al., 2007, Cheng et al, 2019). Moreover, in our study, high fat low carbohydrate diet with zero (LC-0F) does not include fiber. In this regard this diet differs from the high fat diet. This could be the reason for the lower gain weight of the LC-0F diet in this study. The microbial changes and decrease in microbial diversity in the absence of fiber may be the underlying cause of lower gain weight. A study, parallel with our result, has been shown that microbial diversity is negatively correlated with weight gain and while it is positively associated with fiber intake (Menni et al., 2017). Also, in many studies, the increase in body weight in mice fed high fat diet not observed with upon antibiotic administration or in germ free mice which are lack of all microorganisms (Bäckhed et al., 2004; Kuang et al., 2019 and Zhao et al., 2020). This may be an alternative explanation of the association of lower diversity and lower gain weight in LC-0F diet in this study.

The crypt depth in the intestine is an important parameter of intestinal development and animal health. And diet has the potential to reshape the crypt structure (Chwen et al, 2013). Thus, the crypt depth of the hematoxylin and eosin stained colon tissues were measured to study the effects of diet on crypt development and structure. Significant differences were found in the crypt depth of the colon (Figure 3.2A and Figure 3.2B). Significance to standard diet is seen only with the western diet, and WD has significantly lower crypt depth against ketogenic diet, high carbohydrate

(HC) diet, and high protein (HP) diet, along with the standard diet. In addition, low carbohydrate (LC-0F) diet has shown remarkable decreased crypt depth compared to HC, K, and HP groups (Figure3.2A and Figure 3.2B). Absence of fiber in high carbohydrate diet and low carbohydrate diet may be one of the reasons for decreased crypt depth in LC-0F group (Kuzmuk et al, 2005).

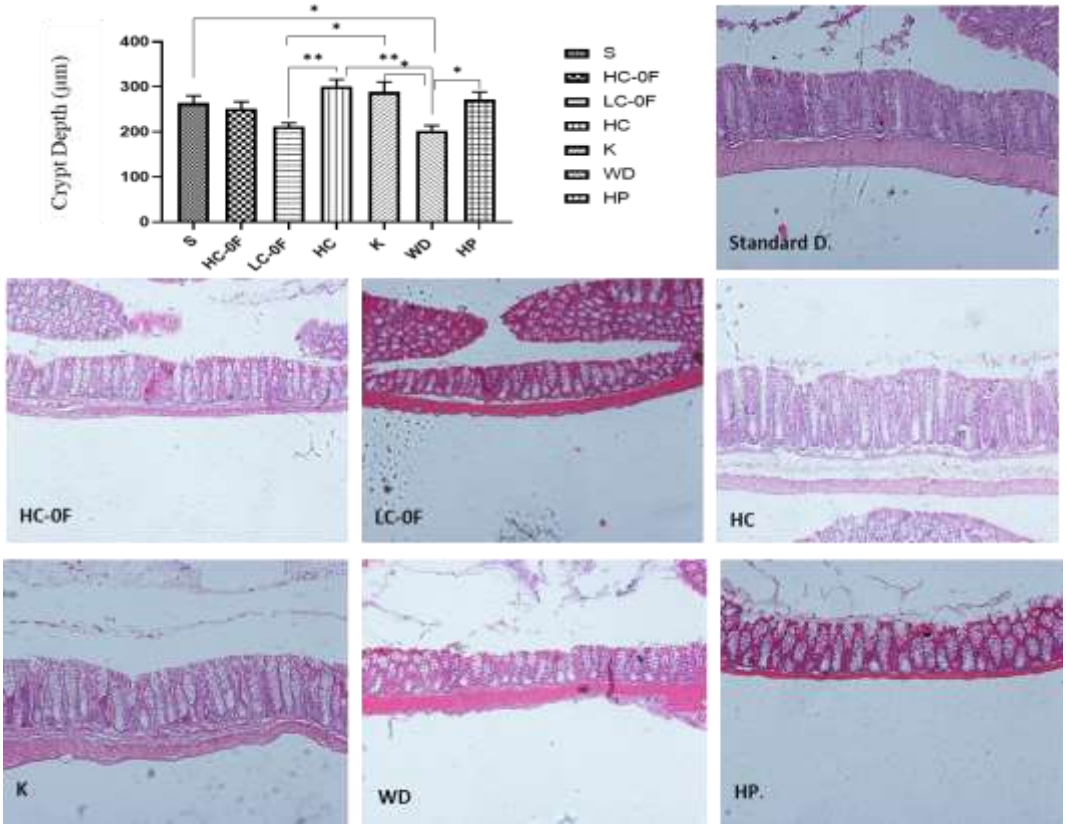


Figure 3.2: Crypt depth of colon samples in diet groups. Quantification and representative images crypts depth of hematoxylin and eosin stained colon tissues of diet groups. Data represents n > 3 images per mouse and n > 3 mice per group. The images are 20X magnification. Statistical analyses were performed with Graph Pad Prism 8. Data are mean ± SEM by Student's t test, unpaired. *p < 0.05, **p < 0.01. (S: Standard chow diet, HC-0F: High carbohydrate with zero fiber, HC: High Carbohydrate diet, LC-0F: Low Carbohydrate diet with zero fiber, K: Ketogenic diet, WD: Western diet, HP: High Protein diet).

Body weights of chow and ketogenic diet fed mice for 4 months were measured before and after the feeding process (Figure 3.3A). There was a slight increase in body weights of the ketogenic diet group compared to the chow diet group (Figure

3.3A). Also, body weights of fasted mice for 24 hours and *ad libitum* group were measured before and after the experiment (Figure 3.3B). There was a decrease in 24 hours fasted group compared to time zero body weight, but there were not any significant differences in ad libitum group (Figure 3.3B).

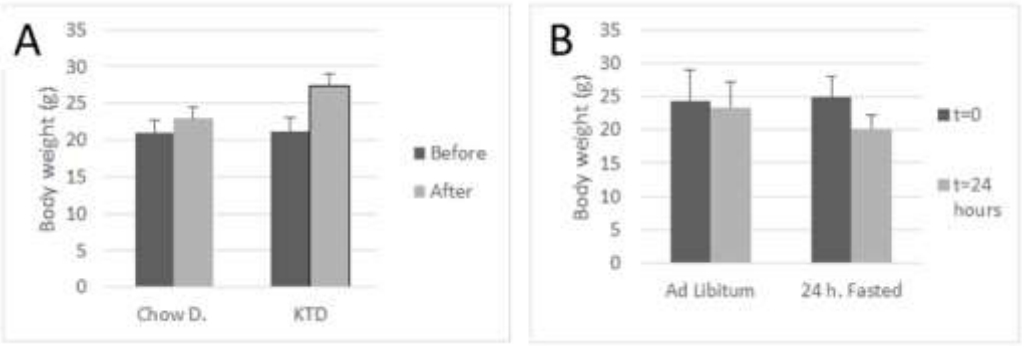


Figure3.3: Body weights. (A) Body weights of mice fed with ketogenic diet and chow diet for 4 months. n > 3 mice per group. (B): Body weight of 24 hours fasted and ad libitum group before and after the fasting. n > 3 mice per group.

3.2 DNA Extraction and PCR Amplification

3.2.1 DNA Extraction

Bacterial DNA was extracted from stool samples which were collected during the feeding of mice at time 0 (zero) and weeks 1, 2, 3, 5, 9, 13, 17. Extracted DNA samples were run with agarose gel electrophoresis and visualized with Gel DoC Xr (Biorad). Moreover, DNA concentration and quantity were analyzed by Nanodrop 2000 (Thermo Scientific, MA, USA). Gel electrophoresis image of extracted DNA samples of time 0 (zero) stools are shown in Figure3.4 and DNA concentrations and quantity of same samples are shown in Table3.1. Extracted DNA were enough for further polymerase chain reaction and 16S DNA amplification.

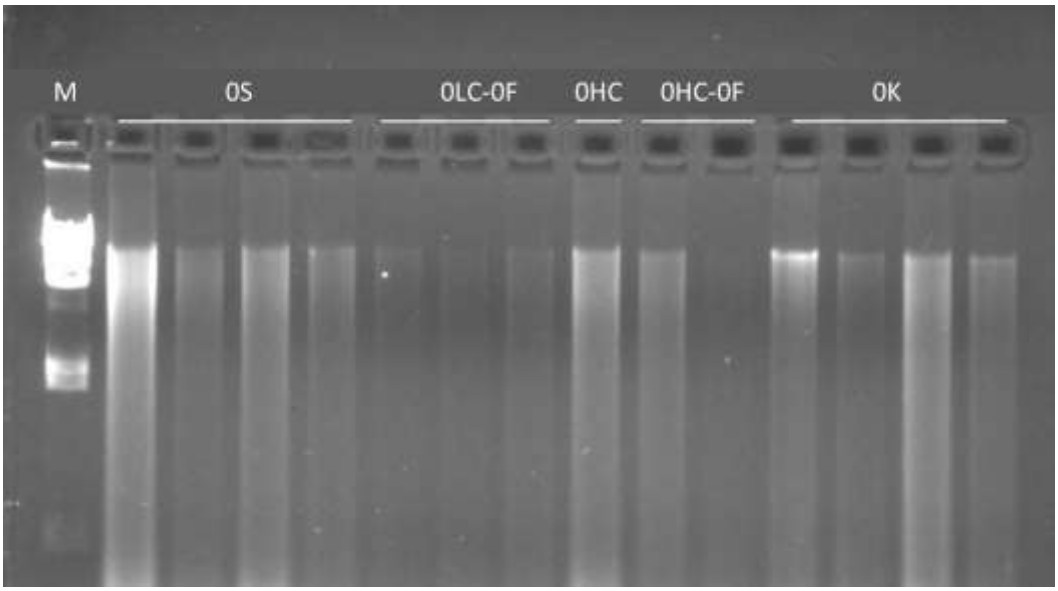


Figure 3.4: Gel electrophoresis view of extracted bacterial DNA samples. Representative agarose gel electrophoresis view of extracted DNA from time zero stool samples, visualized with Gel DoC Xr (Biorad). M: Marker, S: Standard chow diet, LC-0F: Low Carbohydrate diet with zero fiber, HC: High Carbohydrate diet, HC-0F: High carbohydrate with zero fiber, K: Ketogenic diet.

Table 3.1: DNA concentration and quantity of time zero stool samples.

Sample ID	**Nucleic Acid Conc. (ng/µl)**	**260/280**	**260/230**
0S1	49,3	1,78	1,57
0S2	13,6	1,69	1,03
0S3	30	1,82	1,27
0S4	19,7	1,74	0,75
0LC-0F1	7,3	1,6	0,78
0LC-0F2	7,2	1,67	0,74
0LC-0F3	10,3	1,71	0,86
0HC1	28,8	1,79	1,42
0HC-0F1	15	1,83	1,12
0HC-0F2	9,9	1,61	0,66
0K1	18,5	1,75	1,19
0K2	11,4	1,73	0,94
0K3	35,1	1,81	1,55
0K4	15,1	1,74	1,11

3.2.2 PCR amplification

Extracted DNA from stool samples were successfully amplified by hyper variable V3 region of the 16S ribosomal DNA gene complementary GC357F and 518R primer sets. After the amplification, all of the PCR products were of the expected length and PCR products are approximately 230 bp length. Integrity of PCR products were analyzed by agarose gel electrophoresis. Gel electrophoresis image of PCR products of time 0 (zero) stools were shown in Figure3.5. PCR products were used for as template subsequent DGGE analysis.

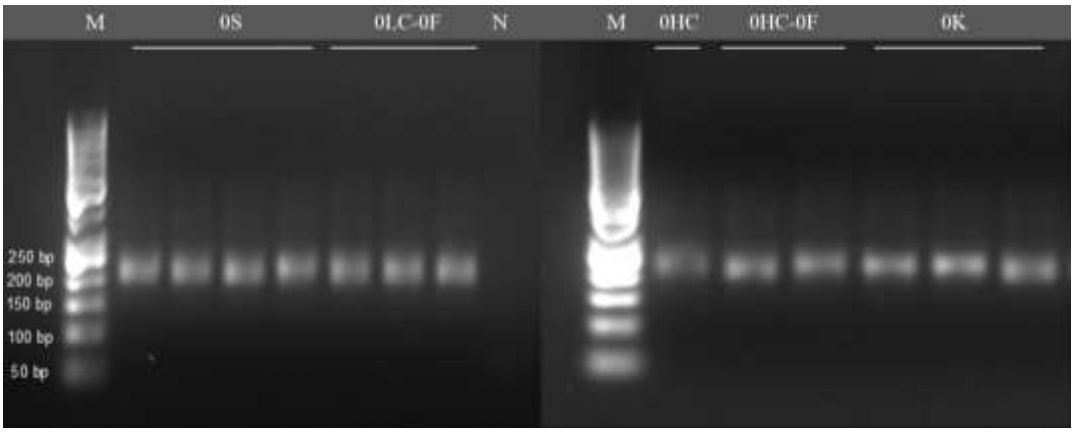


Figure3.5: Gel electrophoresis view of PCR products. Representative agarose gel electrophoresis view of bacterial DNA amplification results from time zero stool samples. visualized with Gel DoC Xr (Biorad). (M: Marker, N: Negative control, S: Standard chow diet, LC-0F: Low Carbohydrate diet with zero fiber, HC: High Carbohydrate diet, HC-0F: High carbohydrate with zero fiber, K: Ketogenic diet.

3.3 Denaturing gradient gel electrophoresis (DGGE)

3.3.1 Sequencing of DGGE bands.

DGGE only shows the clustering and similarity and is widely preferred because of being easy, fast and low cost. But microorganism's identification remains unknown in this technique. To determine bacterial identity of strong DGGE bands and also to prepare a lab made marker for further DGGE running, strong bands on DGGE were

excised and 16S rDNA gene sequencing was performed. Figure 3.6 shows excised strong bands on two DGGE gels. These two gels belonged to 1st week stool samples. 16S bacterial sequences were blasted in NCBI GenBank databases and many of these sequences matched with bacteria which were isolated from stool samples of humans or mice. Table 3.2 shows the closest bacterial species relative to the 16S sequences of strong DGGE bands and it was shown that most of the bacteria belongs to the Firmicutes phylum and Clostridia class. The sequencing results showed that about 70% of the DGGE bands belongs to Firmicutes phylum which is the most abundant phyla in human and mice stools (Ley et al., 2005, Arumugam et al., 2011). About 15% of the DGGE bands belong to Bacteroidetes and 15% of belongs to Proteobacteria. While Bacteroidetes phylum is the second largest bacterial phyla in humans and mice (Rinninella et al., 2019; Ley et al., 2005; Arumugam et al., 2011), it was captured only in a few bands in this DGGE study. The main reason for this may be due to the sampling technique that is used for sequencing the DGGE bands. While, the strong bands on DGGE were further analyzed and sequenced, the weaker (fade) bands difficult to analyze for sequencing and remained as unknown. The lower proportion for Bacteroidetes phyla (15%) likely results from this phyla being represented with weak DGGE bands in this study. (Sequence is shown in Appendix B).

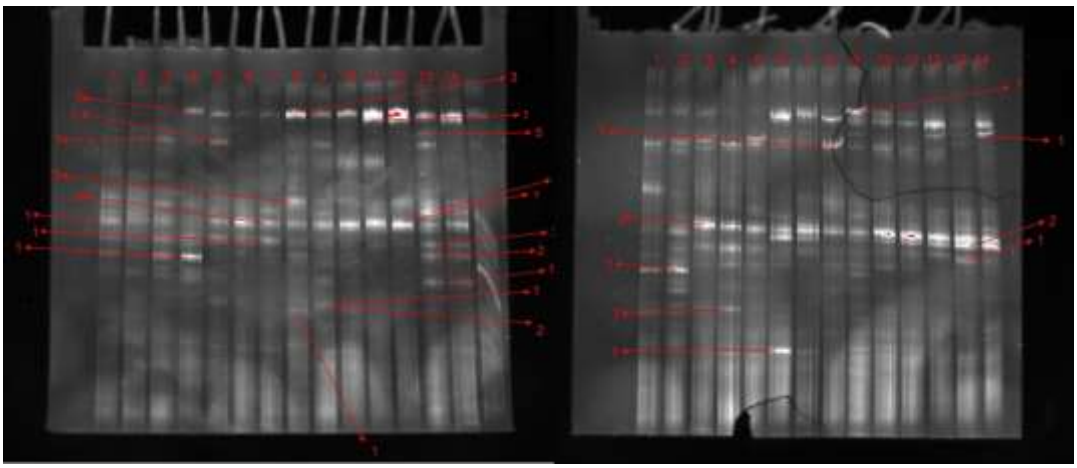


Figure 3.6: Excision of strong DGGE bands. DGGE gel view of 1st week stool samples GC357f/518r PCR products from different diet groups. Red arrows show the excised strong bands.

Table 3.2: The sequence analysis of DGGE bands from 1st and 2nd collection of stool samples.

Phylum	Class	Closest relative	Genbank accession no.	% Identity
Firmicutes (15)	Bacilli (2)	Lactobacillus murinus	MH071125.1	99
		Lactococcus lactis	LT853603.1	99
	Clostridia (12)	Anaerostipes butyraticus	NR_116863.1	97
		Anaerostipes hadrus	MG680450.1	95
		Anaerotaenia torta	NR_151894.1	99
		Clostridium bolteae	CP022464.2	97
		Clostridium indolis	MF188186.1	100
		Coprococcus comes	NR_044048.1	98
		Eisenbergiella tayi	MG680453.1	100
		Kineothrix alysoides	KX356509.1	98
		Lachnoanaerobaculum umeaense	NR_116814.1	100
		Lachnoclostridium pacaense	LT631510.1	100
		Roseburia hominis	NR_074809.1	96
		Paraclostridium bifermentans	MF521461.1	100
	Erysipelotrichia (1)	Faecalibaculum rodentium	NR_146011.1	99
Proteobacteria (3)	Epsilonproteobacteria (2)	Helicobacter fennelliae	GQ867152.1	97
		Helicobacter japonicus	NR_149210.1	100
	Gammaproteobacteria	Microbulbifer elongatus	KY034403.1	95
Bacteroidetes (3)	Bacteroidia (3)	Barnesiella intestinihominis	MF317401.1	99
		Culturomica massiliensis	MF267713.1	99
		Muribaculum intestinale	CP015402.2	89

3.3.2 Microbiota analysis by DGGE

DGGE is a widely used microbial community profile analyzing method in responses to over time or different conditions. However, variability in different gels has always been an obstacle to the proper interpretation of DGGE profiles from multiple gels. To

overcome this problem, an appropriate standard that that used for within- and between-gel alignment has a vital importance (Tourlomousis et al., 2010). To address this problem, a lab made marker (formed from strong DGGE bands) was used in this study and gels were normalized according this marker. Table3.3 shows the marker which was obtained from strong DGGE bands of different bacterial origin and sequenced, identified and used in this study. Sequence results of these bands were given in Apandices B.

Table 3.3: The closest relative of the bands which were used in DGGE study as marker.

Ladder order	Phylum	Closest relative	% Identity	Uncultured case
1	Firmicutes	Lachnoclostridium pacaense	100	
2	Proteobacteria	Helicobacter japonicus	100	
3	Bacteroidetes	Culturomica massiliensis	91	Uncultured (99 %)
4	Firmicutes	Clostridium indolis	100	
5	Firmicutes	Lactococcus lactis	96	
6	Firmicutes	Lactobacillus murinus	99	
7	Firmicutes	Coprococcus comes	98	
8	Firmicutes	Anaerotaenia torta	95	
9	Firmicutes	Anaerostipes caccae	96	Uncultured (99 %)
10	Bacteroidetes	Barnesiella intestinihominis	88	Uncultured (99 %)
11	Bacteroidetes	Muribaculum intestinale	89	
12	Firmicutes	Paraclostridium bifermentans	100	

DGGE gel profile of GC357f/518r PCR products of 1st and 17th stool samples samples are shown in Figure 3.7. DGGE profile of stool samples indicate a dynamic microbial diversity over time and during diet feeding in different diet groups. While DGGE of 1st week stool samples showed more diverse profile in the all diet groups, DGGE profile of 17th stool samples showed a narrow and a reduced diversity in all diet groups except for rhe standard chow diet group. This group has conserved its microbial composition and microbial richness throughout the study (Figure 3.7). *Lactobacillus murinus* was the most common and conserved species during the different diets in this study, except for the low carbohydrate diet (LC-0F). Another

abundantly observed species is *Lactococcus lactis*, which seems higher in low carbohydrate diet and ketogenic diet but not observed in standard chow diet.

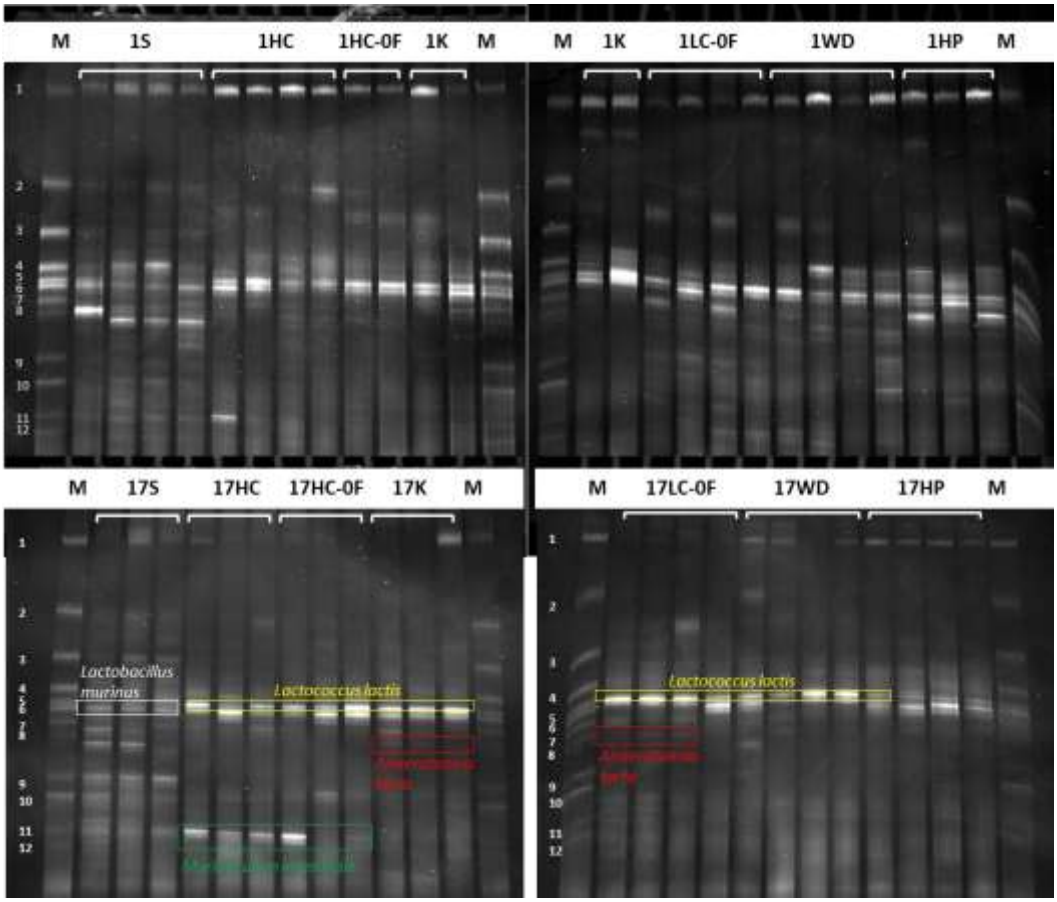


Figure 3.7: DGGE gel profile of GC357f/518r PCR products. DGGE gel profile of the 1st week (above) and 17th week (below) stool samples. The number from 1 to 12 represent marker bacterial species. Color labelled rectangle shows strong bands belong to the specific species in different diet group. White color rectangle stands for *Lactobacillus murinus*, Yellow color rectangle stands for *Lactococcus lactis*, red color rectangle stands for *Anaerotaenia torta* and green color rectangle stands for *Muribaculum intestinale*. (M: Marker, S: Standard chow diet, HC: High Carbohydrate diet, HC-0F: High carbohydrate with zero fiber, K: Ketogenic diet, LC-0F: Low Carbohydrate diet with zero fiber, WD: Western diet, HP: High Protein diet).

Both *Lactobacillus murinus* and *Lactococcus lactis* have probiotic and anti-inflammatory properties and are commonly found in humans and other animals and the abundance of these two species in this study is consistent with recent studies (Figure 3.7; Biddle et al, 2014; Taniguchi et al; 2020; Yuan et al., 2020). But the

proportion of these two species differ both individually and throughout different diet groups. For example, the highest *Lactobacillus murinus / Lactococcus lactis* ratio was observed in standard chow diet (S) whereas the lowest ratio was observed in low carbohydrate diet (LC-0F). (Figure 3.7). Such bacterial ratios were used in recent studies for different aims. For instance, in a clinical study, it was shown that participants with high *Prevotella*/*Bacteroides (*P/B) ratio is associated with loss of body fat, while low P/B ratio were more susceptible to gain body fat (Hjorth et al., 2018). Similarly, the ratio of *Firmicutes/Bacteroidetes* is an indicator for gut dysbiosis, obesity and gut inflammation. Higher *Firmicutes/ Bacteroidetes* ratio is associated with IBD (Magne et al., 2020, Stojanov et al., 2020). The ratio of *Lactobacillus murinus / Lactococcus lactis* can also be used as a diagnostic marker for futher studies to microbiota and diet associated diseases or die such as IBD or obesity.

Anaerotaenia torta, a member of Firmicutes phylum, seems to be highly abundant in LC-0F and K diet groups than S, HC-0F, and HC diet groups (Figure 3,7). A recent study has shown that simple sugar intake in mice increases the risk of colitis by microbial changes, also shown that *Anaerotaenia torta* significantly decreased in mice fed with the high sugar diet (Khan et al., 2020). Thus, *Anaerotaenia torta* may be used as a marker for low glucose diet incidence and intestinal inflammation. Also, in DGGE patterns it was observed that, *Muribaculum intestinale* belonging to Bacteroidetes phylum were considerably increased in HC-0F and HC diet groups. *Muribaculum intestinale* has a higher ability to degrade a variety of complex carbohydrates (Ormerod et al., 2016). Moreover, studies have shown that during the high calorie or high fat, or simple sugar rich diet feeding, the abundance of *Muribaculum intestinale* decrecreased in different animal models (Do et al., 2018, Barouei et al., 2017, Lagkouvardos et al., 2016 and Obanda et al., 2018). High carbohydrates content from different sources in HC-0F and HC diet groups may lead an increase in *Muribaculum intestinale* in this study.

3.3.3 DGGE Profile Analysis

DGGE profile patterns of all stool samples from all diet groups were clustered according to their bacterial 16S rRNA gene products. Unweighted pair group method using arithmetic averages (UPGMA) was used to compare the similarity of samples in a dendrogram. It is assumed that DGGE can capture only 1-2% of the microbial population that reflect the dominant species found in a microbial community (Muyzer et al., 1993). Similarly, PCR-DGGE performed to analyze microbial population is shaped based on dominant species, not the whole species in the intestine (Liu et al., 2010). Figure 3.8 shows the dendrogram of DGGE profile of 1^{st} and 17^{th} week stool samples. Here the 1^{st} week stool samples are the first collected samples after starting different diet feeding and 17^{th} week stool samples are the last collected stool samples. Overall, DGGE profile analysis displayed that, the microbial similarity between the group (individuals) or inter groups were low for stool samples collected at different times (1st week stool samples and 17^{th} week stool samples) except for the standard chow diet group. Profiles of S diet at 17^{th} week stool samples displayed the highest similarity with profiles of S diet at 1^{st} week stool samples. The DGGE pattern also showed that the diversity of 1^{st} week stool samples were higher than the 17^{th} week stool samples; 17^{th} week stool samples within each group clustered together while 1^{st} week stool samples did not show any clusters. For example, 17^{th} week stool samples from LC-0F and HC-0F groups clustered within their groups. Also there is a similarity between western diet (WD) group and the high protein diet (HP) group; these two diet groups are clustered together. Overall, this DGGE dendrogram shows that bacterial richness in the groups has decreased over the time and instead more abundant specific strains were observed.

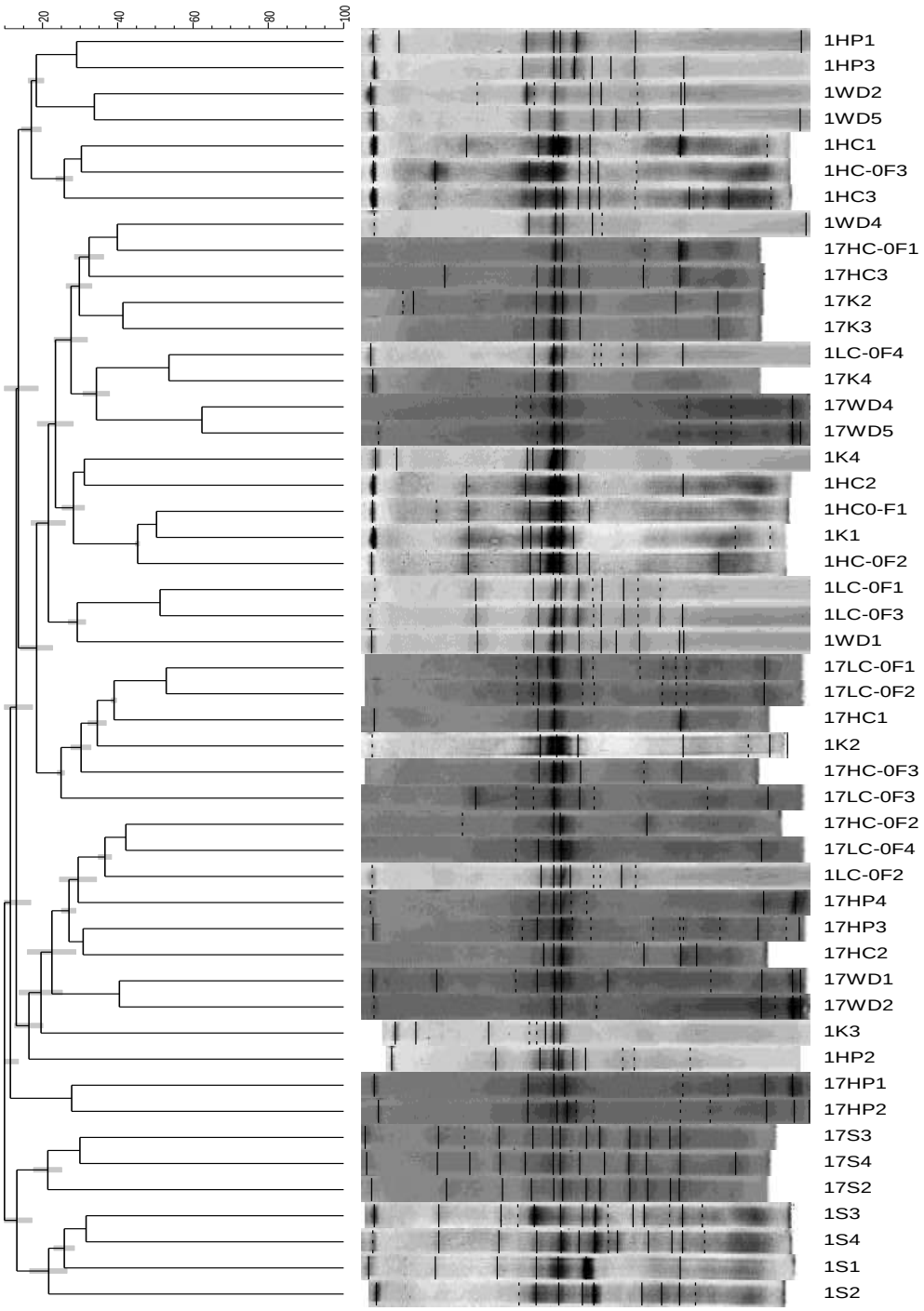


Figure 3.8: DGGE similarity dendograms. Similarity (Pearson correlation coefficient- UPGMA) of the DGGE profile of 1st week and 17th week stool samples. $n \geq 3$ mice per group. (S: Standard chow diet, HC-0F: High carbohydrate with zero fiber, HC: High Carbohydrate diet, LC-0F: Low Carbohydrate diet with zero fiber, K: Ketogenic diet, WD: Western diet, HP: High Protein diet).

In Appendix D, the dendrogram based on denaturing gradient gel electrophoresis (DGGE) of 1st, 13th and 17th week stool samples are shown. Each mouse (individual) has a unique DGGE pattern, DGGE profile within groups resembles each other more than DGGE profile of between groups as expacted. For instance, 1st week stool samples within a group cluster together. Similarly, 13th and 17th week stool samples within each group also clustered together. But the clustering between different time points of stool samples was not observed in DGGE dendrogram from Appendices D. Again the highest similarity is seen in standard chow diet group (S) from three different time points (1st, 13th and 17th) are clustered together, the similarity between different time points is lowest in high carbohydrate with zero fiber diet group (HC-0F).

Figure 3.9 and Figure 3.10 show the average DGGE bands number in each diet group. S diet group relatively has higher numbers of bands (12-13), LC-0F and K diet groups have fewer bands (7-8) and HC-0F and HC diet groups relatively have lowest number of bands (4-5) at the end of the study.

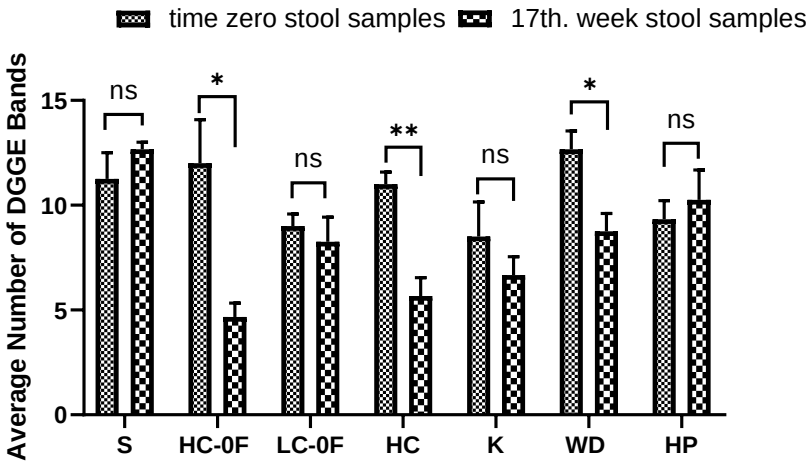


Figure 3.9: Average DGGE band number of time zero and 17th week stool samples. Average DGGE band number of time zero and 17th week stool samples of all diet groups. Statistical analyses were performed with Graph Pad Prism 8. Data are mean ± SEM by Student's t test, unpaired. ns: not significant, *p < 0.05, **p < 0.01. (S: Standard chow diet, HC-0F: High carbohydrate with zero fiber, HC: High Carbohydrate diet, LC-0F: Low Carbohydrate diet with zero fiber, K: Ketogenic diet, WD: Western diet, HP: High Protein diet).

High carbohydrate content in HC-0F and HC diet groups may be the main reason for the low number of bands (Figure 3.10, Figure 3.11 Kim et al., 2011). As can be seen, the S diet group has conserved its band numbers, but especially the HC-0F and HC diet groups have decreased the number of bands during the study. Figure 3.10 showed that there are small differences between the time zero stool samples of different diet groups because of heterogeneity of host microbiota. But these differences were not significantly meaningful. After the end of the feeding (17^{th} week stool samples) HC-0F, HC and W diet groups showed statistically significant reduction ($p < 0.05$) of average DGGE bands number when comparing with their time zero stool samples. But S, LC-0F, K and HP diet groups did not Show any considerable changes. Total band numbers of DGGE are accepted as the indicator of microbial richness (Yuan et al., 2014). These DGGE profile analyses (Figure 3.9 and Figure 3.10) show that the bacterial diversity of the stool samples has decreased significantly in the HC-0F and HC and W diet groups.

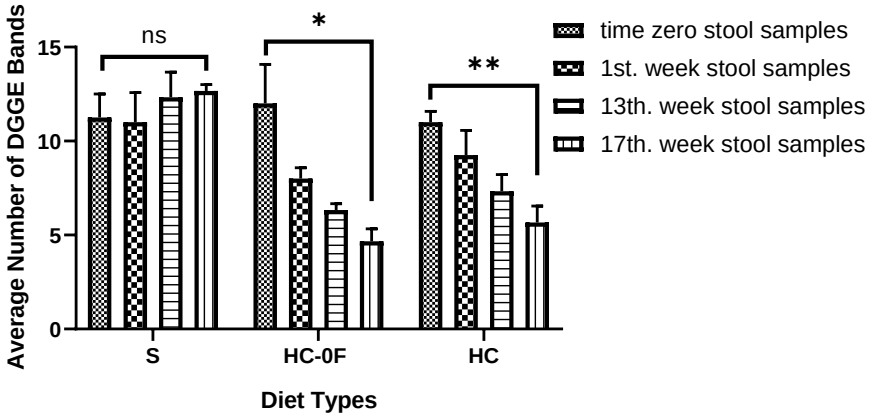


Figure 3.10: Average DGGE bands number of time zero, 1^{st}, 13^{th} and 17^{th} week stool samples. Average DGGE bands number of time zero, 1^{st}, 13^{th} and 17^{th} week stool samples of standard chow (S), high carbohydrate with zero fiber (HC) and high carbohydrate (HC) diet groups. Statistical analyses were performed with Graph Pad Prism 8. Data are mean ± SEM by Student's t test, unpaired. ns: not significant, *$p < 0.05$, **$p < 0.01$.

Figure 3.10 is also shown the average DGGE band number of time zero, 1^{st}, 13^{th} and 17^{th} week stool samples from S, HC-0F and HC diet groups. The gradually significant changes in regard to DGGE bands number can be seen easily. The

reduction of bacterial diversity has been shown as a hallmark of the inflammatory bowel diseases (IBDs) (Ott et al., 2004). HC-0F and HC diets contain 74% and 72% percentages of carbohydrate respectively in this study. The reason for dramatic loss in microbial diversity in the HC-0F, HC and W diet groups may be due to the high content of simple sugar in these three groups. It was previously shown that western diet high in simple sugar caused a significant reduction of bacterial diversity in mice (Turnbaugh et al., 2008). Our study has also shown that PCR-DGGE is an effective way of monitoring the changes of composition of the intestinal microbiota over time during different diets.

3.4 Analysis of Selected Cytokine and Tight Junction mRNA Expression Levels by Quantitative Real-Time PCR

Anti-inflammatory cytokines; IL-10, TGF-β and pro-inflammatory cytokines; IL-1β, IL-6 and TNF-α mRNA levels are analysed to detect intestinal inflammation. In addition, to analyse the permeability of epithelial cell and barrier function, tight junction (TJ) proteins, including Occludin, Claudin-1 and Zona Occludens 1 (ZO-1) mRNA expression levels were measured with quantitative RT-PCR methods. A weak intestinal tight junction (TJ) barrier causes increased foreign antigens including bacteria passage into the underlying lamina propria (DeMeo et al., 2002). These foreign bacteria and antigens are activated inflammatory responses through helper T-lymphocytes and antigen presenting cells. The pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are secreted after the inflammatory response and lead to impairment of intestinal tight junction barrier causing increase in intestinal permeability (Nusrat et al., 2000, Bruewer et al., 2006 and Shen et al., 2006). Anti-inflammatory cytokines such as transforming growth factor-β (TGF-β) and interleukin-10 (IL10) also alleviate the inflammation or maintain the integrity of intestinal barrier by protecting the tight junctions (TJs) (Forsyth et al., 2007). Therefore, to evaluate the effect of different diets on intestinal

inflammation both cytokines and tight junction protein level were analyzed in this study.

Figure 3.11 shows the heat map of the normalized mRNA levels of anti-inflammatory cytokines; IL-10, TGF-β, pro-inflammatory cytokines; IL-1β, IL-6 and TNF-α and tight junction (TJ) protein, includes Occludin, Claudin-1 and Zona Occludens 1 (ZO-1) both colonic and small intestinal tissues. In general, in the colon tissues from the high carbohydrate with zero fiber (HC-0F) diet fed mice, anti-inflammatory, pro-inflammatory cytokines genes and a tight junction gene (ZO-1) were up regulated. But other tight junction genes; Occludin and Claudin-1 from the same group (colon, HC-0F group) were down regulated.

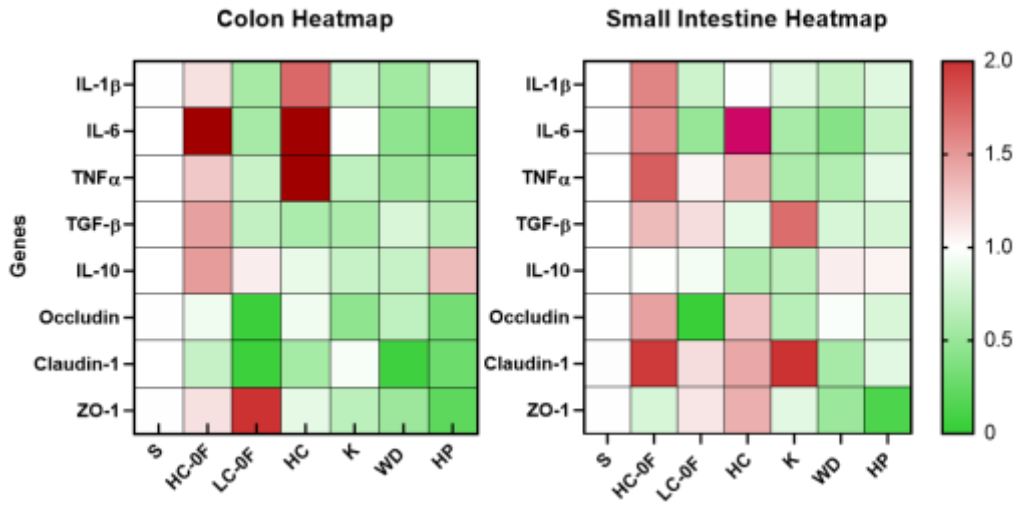


Figure 3.11: Heatmap of gene-expression of inflammatory markers. The heat map of the normalized mRNA fold relative to standard chow diet. Green indicates down regulated mRNA levels and red indicates up regulated mRNA levels and White indicates no change in mRNA levels . (S: Standard chow diet, HC-0F: High carbohydrate with zero fiber, HC: High Carbohydrate diet, LC-0F: Low Carbohydrate diet with zero fiber, K: Ketogenic diet, WD: Western diet, HP: High Protein diet).

The small intestinal tissues from the HC diet gave similar results with colon tissues in anti-inflammatory cytokines and pro-inflammatory cytokines which were up-regulated. Besides Occludin and Claudin-1 genes were up-regulated. Only the ZO-1

gene was down regulated. In the small intestine tissues from the high carbohydrate with zero fiber (HC-0F) and high carbohydrate (HC) diet fed mice, anti-inflammatory, pro-inflammatory cytokines genes were up regulated. Tight junction genes from HC diet fed mice small intestine tissues were also up regulated. But other tight junction genes (Occludin and Claudin-1) from the HC-0F small intetsine tissues were down regulated.

Previous studies regarding how high-carbohydrate diet affects inflammation in different tissues of different animal models have resulted in conflicting results. For example, a study conducted with high fat diet fed mice for 9 months showed there was no activation of inflammatory pathways in small intestinal crypt or organoids by measuring mRNA levels of IL-1β, IL-6 and TNF-α (Beyaz et al., 2016). Another study demonstrated that 30 days treatment of high-fat diet-had induced inflammation by increasing IL-1β, IL-6 and TNF-α. in mouse intestinal tissue (Doerner et al., 2016). But in a recent study, it was shown that high carbohydrate diet (HCD) is more potent to induce inflammation in skeletal muscle than high fat diet (HFD) in mice (Antunes et al., 2020). Our findings showed that the high-carbohydrate diet has an inflammation-enhancing effect, similar to the findings of Antunes et al., (2020). Similar study with mice on a high sugar diet has shown a reduced bacterial diversity and increased intestinal permeability with decreased short chain fatty acid (SCFA) (Laffin et al., 2019).

Figure 3.12 shows the normalized mRNA levels of anti-inflammatory cytokines; IL-10, TGF-β, pro-inflammatory cytokines; IL-1β, IL-6 and TNF-α. Although inflammatory cytokines were increased in colonic tissue of high carbohydrate with zero fiber diet (HC-0F) group, this increase was not significant. Pro-inflammatory cytokine IL-6 ($p<0.1$), TNF-α ($p<0.05$) levels in colonic tissues from high carbohydrate diet (HC) showed significant differences compared to standard chow diet (S). Also, the HC diet group showed considerable increase in IL-1β, TNF-α and IL-6 levels comparison to other diet groups. For instance, IL-1β gene expression was significantly up-regulated in the HC diet group compared to the LC-0F diet ($p<0.1$)

or compared to the western diet (WD, $p<0.05$). But anti-inflammatory cytokines in the HC group did not show any change compared to the standard diet. This may result from high level of pro-inflammatory cytokines suppressing anti-inflammatory cytokine production. Remaining diet groups (LC-0F, K, WD, HP) did not show any significant changes compared with the standard chow diet. In a study with high fat diet fed mice IL-1β mRNA level was elevated after 11 week, TNFα mRNA level was up regulated after 22 week (Gulhane et al., 2016). But in our study, increased expression of cytokines levels were not observed in high fat diets (LC-0F and K) fed mice. mRNA levels of colonic cytokines were complementing with the result of DGGE analysis where ignificant decreases in bacterial diversity are observed in HC-0F and HC diet groups. The studies conducted with high sugar diets show an association between low microbial diversity and inflammation (Ott et al., 2004, Turnbaugh et al., 2008, Laffin et al., 2019, Khan et al., 2020). Therefore it can be said that HC-0F diet may cause low bacterial diversity resulting in an increased inflammation though not significant when compared to the standard chow diet.

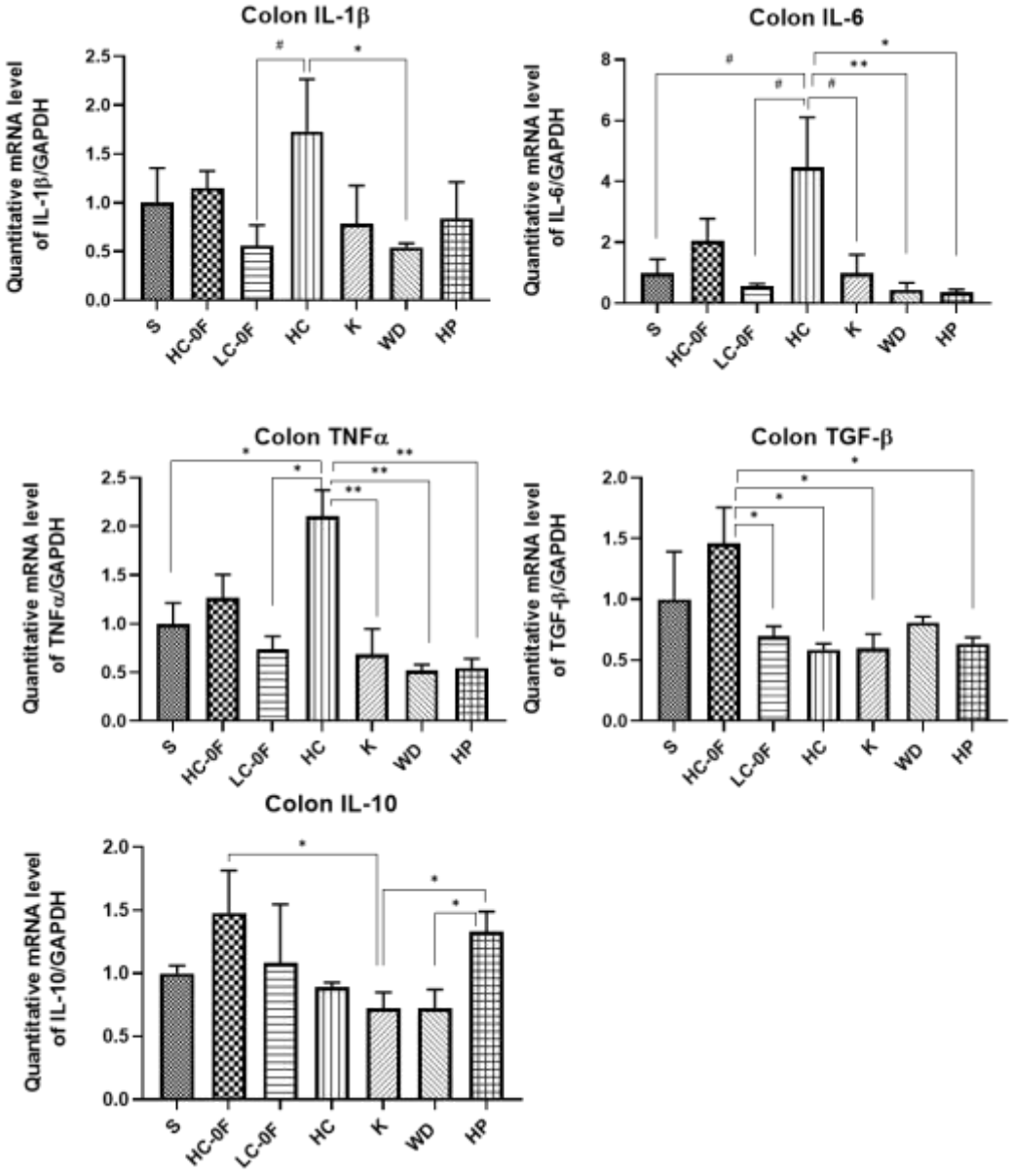


Figure 3.12: Quantitative mRNA level of pro-inflammatory and anti-inflammatory cytokines in colon. Inflammatory cytokines results were expressed as **2-ΔΔCt**. mRNA values were normalized with standard chow diet group. Gapdh gene were used as housekeeping gene. n≥4 mice. Statistical analyses were performed with Graph Pad Prism 8. Data are mean ± SEM. by Student's t test, unpaired. # $p<0.1$, *$p < 0.05$, **$p < 0.01$. (S: Standard chow diet, HC-0F: High carbohydrate with zero fiber, HC: High Carbohydrate diet, LC-0F: Low Carbohydrate diet with zero fiber, K: Ketogenic diet, WD: Western diet, HP: High Protein diet).

Tight Junction proteins support the intercellular barrier between epithelial cells and are crucial to regulate selective permeability of solutes among the epithelium and are important for the maintenance of epithelial barrier integrity. Impaired structure and integrity of tight junction proteins cause inflammation (Anderson et al., 2009 and Chelakkot et al., 2018). Figure 3.13 shows the normalized mRNA levels of tight junction (TJ) proteins Occludin, Claudin-1 and Zona Occludens 1 (ZO-1) to study differences in gut permeability between different diets.

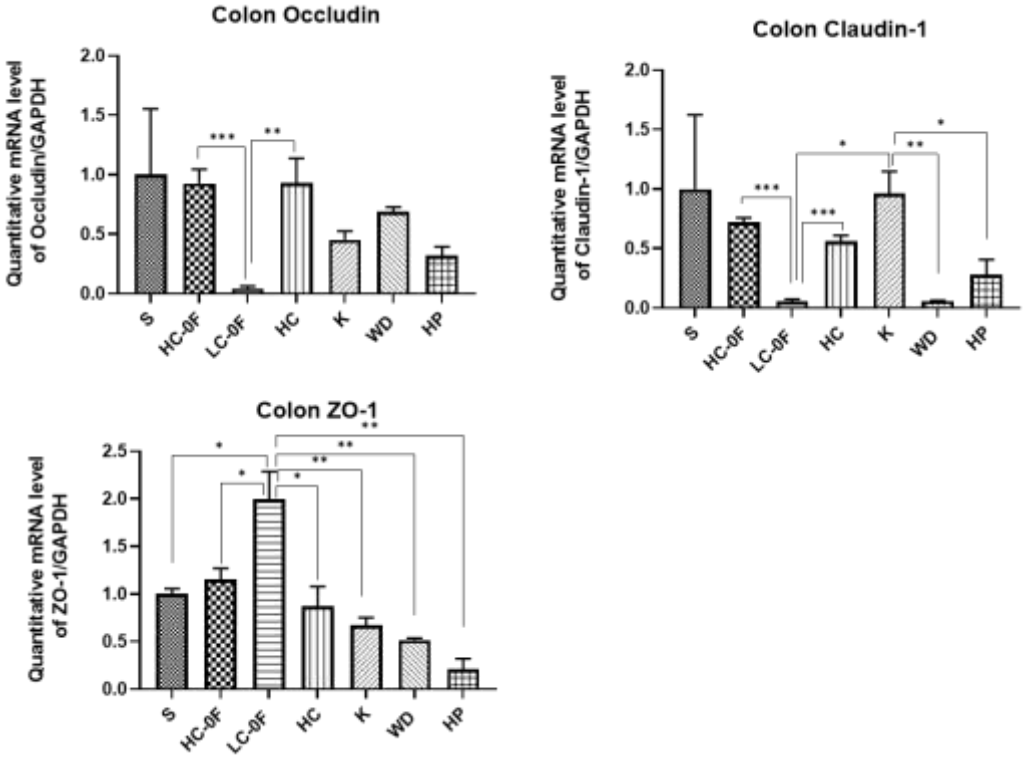


Figure 3.13: Quantitative mRNA level of tight junctions in colon. Tight Junction proteins results were expressed as **2-ΔΔCt**. mRNA values were normalized with standard chow diet group. Gapdh gene were used as housekeeping gene. . n≥4 mice. Statistical analyses were performed with Graph Pad Prism 8. Data are mean ± SEM. by Student's t test, unpaired. # p<0.1, *p < 0.05, **p < 0.01 . (S: Standard chow diet, HC-0F: High carbohydrate with zero fiber, HC: High Carbohydrate diet, LC-0F: Low Carbohydrate diet with zero fiber, K: Ketogenic diet, WD: Western diet, HP: High Protein diet).

Colonic Occludin and Claudin-1 mRNA levels in LC-0F fed mice were significantly down-regulated compared to HC-0F (***$p < 0.001$) and HC diet (**$p < 0.01$). In contrast, Zona Occludens 1 (ZO-1) mRNA level of LC-0F fed mice up regulated (*$p < 0.05$) compared to all diet groups. There was no significant change in TJ mRNA levels for high carbohydrate diet fed mice compared with standard chow diet. A study with mice on a high sugar diet has shown a reduced bacterial diversity and increased intestinal permeability and reduced tight junction protein levels (Laffin et al., 2019). Similarly it was shown that expression level of claudin-1, occludin increased in the intestinal mucosa of animals fed high-fat diets (de La Serre et al., 2010 and Suzuki et al., 2010).

Figure 3.14 shows the normalized mRNA levels of anti-inflammatory cytokines; IL-10 and TGF-β, pro-inflammatory cytokines; IL-1β, IL-6 and TNF-α in small intestine tissue. Expression of inflammatory cytokines; IL-1β, IL-6, TNF-α, and TGF-β increased in small intestine tissue of HC-0F diet group. While IL-6 was significantly increased ($p<0.05$) compared to standard chow diet, the increase in expression of IL-1β, TNF-α, and TGF-β were not significant compared to standard chow diet. Anti-inflammatory cytokine TGF-β from K diet showed significant increase compared to standard chow diet (S), HC, WD, HP diet groups as well. Also, HC diet group showed considerable increase in IL-6 ($p<0.05$) compared to K and WD diet groups. Remaining diet groups (LC-0F, K, WD, HP) did not showed any significant changes compared to standard chow diet in small intestine tissues. A study conducted with high fat diet fed mice for 9 months showed that there were no activation of inflammatory pathways in small intestinal crypt or organoids by measured mRNA levels of IL-1β, IL-6 and TNF-α (Beyaz et al., 2016). Inflammatory cytokines are mainly produced by immune cells such as macrophages, T cells, dendritic cells (DCs) in lamina propria or in epithelium mucosa. But epithelial cells also produced cytokines although low quantities (Neurath, 2014; Andrews et al., 2018). In this study, total intestinal tissue which includes both intestinal epithelium and lamina propria were analyzed together. So it is hard to see the source of cytokines. For a better understanding intestinal crypt can be isolated and further analyzed. Similarly, in our study, pro-inflammatory and anti-inflammatory cytokines in high fat diet fed mice were not changed compared to standard chow diet in small intestine (Figure 3.14). But a slight increase of

inflammatory markers in high carbohydrate diets, like colonic tissues, were observed.

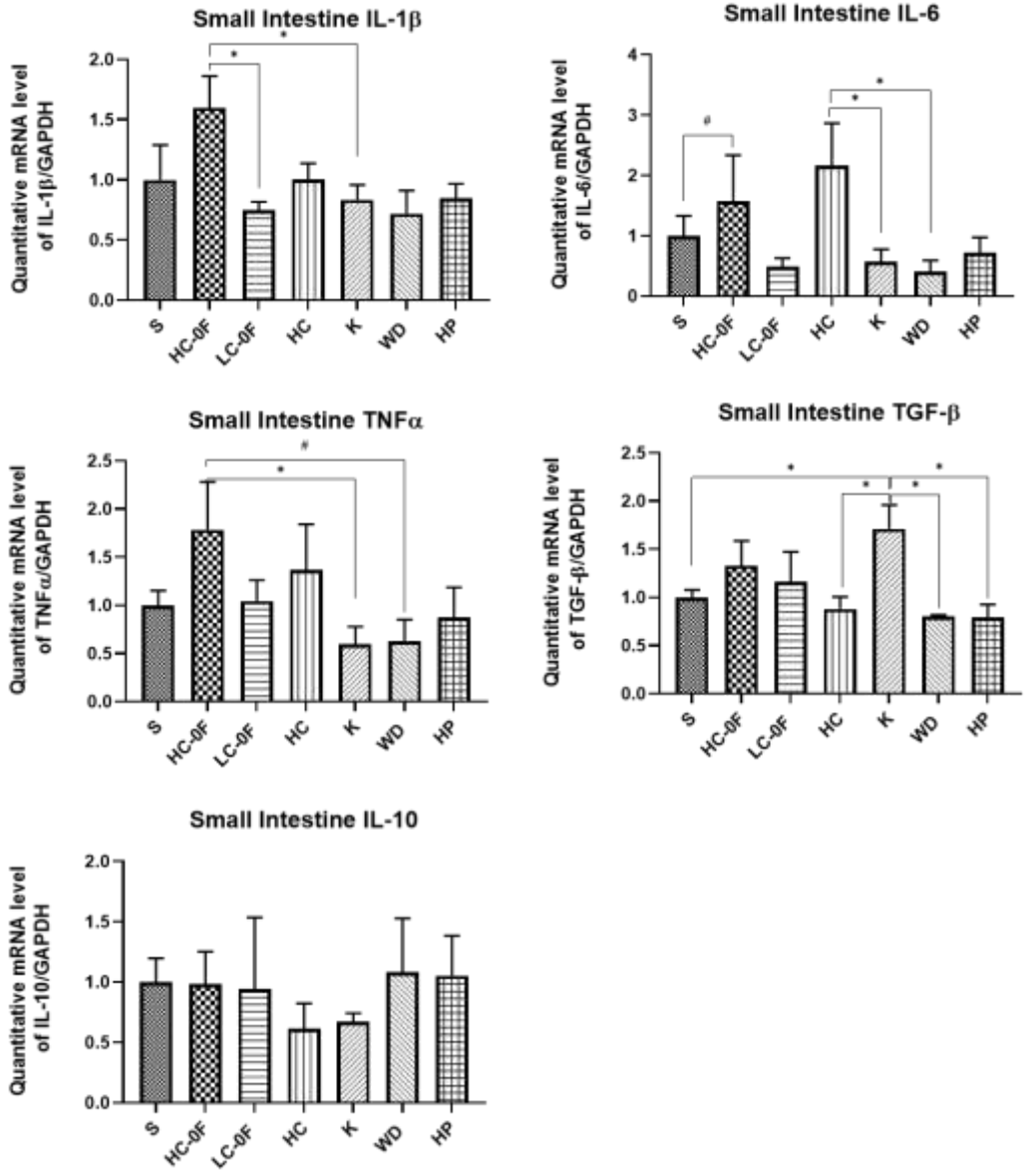


Figure 3.14: Quantitative mRNA level of pro-inflammatory and anti-inflammatory cytokines in small intestine. Inflammatory cytokines results were expressed as **2-ΔΔCt**. mRNA values were normalized with standard chow diet group. Gapdh gene were used as housekeeping gene. . n≥4 mice. Statistical analyses were performed with Graph Pad Prism 8. Data are mean ± SEM. by Student's t test, unpaired. # $p<0.1$, *$p < 0.05$, **$p < 0.01$.

Figure 3.15 shows the normalized mRNA levels of tight junction (TJ) protein, including Occludin, Claudin-1 and Zona Occludens 1 (ZO-1). Small intestinal Occludin and Claudin-1 mRNA levels in LC-0F fed mice were significantly down regulated (*p < 0.05) compared to HC-0F. In contrast, Zona Occludens 1 (ZO-1) mRNA levels of low carbohydrate diet (LC) fed mice were up-regulated compared with high carbohydrate diet (HC) groups.

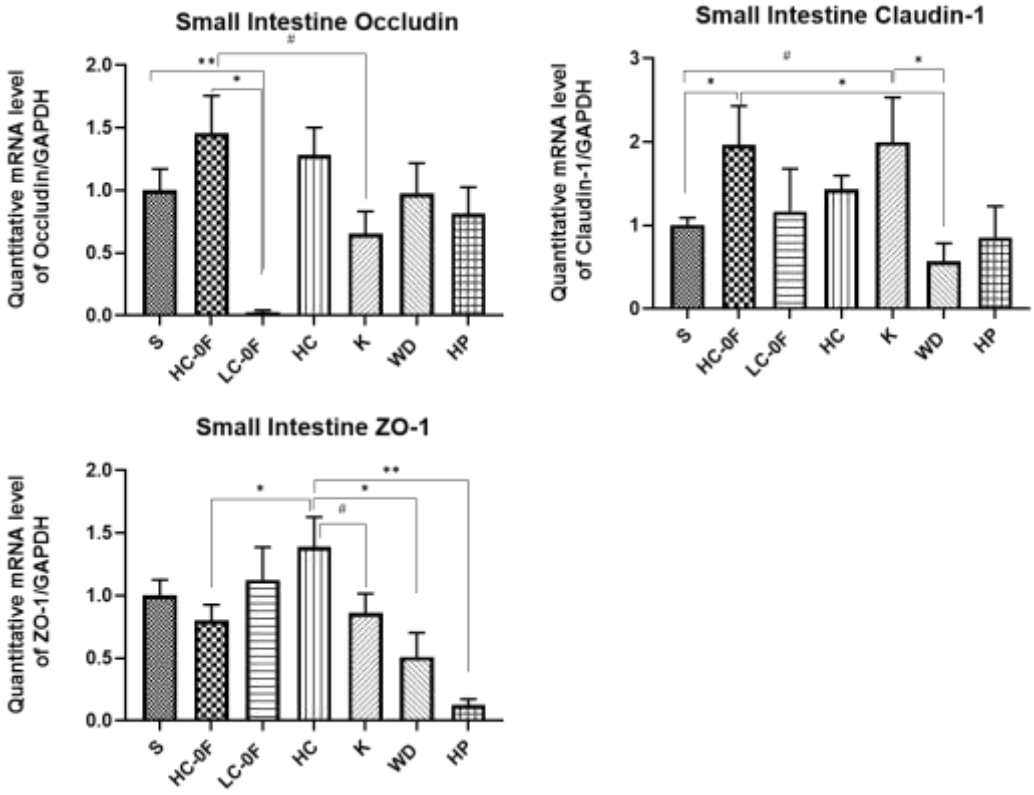


Figure 3.15: Quantitative mRNA level of tight junctions in small intestine. Tight Junction proteins results were expressed as **2-ΔΔCt**. mRNA values were normalized with standard chow diet group. Gapdh gene were used as housekeeping gene. . n≥4 mice. Statistical analyses were performed with Graph Pad Prism 8. Data are mean ± SEM. by Student's t test, unpaired. # p<0.1, *p < 0.05, **p < 0.01 . (S: Standard chow diet, HC-0F: High carbohydrate with zero fiber, HC: High Carbohydrate diet, LC-0F: Low Carbohydrate diet with zero fiber, K: Ketogenic diet, WD: Western diet, HP: High Protein diet).

Claudin-1 mRNA level of high carbohydrate diet fed mice also significantly up regulated (*$p < 0.05$) compared to standard chow diet. But other tight junctions (Occludin and Zona Occludens 1) were not changed considerably. Similar results were observed in mRNA level of tight junctions in colon and small intestine in this study.

Overall, mRNA level of cytokines and tight junction show that the inflammation in high carbohydrate diet (HC) and low fat diet (LF) is higher than standard chow diet (S) or low carbohydrate diet (LC). There is also a negative correlation between bacterial richness and colonic inflammation in our results. The bacterial diversity was less in HC and LF mice groups than in other diet groups and there is an increase in colonic inflammation. A recent study showed that individuals with low bacterial diversity were associated with more significant inflammation than individuals with high bacterial diversity (Le Chatelier et al., 2013) which is in line with high carbohydrate induced low bacterial diversity and inflammation in this study. Moreover, higher inflammation in the colon than the small intestine may be associated with the high bacterial load and dramatic microbial changes in the colon in this study.

3.5 Analysis of Selected Cytokine and Tight Junction Protein Expression Levels by Western Blotting

Protein expression level of two pro-inflammatory cytokines; IL-1β, TNFα and two tight junction (TJ) proteins; Occludin and Claudin-1 of colon samples from different diet fed mice were performed by western blotting technique (Figure 3.16). Western blot quantification of protein expression levels of IL-1β, TNFα, Occludins and Claudins-1 in in colon tissues can be seen in Figure 3.18. In the high carbohydrate diet (HC) fed mice, while TNFα protein expression did not significantly change in colonic tissues, while IL-1β protein expression significantly decreased (*$p < 0.05$) compared with standard chow diet. Also, TJs; Occludin and Claudin-1 protein expression did not change considerably in colonic tissues. Conversely, in low

carbohydrate diet (LC) fed mice, both pro-inflammatory IL-1β and TNFα and TJs Occludin and Claudin-1 protein expression significantly decreased compared with standard chow diet.

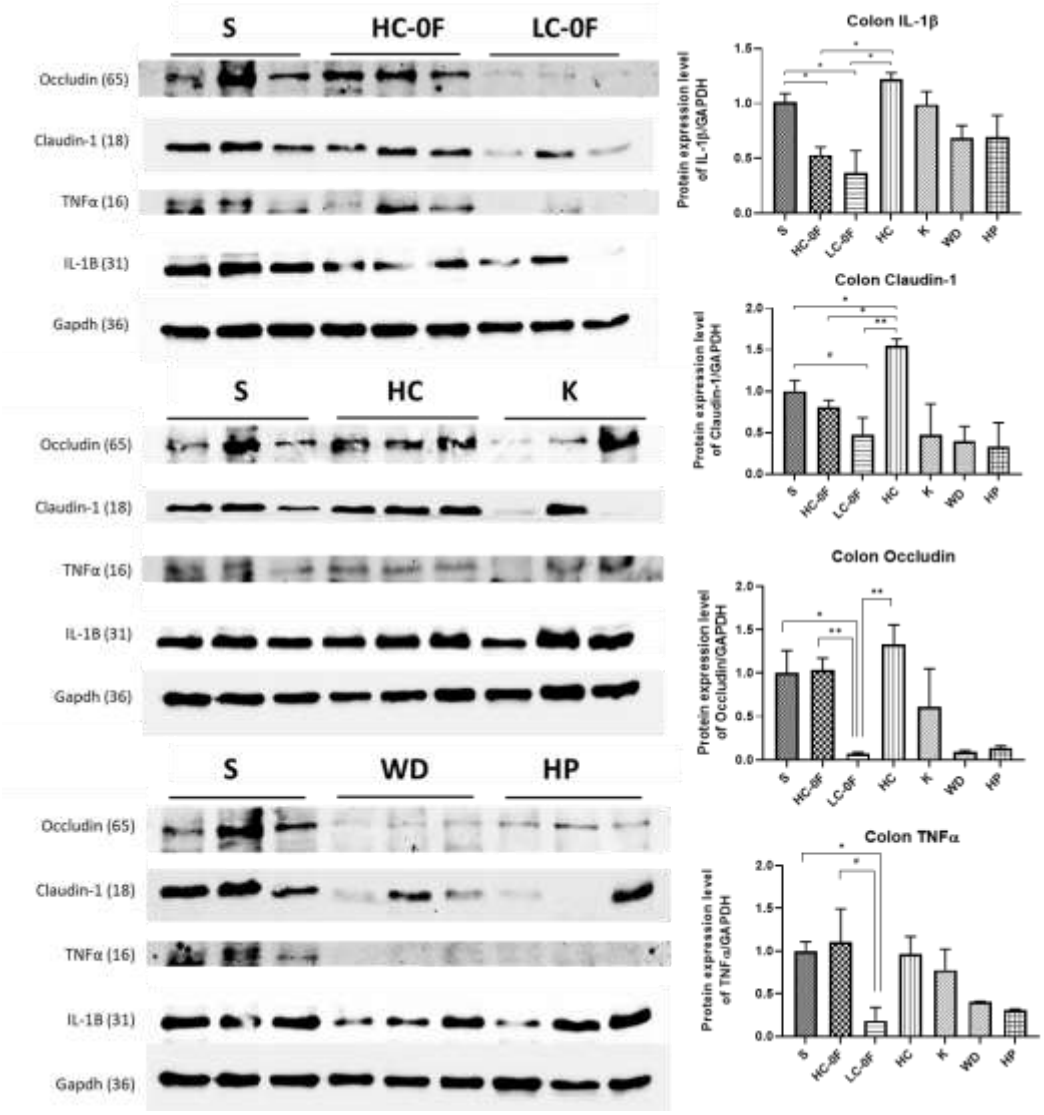


Figure 3.16: Protein expression levels of IL-1β, TNFα, Occludins and Claudins-1 in colon. Protein expression and quantification of IL-1β, TNFα, Occludins and Claudins-1proteins in colon tissue. Relative expression levels were measured by normalized gray values of IL-1β, TNFα, Occludins and Claudins-1/Gapdh images. n≥3 mice. Images were analyzed with image J. Statistical analyses were performed with Graph Pad Prism 8. Data are mean ± SEM. by Student's t test, unpaired. # $p<0.1$, *$p < 0.05$, **$p < 0.01$. (S: Standard chow diet, HC-0F: High carbohydrate with zero fiber, HC: High Carbohydrate diet, LC-0F: Low Carbohydrate diet with zero fiber, K: Ketogenic diet, WD: Western diet, HP: High Protein diet).

TJ proteins provide a protective barrier between epithelial cells, allow the selective transformation of nutrients and solutes and are crucial for the maintenance of intestinal barrier integrity and function. Impaired structure and integrity of tight junction proteins cause inflammation (Anderson et al., 2009 and Chelakkot et al., 2018). The results of the low carbohydrate diet fed mice pro-inflammatory cytokines; IL-1β, TNFα and two tight junction (TJ) proteins; Occludin and Claudin-1 was inversely correlated. The IL-1β and TNFα protein expression levels were lower than standard chow diet, meaning lower inflammation. In contrast, tight junction protein expression was also decreased, meaning impaired integrity of tight junction and a higher inflammation. There may be a few explanations for such contradictory results in the studies; such as the action time of cytokines, the animal model and method used (Schreiber et al., 1995 and Waddell et al., 2015). Overall western blot results fit qRT-PCR results and both studies show an increased inflammation in high carbohydrate diet than standard chow diet in mice.

Inflammatory cytokines are mainly produced by immune cells such as macrophages, T cells, dendritic cells (DCs) in lamina propria or in epithelium mucosa. But epithelial cells also produced cytokines although low quantities (Neurath, 2014; Andrews et al., 2018). In this study, total intestinal tissue which includes both intestinal epithelium and lamina propria were analyzed together. So it is hard to see the source of cytokines. For a better understanding intestinal crypt can be isolated and further analyzed.

3.6 Next Generation Sequencing Analysis of Microbiota

All DNA samples from luminal content of the small intestine and colon were processed with Next Generation Sequencing (NGS) for microbial composition. Figure 3.17 shows the next generation sequencing analysis of colon lumen samples from mice fed with ketogenic diet and control (chow) diet for 4 months based on 16S RNA bacterial gene. The mice fed with ketogenic diet showed that abundance of *Muribaculaceae* family (Bacteroidetes phylum) and *Faecalibaculum* family

(Firmicutes phylum) decreased and *Bacteroides* family (Bacteroidetes phylum) increased compared to control chow diet (Figure 3.17A). Similarly, a recent study showed that high levels of *Bacteroides* accumulation was observed after one-week ketogenic diet treatment of mice (Xie et al., 2017). Also, another study showed that a high fat rich diet selectively enriched *Bacteroides* in humans (Arumugam et al., 2011).

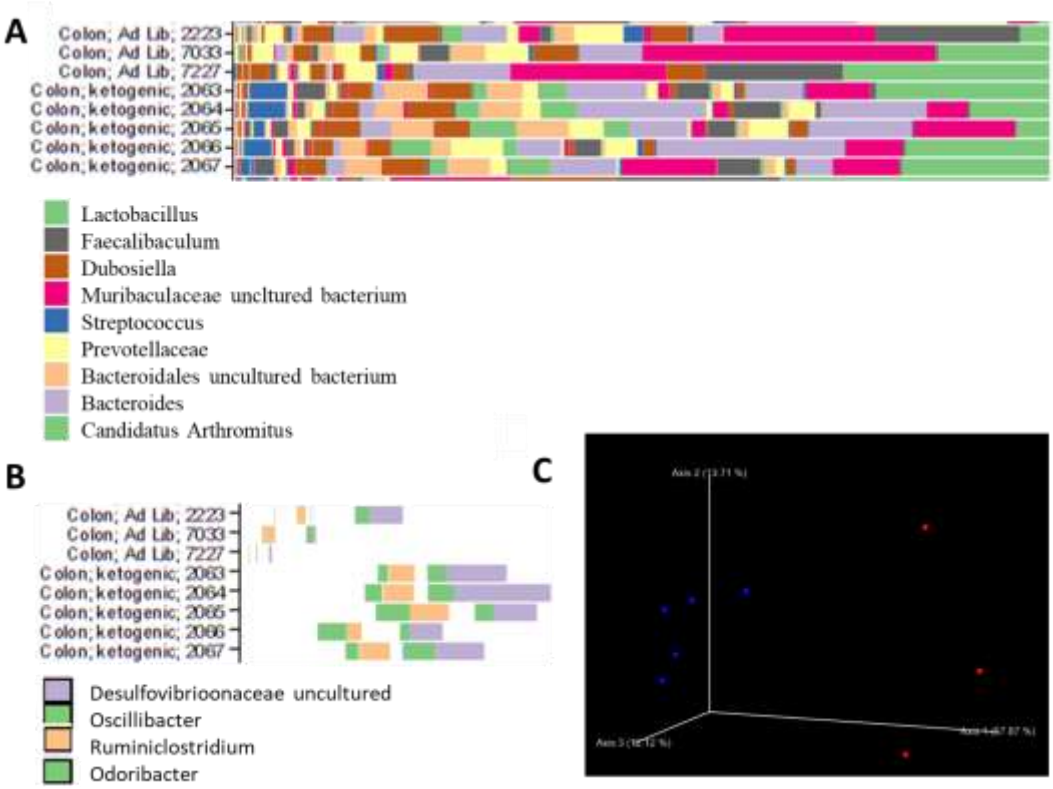


Figure3.17: 16S colonic microbiota analysis of ketogenic diet fed mice. Next generation sequencing (NGS) analysis of the colon lumen samples of mice fed with ketogenic diet and chow for 4 months. (A) Genus-level microbiota analysis of colon lumen samples. (B) Genus level of increased bacteria in ketogenic diet. (C) Principal Component Analysis (PCA) Red Dots: Mice fed with chow diet. Blue dots: Mice fed with ketogenic diet. n≥3 mice for each group.

Figure 3.17B, shows the increased bacterial genera due to ketogenic diet. Humans and other mammals host a complex gut microbiota whose composition differs throughout the gastrointestinal tract (Zoetendal et al., 2012). So it is important to

analyze microbial composition and diversity of not only stool content but also lumenic content. This study was important as being the first that analysed the colon lumen microbiota of ketogenic diet fed mice. Focusing on bacteria increased due to ketogenic diet, we observe changes in four genera. These bacteria are *Desulfovibrionaceae* (Proteobacteria phylum), *Oscillibacter* (Firmicutes phylum), *Ruminiclostridium* (Firmicutes phylum), *Odoribacter* (Bacteroidetes phylum). Also, by using principal component analysis (PCA, Figure 3.17C), it can be seen the clear separation between ketogenic diet and chow diet groups. Therefore, it can be concluded that microbial composition of colon lumen of ketogenic diet fed mice is different from chow diet fed mice.

Figure 3.18 shows NGS analysis of small intestine and colon lumen samples of mice which fasted for 24 hours. In fasting, it was observed that there was an increase in *Lactobacillus* and a decrease in *Dubosiella* in small intestinal lumen. Furthermore, there was an increase in *Lactobacillus* and *Prevotellaceae* and and a decrease in *Dubosiella* and *Muribaculaceae* after fasting for 24 hours in colon lumen. *Lactobacillus* genus were significantly increased both in the small intestine and colon lumen of fasted mice. Similarly, it has been shown that *Lactobacillus* increased considerably after 8 weeks of calorie restriction (which include 70% of ad libitum diet) treatment in rats (Fraumene et al., 2018).

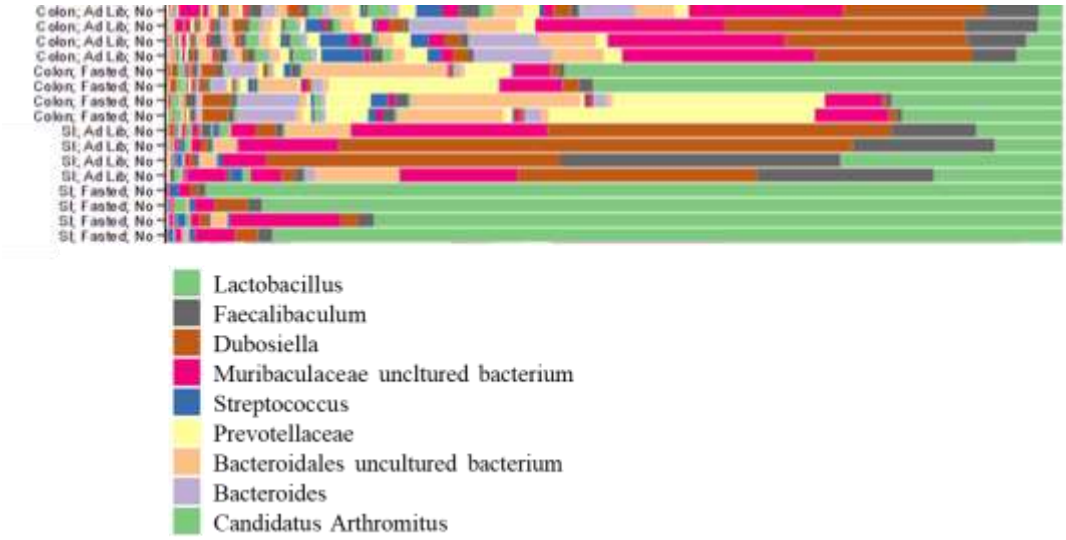


Figure 3.18: 16S colon and small intestine microbiota analysis of fasted mice. Next generation sequencing (NGS) analysis of the colon and small intestine lumen samples from mice fasted 24 hours and mice fed ad libitum as control group. Microbiota analysis was performed at genus-level. n=4 for each group.

Taken together, data showed that only 24 hours are enough for colonization of *Lactobacillus* and dominate the gastrointestinal tract. Bacterial mapping of gastrointestinal tract of mice has revealed that *Lactobacillaceae* family was dominated at stomach and small intestine at about 40% level and reversely *Lactobacillus* family was found in cecum and colon at about 5% level (Gu et al., 2013). In the same study, they have shown that *Prevotellaceae* family was dominated in the colon at approximately 50 % level (Gu et al., 2013). But in this study, the amount of *Prevotellaceae* remained little in the colon of the *ad libitum* group. These differences might be because different mice strain in these two studies. However, both *Lactobacillus* and *Prevotellaceae* were dominated in their specific regions (small intestine and colon respectively) after 24 hours fasting. This may be because these two bacterial families are more competitive and persistent in their respective regions. While comparing the fasting regime with the ketogenic diet in our study, both ketogenic diet fed mice and 24 hours fasting mice showed changes of microbiota in lumen. But, it can be said that the microbial diversity in the gastrointestinal lumen of mice in the fasting regim decreased dramatically, while the diversity is preserved in the ketogenic diet fed mice.

Lactobacillus, which was dominantly present in stool samples of the mouse in the DGGE profile, was also found at the same level in NGS results. However, the decrease in microbial diversity observed in DGGE profiles of mice fed ketogenic diets was not observed in NGS results of colon lumen samples mice fed with ketogenic diet. Different results may be due to the using different samples in microbial analysis. The DGGE is a useful method to evaluate the microbial diversity of a community, especially to evaluate the batch-to-batch differentiation and the stability of the community over time. However, DGGE as a single used technique may be limited in detecting the small number of bacteria in the community (Samarajeewa et al., 2015). The use of NGS, which can read and detect all bacteria in a community, after the DGGE technique can be complementary. Therefore, DGGE can be used to monitor changes happening in the bacterial community, and NGS can be used for first and last samples for a more detailed analysis.

3.7 Organoid Formation Assay Results

Organoid forming capacity of distal colon was greater than proximal colon in this study. It is known that proximal colon and distal colon have distinct physical and metabolic differences. Firstly, it has been reported that microbial diversity increases from proximal colon to distal colon (Wang et al., 2003; Müller et al., 2020; James et al., 2020). Secondly butyrate concentration decreases from proximal colon to distal colon (Müller et al., 2020) and it was reported that lumen butyrate concentration is about 5 mM in mouse and 70 mM in human (Louis et al., 2007) and 0.5 mM in mice stool (Zarrinpar et al., 2018). Lastly there are many differentially expressed genes in proximal and distal colon such as peroxisome proliferator-activated receptor γ (PPAR-γ) (Su et al., 2007) and its target genes like Hmgcs2 (Helenius et al., 2015). Both PPAR-γ and Hmgcs2 expression are higher in the proximal colon than the distal colon. We also showed this regional differential expressions in our study (Figure 3.19). The difference in organoid forming capacity in the proximal colon and distal colon may be due to one of the above-mentioned differences or may be due to the cumulative effect of these regional differences. The differences in

organoid forming capacity of proximal and distal colon also showed that proximal and distal ISCs need different media conditions in organoid assay.

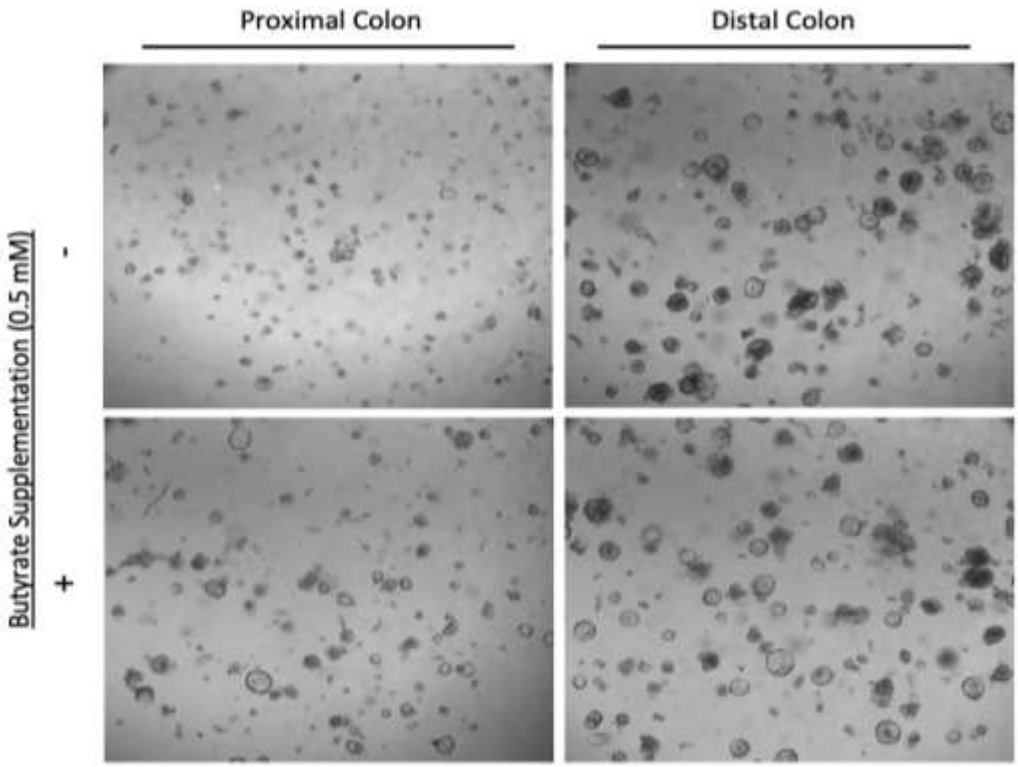


Figure 3.19: Organoid-forming assay for proximal and distal colon with and without butyrate. 0.5 mM butyrate were added to organoid media. Images are representative images of day 4 organoids. n > 10 mice per group. (4x magnification).

Organoid assay and subsequent studies were performed to see the effect of microbial derived short chain fatty acid (SCFA) butyrate in intestinal stem cells function and on the ketogenic pathway. Results showed that butyrate increased the number of organoids both in proximal and distal colon. PPAR-γ is a subfamily of nuclear receptors and regulates transcription of various genes. SCFA butyrate regulates the PPAR-γ in diverse tissue (Alex et al, 2012 and Byndloss et al 2017, Harmon et al, 2010, Kim et al, 2019). In the current study, we showed that butyrate increased the PPAR-γ expression in proximal and distal colon with in vitro organoid assay. We also observed that Hmgcs2 and Cpt1a, which are considered to be PPARg target genes, increased both at the mRNA level and at the protein level.

Butyrate, which is a microbial derived SCFA, boosts the organoid formation capacity at an optimum 0.5 mM concentration while higher butyrate concentration (1 mM) has inhibition effects on organoid formation (Figure 3.19). The optimum butyrate concentration was 0.5 M for culture organoids in vitro and the number of distal colon organoids were more than the number of proximal colon organoids (Figure3.19 and Figure 3.20). As can be seen from Figure 3.20, low concentration of butyrate is not enough, and high concentration of butyrate shows an inhibition effect on organoid formation. Moreover, high concentration of butyrate supplementation can inhibit growth of both proximal and distal organoids.

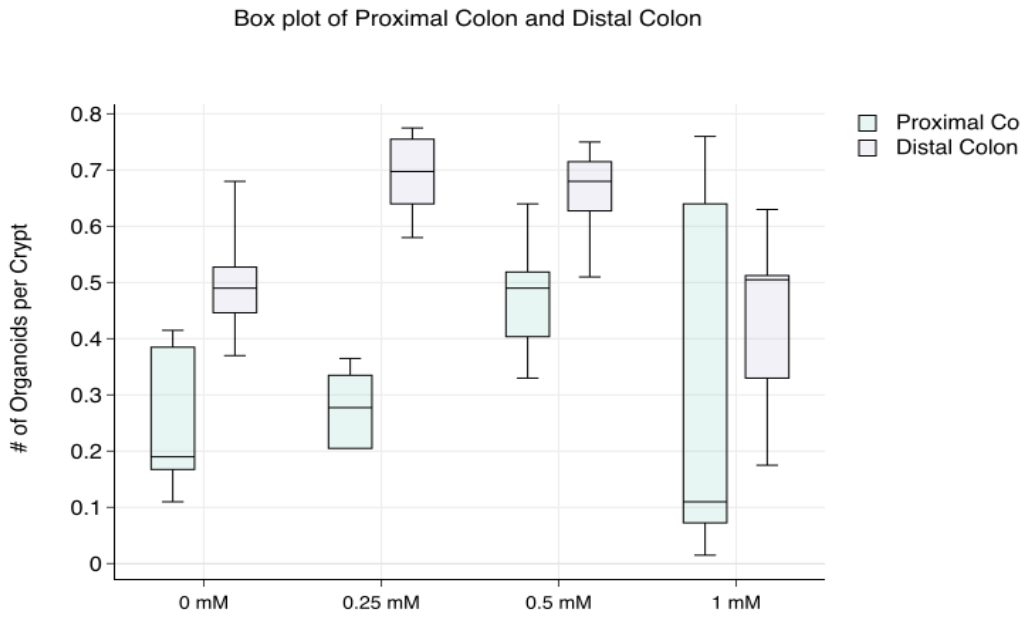


Figure 3.20: Characterization of colon organoid-formation capacity with different butyrate concentrations. Organoid-forming assay for proximal and distal colon with different concentration of butyrate (0, 0.25, 0.5 and 1 mM). n=5 mice per group. Organoid numbers were normalized to the started crypt number.

Eight hours fasting has elevated the βOHB concentration in serum (Geisler et al., 2016). However, the response of intestinal stem cells to fasting occurs in a longer time. Studies have used 24 hours fasted mice to see the effect of fasting on hematopoietic stem cells (Cheng et al 2014) and intestinal stem cells (Mihaylova et al, 2018).

To see the effect of fasting on organoid formation capacity of proximal and distal colon crypt, fasted mice were used in this study. 8 hours fasting has elevated the βOHB concentration in serum (Geisler et al., 2016). However, the response of stem cells to fasting takes a longer time. Studies have used 24 hours fasted mice to see the effect of fasting on hematopoietic stem cells (Cheng et al 2014) and intestinal stem cells (Mihaylova et al, 2018). 24 hours fasted mice were sacrificed and crypt were cultured as organoid assay. Figure3.21 shows day 4 colon organoids of ad libitum fed and fasted mice. Fasting improves adult stem cell function in diverse tissues (Longo et al, 2014; Cheng et al 2014; Mihaylova et al, 2018). It has also shown that diet 24 hours fasting improves LGR5+ ISCs function in the mouse intestine. They have shown increased ISCs activity in 24 hours fasted mice by using organoid cultures. Crypt organoid-forming capacity of small intestine of 24 hours fasted mice significantly increases as compared with mice fed standard chow *ad libitum* (Mihaylova et al 2018, Cheng et al 2019). Fasting increased the organoid-forming capacity of both proximal and distal colon (Figure3.21). Especially in the proximal colon, there was a significant increase of organoid formation after 24 hours fasting (Figure 3.21).

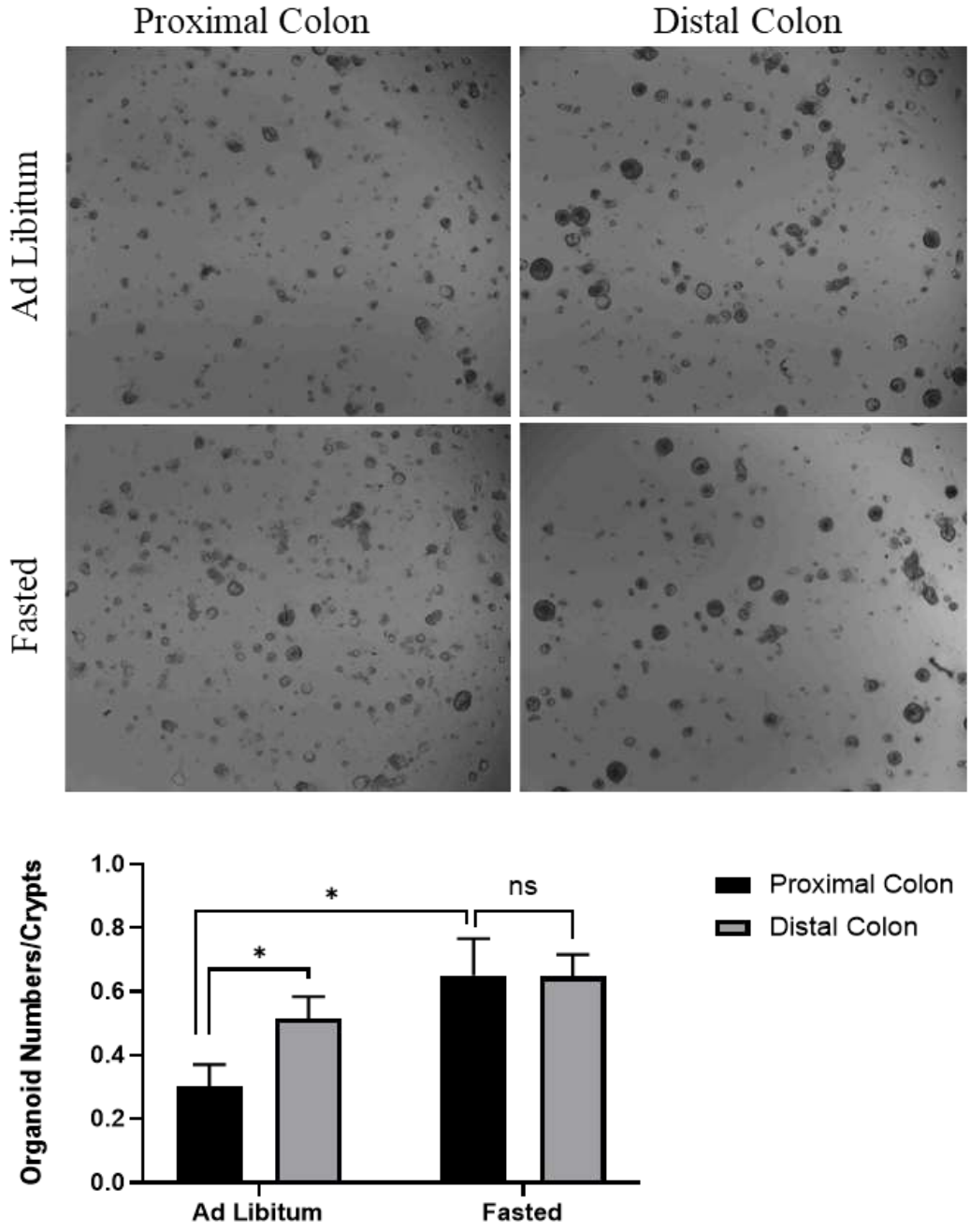


Figure 3.21: Organoid-forming assay for proximal and distal colon of fasted and ad libitum mice. Images are representative images of day 4 organoids. Graph shows the quantification of organoid numbers. n=4 mice per group. (4x magnification).

3.7.1 QPCR and Western Blot Results of Organoid Samples.

After the organoid assay, organoid samples from day4 were collected for qPCR and western blot experiment. We analysed two important genes in ketogenesis; Hmgcs2 and Cpt1a. The latter, Cpt1a (Carnitine Palmitoyltransferase 1A) is a mitochondrial outer membrane protein that converts activated fatty acids into acylcarnitines and is important for the ketogenic pathway. Results show that butyrate addition increases the expression of Hmgcs2 and Cpt1a both in proximal and distal colon in in vitro organoids. Similar results were obtained by qRT PCR technique (Figure 3.22) and western blot technique (Figure 3.23).

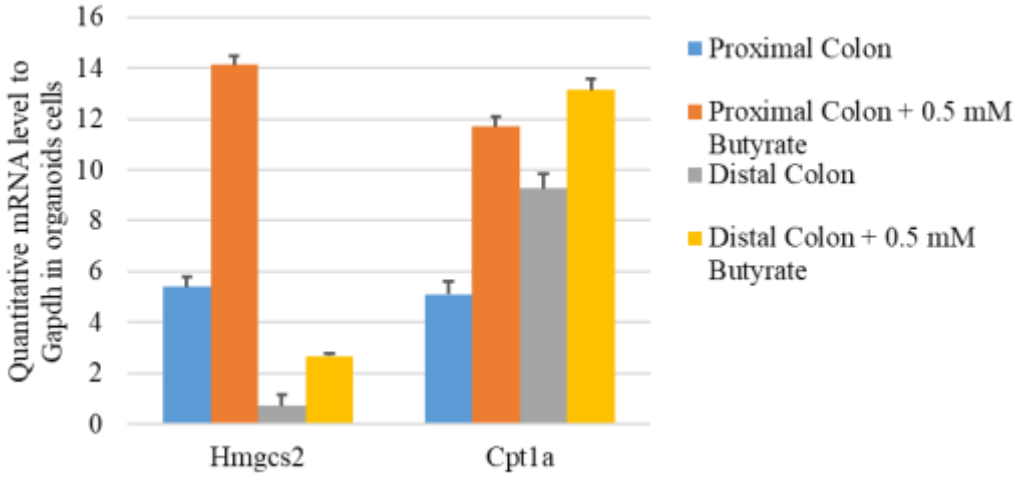


Figure 3.22: Quantitative mRNA level of Hmgcs2 and Cpt1a in butyrate treated colon organoids. Hmgcs2 and Cpt1a mRNA levels in butyrate treated/untreated organoids of proximal and distal colon. Hmgcs2 and Cpt1a quantitative PCR results were expressed $2^{-\Delta Ct}$. Gapdh gene were used as housekeeping gene. Bars show the standard deviations.

Microbial derived butyrate is a well-known short chain fatty acid (SCFA) by upregulating the PPAR-γ in the intestine (Alex et al, 2012 and Byndloss et al 2017). Butyrate has a dose dependent responsive effect on activity of PPAR-γ and its target genes. Increased butyrate until a certain concentration strongly activated PPAR-γ but not activated other PPARs. Because microbial derived SCFA butyrate regulate the PPAR-γ in diverse tissue (Alex et al, 2012 and Byndloss et al 2017, Harmon et al,

2010, Kim et al, 2019), (Figure 3.23B) we tested whether microbial induced PPAR-γ by butyrate up regulates the Hmgcs2 in organoid culture from proximal and distal colon crypt. Butyrate (0.5 mM) increases the expression of ketogenic enzymes, Hmgcs2 and Cpt1a in proximal and distal colon crypt organoids both in mRNA level (Fig 3.22) and protein level (Figure 3.23A). Therefore, this showed that induction of the ketogenic pathway by butyrate was mediated by PPAR-γ.

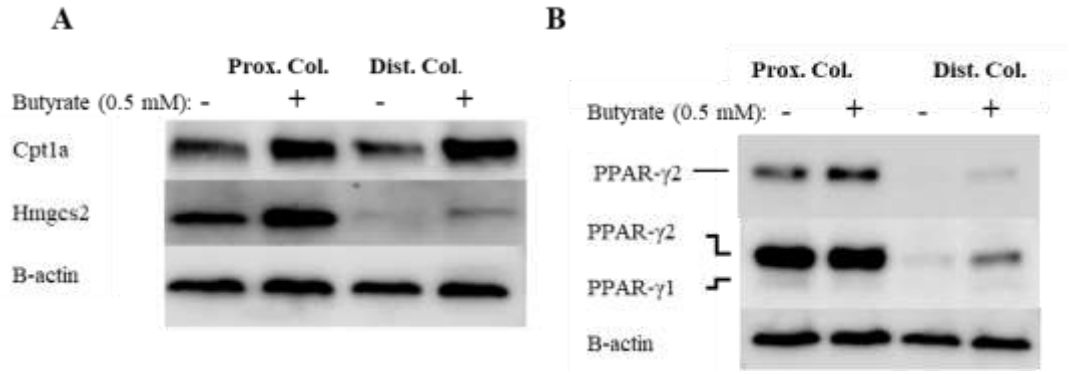


Figure 3.23:Protein expression of Hmgcs2, Cpt1a and PPAR-γ in butyrate treated colon organoids. Hmgcs2 and Cpt1a (A) and PPAR-γ1 and PPAR-γ2 (B) protein expression levels in 0.5 mM butyrate treated/untreated organoids of proximal and distal colon by by imminoblotting. Protein levels were normalized to B-actin. Quantifications were performed with Image J and B-actin protein was used as internal control.

3.8 Antibiotic Treatment

In a recent study, (Zarrinpar et al, 2018) treated mice with antibiotics resulted in a dramatic decrease of butyrate in the cecal content of mice. Furthermore, genes involved in ketogenesis were down-regulated Hmgcs2 and Cpt1a in the cecum and anaerobic glycolysis was up-regulated. In another previous study, it was shown that 24 days antibiotic treatment significantly reduced the copy number of 16S rRNA genes in stool samples of mice (Reikvam et al., 2011). To understand how colonic Hmgcs2 changes during microbiota depletion, we have treated C57B/6 mice with antibiotics for 24 days (Figure 3.24A). The cecum of antibiotics-treated mice was larger than its normal size (Figure 3.23B). This is a well-known characteristic of

antibiotic treated mouse cecum (Ge et al, 2017; Murakami et al, 2016). Microbial depletion was verified by real-time PCR (Figure 3.23C). Normalized mean bodyweight change compared to the initial weight during the antibiotic treatment is shown in Figure 3.23D. There isn't any significant change in body weight between control and antibiotic treatment groups.

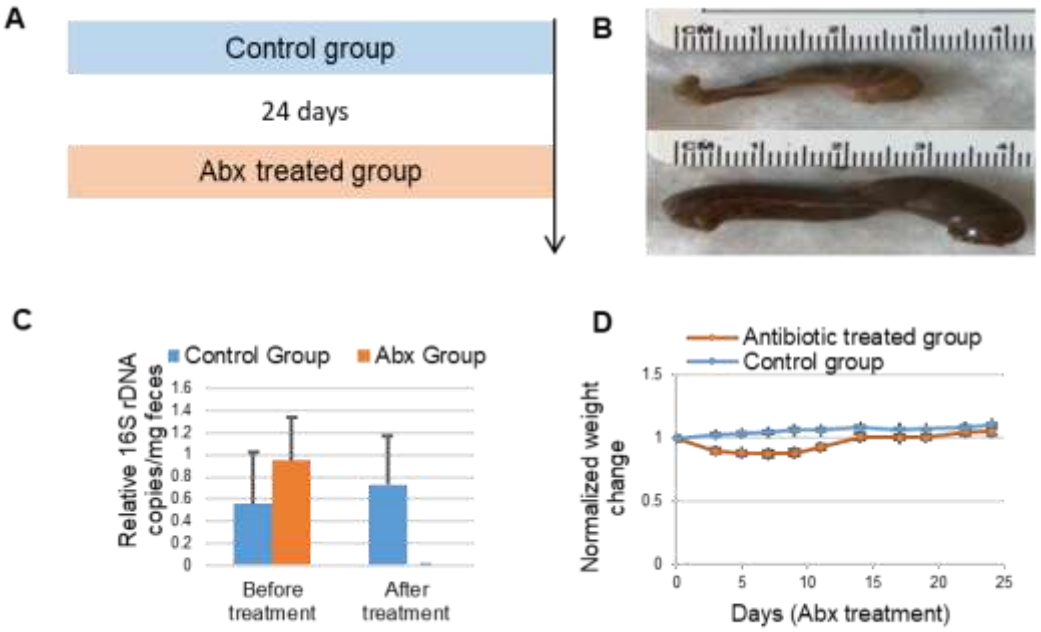


Figure 3.24: Antibiotic treatment of C57B/6 mice. (A) Schematic representation of experimental setup of antibiotic treatment. (B) Representative images of cecum were showed for each group. Upper image: The cecum of control group, The bottom image: The cecum of antibiotics-treated group. (C)Relative abundance 16S rDNA gene copies were determined by quantitative real-time polymerase chain reaction (PCR) using universal bacterial primer for 16S ribosomal RNA genes from stool samples before and after the antibiotic treatment. (D) Normalized mean bodyweight change compared to the initial weight during the antibiotic treatment is shown.

Average crypt length of both proximal colon and distal colon were decreased in antibiotic treated group when compared with control group (Figure 3.25B). At 4 hours after BrdU injection, the number of BrdU-positive epithelial cells in proximal and distal colon crypts was greatly reduced in the antibiotic-treated mice compared with control mice (Park et al, 2016, Figure 3.25A and Figure 3.25C). BrdU

(Bromodeoxyuridine;5-bromo-2'-deoxyuridine) is used in detection of rapidly proliferating cells *in vivo*. Therefore, microbial depletion by antibiotic treatment caused a decrease in proliferation activity of intestinal stem cells.

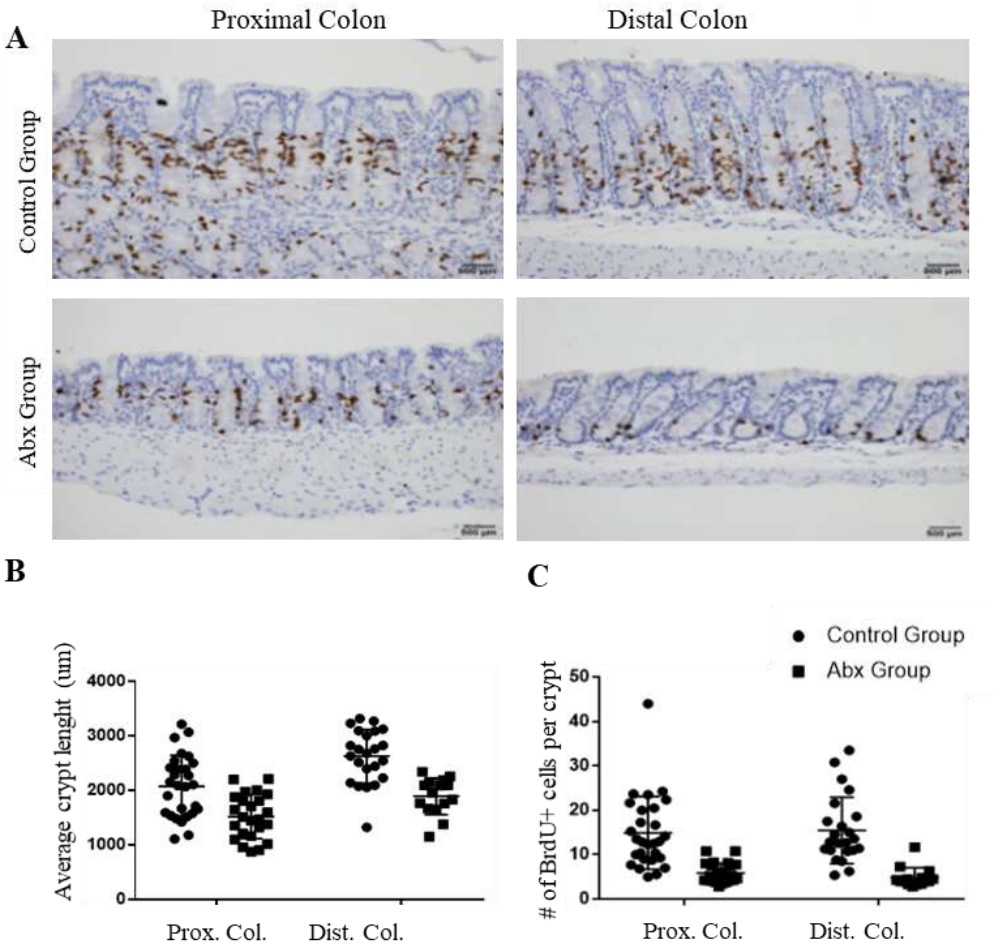


Figure 3.25:Immunohistochemistry analysis of antibiotic treated/untreated mice. (A) BrdU staining of proximal and distal colon by immunohistochemistry (IHC) after 4 hours after BrdU administration. BrdU positive cells show brown nuclear stain. Scale bar: 500 μm. (B) Average crypt depth (μm) was quantified in control and antibiotic treated mice both in proximal and distal colon. Each dot represents crypt depth of a crypt. Data represents mean ± SD of n = 4 mice per group. (C) Quantitative analysis of BrdU positive cells per crypt. Each dot represents BrdU positive cells in one crypt. Data represents mean ± SD of n = 4 mice per group.

Preliminary IHC analysis shows that Hmgcs2 protein expression highly decreased in distal colon of antibiotic treated mice. Furthermore, the extent of decrease is similar

and limited to the crypt bottom (ISC zone) in both proximal and distal colon in antibiotic treated mice (Figure 3.26). Antibiotic treated mice have decreased butyrate in cecal (Zarrinpar et al, 2018). Overall butyrate reduction caused a decrease in Hmgcs2 and expression and proliferation activity of stem cells which also affect crypt length of both proximal and distal colon (Figure 3.25B).

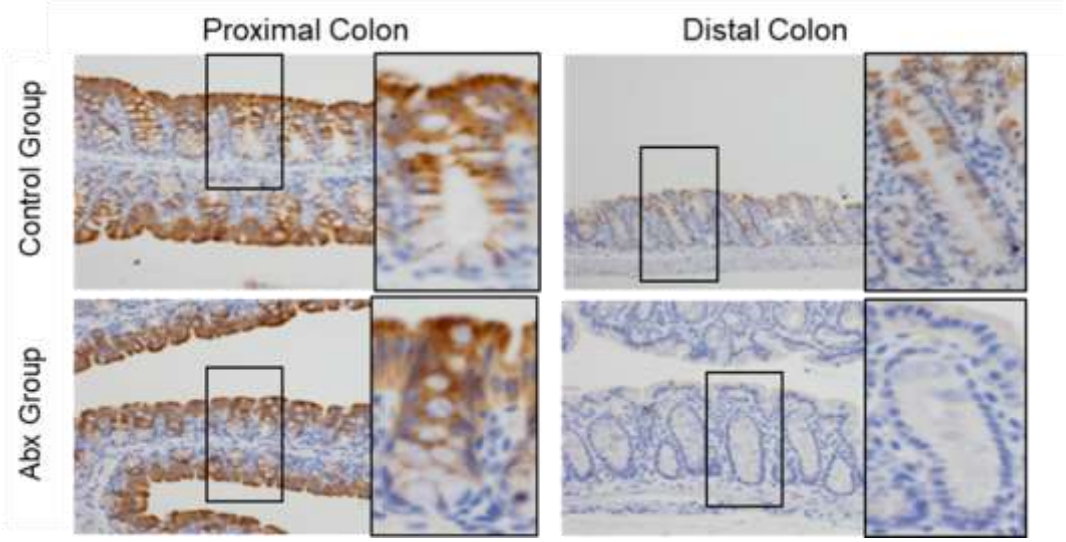


Figure 3.26: Hmgcs2 expression in proximal and distal colon of antibiotic treated/untreated mice. Mouse colon HMGCS2 protein expression by IHC (brown). The image represents one of 4 biological replicates. Scale bar, 500 μm.

It was obvious from our study that butyrate availability in colon lumen affects the intestinal stem cell activity and changes the gene expression level of some certain genes such as PPAR-γ, Hmgcs2 and Cpt1a. The concentration of butyrate in lumen crucial, in a study it was claimed that intestinal stem cells are protected from butyrate by crypt structure (butyrate concentration decreases from lumen to crypt base) and butyrate suppresses the intestinal stem cell proliferation after exposure of ISCs to lumen (Kaiko et al., 2016). We also observed this phenomenon in our study, by using high butyrate concentration (1 mM). This concentration may seem lower than the reported lumenic butyrate concentration (5 mM) in mice, but it is clear that ISCs are more open to the butyrate exposure in the petri dish.

Ketogenesis contributes to intestinal stem cell proliferation and differentiation via the rate limiting enzyme, Hmgcs2 (Wang et al., 2017; Cheng et al., 2019). Also we found that butyrate availability by butyrate addition to organoid media or butyrate

unavailability by antibiotic treatment robustly affected the colon stem cell proliferation and Hmgcs2 expression. Ketogenesis can be activated by ketogenic diet fed, short term fasting like 24 hour or less and butyrate supplementation in the mice. In humans, this can be achieved by long term fastıng such as intermittent fasting (IF) or periodic fasting (PF) (Longo et al., 2021), or by consuming foods suitable for a ketogenic diet, such as non starchy vegetables, nuts, butter, seeds, avocado, olive oil, fish, eggs, red meat, cheese (Taylor et al., 2019) to keep ketogenesis active.

CHAPTER 4

CONCLUSION

Microbiota is one of the most important variables shaped by nutritional habits. Our microbiota analyzing results show that microbial composition is rapidly changed during the different diets which are consistent with many diet and microbiota studies (Conlon et al., 2014 and David et al., 2014). Nevertheless, mice fed with standard chow diet, which was used as a control, has conserved fecal microbial composition and microbial richness throughout the study.

A significant decrease in the number of bands was observed in the DGGE profiles of the stool samples in high carbohydrate with zero fiber (HC-0F) and high carbohydrate (HC) diet groups compared to the standard chow diet (S). This showed that the microbial diversity in these groups reduced. *Lactococcus lactis* and *Lactobacillus murinus* species are common and mostly conserved their viability among the different diets in this study (Biddle et al, 2014; Taniguchi et al, 2020). *Anaerotaenia torta*, belongs to firmicutes phylum, seems higher in low carbohydrate with zero fiber (LC-0F) and ketogenic (K) diets than standard chow (S), high carbohydrate with zero fiber (HC) and high carbohydrate (HC) diet groups. DGGE patterns also showed that, *Muribaculum intestinale* from Bacteroidetes phylum were consoderably increased in HC-0F and HC diet groups. DGGE was succesfuly used for comparision of microbial population in different diet fed mice.

In colon tissues from mice fed with HC-0F diet, anti-inflammatory and pro-inflammatory cytokines genes expression were up-regulated and tight junction genes expression (Occludin, Claudin-1) were down-regulated. Similar results were seen in the small intestinal tissues of HC-0F group. In contrast, cytokines genes expression in colon tissues from mice fed LC-0F diet were down-regulated. Western blotting results support qRT PCR results. Overall, the mRNA levels of cytokines and tight

junctions showed that inflammation was greater in the HC-0F and HC groups than in the S or LC-0F groups. Pro-inflammatory cytokines level in colonic tissues from HC group showed significant increases in IL-1β, IL-6 and TNF-α compared to S, LC-0F and K diet groups. But anti-inflammatory cytokines in the HC group did not show any change compared to the standard diet. This may result from high level of pro-inflammatory cytokines suppressing anti-inflammatory cytokine production in the HC group.

Also it was observed a negative correlation between bacterial diversity and colonic inflammation. While bacterial diversity decreased in HC-0F and HC diet groups compared to the standard chow diet (S) group, colonic inflammation increased. This correlation has been reported before (Le Chatelier et al., 2013). High carbohydrate with zero fiber (HC-0F, include 74% carbohydrate) and high carbohydrate (HC, include 72% carbohydrate) diets that used in this study were similar, the only difference was HC did not include fiber. Although the effects of these two diets on microbiota diversity were the same (both decreased the diversity), their effects on inflammation were different. Therefore, the effect of HC and LF diet, and thus the effect of the absence/presence of fiber on microbial changes and further inducing inflammation can be seen clearly in our study. Collectively, high carbohydrate including diets (HC and LF) in our study are associated with less diverse microbiota and high inflammation.

Next generation sequencing results showed that ketogenic diet changes the intestinal microbiota importantly. Samples from mice fed with ketogenic diet for 4 months showed a decrease in family *Muribaculaceae* and increase in *Desulfovibrionaceae, Oscillibacter, Ruminiclostridium, Odoribacter.* Also, in fasting, it was observed that there was an increase in *Lactobacillus* and a decrease in *Dubosiella* in small intestinal lumen. Furthermore, there was an increase in *Lactobacillus* and *Prevotellaceae* and a decrease in *Dubosiella* and *Muribaculaceae* after fasting for 24 hours in colon lumen. *Lactobacillus* genus were significantly increased both in the small intestine and colon lumen of fasted mice. Taken together, fasting experiment showed that 24 hours is enough for colonization of *Lactobacillus* and dominate the

gastrointestinal tract. Both *Lactobacillus* and *Prevotellaceae* were dominated in their specific regions (small intestine and colon respectively, Gu et al., 2013;) after 24 hours fasting. This may be because these two bacterial families are more competitive and persistent in their respective regions.

While comparing the fasting regime with the ketogenic diet in our study, both ketogenic diet fed mice and 24 hours fasting mice showed changes of microbiota in lumen. But, it can be said that the microbial diversity in the gastrointestinal lumen of mice in the fasting regim decreased dramatically, while the diversity is preserved in the ketogenic diet fed mice. Lack of food may lead to enrichment of more compatible and resilient bacterial species and cause decrease of diversity in short term fasting (24 hour). But studies have shown that long term fasting such as intermittent fasting has increased microbial biodiversity (Su et al., 2021; Shi et al., 2021). This showed that while 24-hour fasting allows enough time to shape microbiota rapidly and increase of some specific species like *Lactobacillus* and *Prevotellaceae*, it is not enough time for biodiversity to come to balance again. Diversity is a substantial parameter of the human and other mammalian microbiota (Pasolli et al., 2019) and more diverse microbiota represent a healthier body (Human Microbiome Project Consortium, 2012).

Organoid forming capacity of distal colon was greater than proximal colon in this study. It is known that proximal colon and distal colon have distinct physical and metabolic differences. We also showed this regional differential expressions in our study. The difference in organoid forming capacity in the proximal colon and distal colon may be due to one of the above-mentioned differences or may be due to the cumulative effect of these regional differences. The differences in organoid forming capacity of proximal and distal colon also showed that proximal and distal ISCs need different media conditions in organoid assay. Organoid assay and subsequent studies were performed to see the effect of microbial derived short chain fatty acid (SCFA) butyrate in intestinal stem cells function and on the ketogenic pathway. Results showed that butyrate increased the number of organoids both in proximal and distal colon. PPAR-γ is a subfamily of nuclear receptors and regulates transcription of

various genes. We observed that Hmgcs2 and Cpt1a, which are considered to be PPARg target genes, increased both at the mRNA level and at the protein level.

Contrastly, after the antibiotic treatment and depletion of microbiota, it was observed that Hmgcs2 expression significantly decreased, proliferation ratio of intestinal stem cells and crypt length were decreased in antibiotic treated group. It was obvious from our study that butyrate availability in colon lumen affects the intestinal stem cell activity and changes the gene expression level of some certain genes such as PPAR-γ, Hmgcs2 and Cpt1a. We found that butyrate availability by butyrate addition to organoid media or butyrate unavailability by antibiotic treatment robustly affected the colon stem cell proliferation and Hmgcs2 expression. Ketogenesis can be activated by ketogenic diet fed, short term fasting like 24 hour or less and butyrate supplementation in the mice. In humans, this can be achieved by long term fastıng such as intermittent fasting (IF) or periodic fasting (PF) (Longo et al., 2021), or by consuming foods suitable for a ketogenic diet, such as non starchy vegetables, nuts, butter, seeds, avocado, olive oil, fish, eggs, red meat, cheese (Taylor et al., 2019) to keep ketogenesis active.

This study is important in regard to allowing us to screen and compare the effect of different diets on microbiota and intestinal inflammation. Also it is significant to see the effect of microbiota and microbiota derived butyrate on intestinal stem cell proliferation and genes in the ketogenic pathway. In this study, we demonstrated that high carbohydrate with zero fiber (HC-0F) and high carbohydrate diet (HC) diet fed mice reduced microbial diversity and increased the inflammation level compared to the low carbohydrate with zero fiber (LC-0F) or standard chow (S) diet fed mice. Moreover, we showed that butyrate induced the expression of genes in ketogenic pathways in in vitro organoid experiment while intestinal stem cell proliferation and expression of genes in ketogenic pathways decreased after antibiotic treatment. Also our data support that, the changes in ketogenic pathway and intestinal stem cell proliferation is driven by PPARg which is modulated by microbiota.

REFERENCES

Adijanto, J., Du, J., Moffat, C., Seifert, E. L., Hurle, J. B., & Philp, N. J. (2014). The retinal pigment epithelium utilizes fatty acids for ketogenesis. The Journal of biological chemistry, 289(30), 20570–20582. doi: 10.1074/jbc.M114.565457

Alex S, Lange K, Amolo T, et al. Short-chain fatty acids stimulate angiopoietin-like 4 synthesis in human colon adenocarcinoma cells by activating peroxisome proliferator-activated receptor γ. Mol Cell Biol. 2013;33(7):1303-1316. doi:10.1128/MCB.00858-12

Alonso, S., & Yilmaz, Ö. H. (2018). Nutritional Regulation of Intestinal Stem Cells. Annual review of nutrition, 38, 273–301. doi: org/10.1146/annurev-nutr-082117-051644

Amre, D. K., D'Souza, S., Morgan, K., Seidman, G., Lambrette, P., Grimard, G., Israel, D., Mack, D., Ghadirian, P., Deslandres, C., Chotard, V., Budai, B., Law, L., Levy, E., & Seidman, E. G. (2007). Imbalances in dietary consumption of fatty acids, vegetables, and fruits are associated with risk for Crohn's disease in children. The American journal of gastroenterology, 102(9), 2016–2025. doi: 10.1111/j.1572-0241.2007.01411.x

Anderson, J. M., & Van Itallie, C. M. (2009). Physiology and function of the tight junction. Cold Spring Harbor perspectives in biology, 1(2), a002584. doi: 10.1101/cshperspect.a002584

Andrews, C., McLean, M. H., & Durum, S. K. (2016). Interleukin-27 as a Novel Therapy for Inflammatory Bowel Disease: A Critical Review of the Literature. Inflammatory bowel diseases, 22(9), 2255–2264. doi: 10.1097/MIB.0000000000000818

Andrews, C., McLean, M. H., & Durum, S. K. (2018). Cytokine Tuning of Intestinal Epithelial Function. Frontiers in immunology, 9, 1270. doi: 10.3389/fimmu.2018.01270

Antunes, M. M., Godoy, G., de Almeida-Souza, C. B., da Rocha, B. A., da Silva-Santi, L. G., Masi, L. N., Carbonera, F., Visentainer, J. V., Curi, R., & Bazotte, R. B. (2020). A high-carbohydrate diet induces greater inflammation than a high-fat diet in mouse skeletal muscle. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas, 53(3), e9039. doi: 10.1590/1414-431X20199039

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., Fernandes, G. R., Tap, J., Bruls, T., Batto, J. M., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., Kurokawa, K., … Bork, P. (2011). Enterotypes of the human gut microbiome. Nature, 473(7346), 174–180. doi: org/10.1038/nature09944

Ayabe, T., Satchell, D. P., Wilson, C. L., Parks, W. C., Selsted, M. E., & Ouellette, A. J. (2000). Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. Nature immunology, 1(2), 113–118. doi: 10.1038/77783

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., Semenkovich, C. F., & Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. Proceedings of the National Academy of Sciences of the United States of America, 101(44), 15718–15723. doi: 10.1073/pnas.0407076101

Barker, N., van Es, J. H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P. J., & Clevers, H. (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature, 449(7165), 1003–1007. doi: 10.1038/nature06196

Barouei, J., Bendiks, Z., Martinic, A., Mishchuk, D., Heeney, D., Hsieh, Y. H., Kieffer, D., Zaragoza, J., Martin, R., Slupsky, C., & Marco, M. L. (2017). Microbiota, metabolome, and immune alterations in obese mice fed a high-fat diet containing type 2 resistant starch. Molecular nutrition & food research, 61(11), 10.1002/mnfr.201700184. doi: 10.1002/mnfr.201700184

Baumgart, D. C., & Sandborn, W. J. (2012). Crohn's disease. Lancet (London, England), 380(9853), 1590–1605. doi: 10.1016/S0140-6736(12)60026-9

Behjati, S., & Tarpey, P. S. (2013). What is next generation sequencing?. Archives of disease in childhood. Education and practice edition, 98(6), 236–238. doi: 10.1136/archdischild-2013-304340

Beumer, J., & Clevers, H. (2021). Cell fate specification and differentiation in the adult mammalian intestine. Nature reviews. Molecular cell biology, 22(1), 39–53. doi: 10.1038/s41580-020-0278-0

Beyaz, S., & Yilmaz, Ö. H. (2016a). Molecular Pathways: Dietary Regulation of Stemness and Tumor Initiation by the PPAR-δ Pathway. Clinical cancer research : an official journal of the American Association for Cancer Research, 22(23), 5636–5641. doi: 10.1158/1078-0432.CCR-16-0775

Beyaz, S., Mana, M. D., Roper, J., Kedrin, D., Saadatpour, A., Hong, S. J., Bauer-Rowe, K. E., Xifaras, M. E., Akkad, A., Arias, E., Pinello, L., Katz, Y., Shinagare, S., Abu-Remaileh, M., Mihaylova, M. M., Lamming, D. W., Dogum, R., Guo, G., Bell, G. W., Selig, M., … Yilmaz, Ö. H. (2016b). High-fat diet enhances stemness and tumorigenicity of intestinal progenitors. Nature, 531(7592), 53–58. doi: 10.1038/nature17173

Biddle, A. S., Leschine, S., Huntemann, M., Han, J., Chen, A., Kyrpides, N., Markowitz, V., Palaniappan, K., Ivanova, N., Mikhailova, N., Ovchinnikova, G., Schaumberg, A., Pati, A., Stamatis, D., Reddy, T., Lobos, E., Goodwin, L., Nordberg, H. P., Cantor, M. N., Hua, S. X., … Blanchard, J. L. (2014). The complete genome sequence of Clostridium indolis DSM 755(T.). Standards in genomic sciences, 9(3), 1089–1104. doi: 10.4056/sigs.5281010

Brown, K., DeCoffe, D., Molcan, E., & Gibson, D. L. (2012). Diet-induced dysbiosis of the intestinal microbiota and the effects on immunity and disease. Nutrients, 4(8), 1095–1119. doi: 10.3390/nu4081095

Browning, J. D., Baxter, J., Satapati, S., & Burgess, S. C. (2012). The effect of short-term fasting on liver and skeletal muscle lipid, glucose, and energy metabolism in healthy women and men. Journal of lipid research, 53(3), 577–586.

Bruewer, M., Samarin, S., & Nusrat, A. (2006). Inflammatory bowel disease and the apical junctional complex. Annals of the New York Academy of Sciences, 1072, 242–252. doi: 10.1196/annals.1326.017

Buettner, R., Schölmerich, J., & Bollheimer, L. C. (2007). High-fat diets: modeling the metabolic disorders of human obesity in rodents. Obesity (Silver Spring, Md.), 15(4), 798–808. doi: 10.1038/oby.2007.608

Buhner, S., Buning, C., Genschel, J., Kling, K., Herrmann, D., Dignass, A., Kuechler, I., Krueger, S., Schmidt, H.H., and Lochs, H. (2006). Genetic basis for increased intestinal permeability in families with Crohn's disease: role of CARD15 3020insC mutation? Gut 55, 342-347.

Byndloss, M. X., Olsan, E. E., Rivera-Chávez, F., Tiffany, C. R., Cevallos, S. A., Lokken, K. L., Torres, T. P., Byndloss, A. J., Faber, F., Gao, Y., Litvak, Y., Lopez, C. A., Xu, G., Napoli, E., Giulivi, C., Tsolis, R. M., Revzin, A., Lebrilla, C. B., & Bäumler, A. J. (2017). Microbiota-activated PPAR-γ signaling inhibits dysbiotic Enterobacteriaceae expansion. Science (New York, N.Y.), 357(6351), 570–575. doi: 10.1126/science.aam9949

Cahill G. F., Jr (1970). Starvation in man. The New England journal of medicine, 282(12), 668–675. doi: 10.1056/NEJM197003192821209

Can, G., Poşul, E., Yılmaz, B., Can, H., Korkmaz, U., Ermiş, F., Kurt, M., & Dağlı, Ü. (2019). Epidemiologic features of ınflammatory bowel disease in Western Blacksea region of Turkey for the last 10 years: retrospective cohort study. The Korean journal of internal medicine, 34(3), 519–529. doi: 10.3904/kjim.2015.310

Cangelosi, A. L., & Yilmaz, Ö. H. (2016). High fat diet and stem cells: Linking diet to intestinal tumor formation. Cell cycle (Georgetown, Tex.), 15(13), 1657–1658. doi: 10.1080/15384101.2016.1175275

Chelakkot, C., Ghim, J., & Ryu, S. H. (2018). Mechanisms regulating intestinal barrier integrity and its pathological implications. Experimental & molecular medicine, 50(8), 103. doi: 10.1038/s12276-018-0126-x

Chen, Y., Liu, H., Wang, L., Zhou, T., Liang, Z., Li, W., Shang, X., Leng, J., Shen, Y., Hu, G., & Qi, L. (2019). Lifestyle intervention modifies the effect of the MC4R genotype on changes in insulin resistance among women with prior gestational diabetes: Tianjin Gestational Diabetes Mellitus Prevention Program. The American journal of clinical nutrition, 110(3), 750–758. doi: 10.1093/ajcn/nqz121

Cheng, C. W., Adams, G. B., Perin, L., Wei, M., Zhou, X., Lam, B. S., Da Sacco, S., Mirisola, M., Quinn, D. I., Dorff, T. B., Kopchick, J. J., & Longo, V. D. (2014). Prolonged fasting reduces IGF-1/PKA to promote hematopoietic-stem-cell-based regeneration and reverse immunosuppression. Cell stem cell, 14(6), 810–823. doi: 10.1016/j.stem.2014.04.014

Cheng, C. W., Biton, M., Haber, A. L., Gunduz, N., Eng, G., Gaynor, L. T., Tripathi, S., Calibasi-Kocal, G., Rickelt, S., Butty, V. L., Moreno-Serrano, M., Iqbal, A. M., Bauer-Rowe, K. E., Imada, S., Ulutas, M. S., Mylonas, C., Whary, M. T., Levine, S. S., Basbinar, Y., Hynes, R. O., … Yilmaz, Ö. H. (2019). Ketone Body Signaling Mediates Intestinal Stem Cell Homeostasis and Adaptation to Diet. Cell, 178(5), 1115–1131.e15.

Chwen, L. T., Foo, H. L., Thanh, N. T., & Choe, D. W. (2013). Growth performance, plasma Fatty acids, villous height and crypt depth of preweaning piglets fed with medium chain triacylglycerol. Asian-Australasian journal of animal sciences, 26(5), 700–704. doi: 10.5713/ajas.2012.12561

Clevers H. (2013). The intestinal crypt, a prototype stem cell compartment. Cell, 154(2), 274–284. doi: 10.1016/j.cell.2013.07.004

Conlon, M. A., & Bird, A. R. (2014). The impact of diet and lifestyle on gut microbiota and human health. Nutrients, 7(1), 17–44. doi: 10.3390/nu7010017

Costello, E. K., Lauber, C. L., Hamady, M., Fierer, N., Gordon, J. I., & Knight, R. (2009). Bacterial community variation in human body habitats across space and time. Science 326(5960), 1694–1697. doi: 10.1126/science.1177486

Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J., & Relman, D. A. (2012). The application of ecological theory toward an understanding of the human microbiome. Science (New York, N.Y.), 336(6086), 1255–1262. doi: 10.1126/science.1224203

Crespo-Piazuelo, D., Estellé, J., Revilla, M., Criado-Mesas, L., Ramayo-Caldas, Y., Óvilo, C., Fernández, A. I., Ballester, M., & Folch, J. M. (2018). Characterization of bacterial microbiota compositions along the intestinal tract in pigs and their interactions and functions. Scientific reports, 8(1), 12727.

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V., Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J., & Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. Nature, 505(7484), 559–563. doi: 10.1038/nature12820

de Britto, M. A., Soletti, R. C., Schanaider, A., Madi, K., de Souza, H. S., & Machado, J. C. (2013). Endoluminal ultrasound biomicroscopy as a reliable tool for in vivo assessment of colonic inflammation in rats. International journal of colorectal disease, 28(12), 1613–1620.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., Collini, S., Pieraccini, G., & Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proceedings of the National Academy of Sciences of the United States of America, 107(33), 14691–14696. doi: 10.1073/pnas.1005963107

de La Serre, C. B., Ellis, C. L., Lee, J., Hartman, A. L., Rutledge, J. C., & Raybould, H. E. (2010). Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. American journal of physiology. Gastrointestinal and liver physiology, 299(2), G440–G448. doi: 10.1152/ajpgi.00098.2010

de Lau, W., Kujala, P., Schneeberger, K., Middendorp, S., Li, V. S., Barker, N., Martens, A., Hofhuis, F., DeKoter, R. P., Peters, P. J., Nieuwenhuis, E., & Clevers, H. (2012). Peyer's patch M cells derived from Lgr5(+) stem cells

require SpiB and are induced by RankL in cultured "miniguts". Molecular and cellular biology, 32(18), 3639–3647. doi: 10.1128/MCB.00434-12

DeMeo, M. T., Mutlu, E. A., Keshavarzian, A., & Tobin, M. C. (2002). Intestinal permeation and gastrointestinal disease. Journal of clinical gastroenterology, 34(4), 385–396. doi: 10.1097/00004836-200204000-00003

Divisi, D., Di Tommaso, S., Salvemini, S., Garramone, M., & Crisci, R. (2006). Diet and cancer. Acta bio-medica : Atenei Parmensis, 77(2), 118–123.

Do, M. H., Lee, E., Oh, M. J., Kim, Y., & Park, H. Y. (2018). High-Glucose or -Fructose Diet Cause Changes of the Gut Microbiota and Metabolic Disorders in Mice without Body Weight Change. Nutrients, 10(6), 761. doi: 10.3390/nu10060761

Doerner, S. K., Reis, E. S., Leung, E. S., Ko, J. S., Heaney, J. D., Berger, N. A., Lambris, J. D., & Nadeau, J. H. (2016). High-Fat Diet-Induced Complement Activation Mediates Intestinal Inflammation and Neoplasia, Independent of Obesity. Molecular cancer research : MCR, 14(10), 953–965.

Donskey, C. J., Hujer, A. M., Das, S. M., Pultz, N. J., Bonomo, R. A., & Rice, L. B. (2003). Use of denaturing gradient gel electrophoresis for analysis of the stool microbiota of hospitalized patients. Journal of microbiological methods, 54(2), 249–256. doi: 10.1016/s0167-7012(03)00059-9

Eijkelkamp, N., Steen-Louws, C., Hartgring, S. A., Willemen, H. L., Prado, J., Lafeber, F. P., Heijnen, C. J., Hack, C. E., van Roon, J. A., & Kavelaars, A. (2016). IL4-10 Fusion Protein Is a Novel Drug to Treat Persistent Inflammatory Pain. The Journal of neuroscience : the official journal of the Society for Neuroscience, 36(28), 7353–7363. doi: 10.1523/JNEUROSCI.0092-16.2016

Fellows, R., & Varga-Weisz, P. (2020). Chromatin dynamics and histone modifications in intestinal microbiota-host crosstalk. Molecular metabolism, 38, 100925. doi: 10.1016/j.molmet.2019.12.005

Feuerstein, J. D., & Cheifetz, A. S. (2017). Crohn Disease: Epidemiology, Diagnosis, and Management. Mayo Clinic proceedings, 92(7), 1088–1103. doi: 10.1016/j.mayocp.2017.04.010

Forsyth, C. B., Banan, A., Farhadi, A., Fields, J. Z., Tang, Y., Shaikh, M., Zhang, L. J., Engen, P. A., & Keshavarzian, A. (2007). Regulation of oxidant-induced intestinal permeability by metalloprotease-dependent epidermal growth factor receptor signaling. The Journal of pharmacology and experimental therapeutics, 321(1), 84–97.

Foster D. W. (1967). Studies in the ketosis of fasting. The Journal of clinical investigation, 46(8), 1283–1296. doi: 10.1172/JCI105621

Frank, D. N., St Amand, A. L., Feldman, R. A., Boedeker, E. C., Harpaz, N., & Pace, N. R. (2007). Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proceedings of the National Academy of Sciences of the United States of America, 104(34), 13780–13785. doi:10.1073/pnas.0706625104

Fraumene, C., Manghina, V., Cadoni, E., Marongiu, F., Abbondio, M., Serra, M., Palomba, A., Tanca, A., Laconi, E., & Uzzau, S. (2018). Caloric restriction promotes rapid expansion and long-lasting increase of Lactobacillus in the rat fecal microbiota. Gut microbes, 9(2), 104–114. doi: 10.1080/19490976.2017.1371894

Fu, T., Coulter, S., Yoshihara, E., Oh, T. G., Fang, S., Cayabyab, F., Zhu, Q., Zhang, T., Leblanc, M., Liu, S., He, M., Waizenegger, W., Gasser, E., Schnabl, B., Atkins, A. R., Yu, R. T., Knight, R., Liddle, C., Downes, M., & Evans, R. M. (2019). FXR Regulates Intestinal Cancer Stem Cell Proliferation. Cell, 176(5), 1098–1112.e18.

Ge, X., Ding, C., Zhao, W., Xu, L., Tian, H., Gong, J., Zhu, M., Li, J., & Li, N. (2017). Antibiotics-induced depletion of mice microbiota induces changes in host serotonin biosynthesis and intestinal motility. Journal of translational medicine, 15(1), 13. doi: 10.1186/s12967-016-1105-4

Gebert, A., Rothkötter, H. J., & Pabst, R. (1996). M cells in Peyer's patches of the intestine. International review of cytology, 167, 91–159. doi: 10.1016/s0074-7696(08)61346-7

Geisler, C. E., Hepler, C., Higgins, M. R., & Renquist, B. J. (2016). Hepatic adaptations to maintain metabolic homeostasis in response to fasting and refeeding in mice. Nutrition & metabolism, 13, 62. doi: 10.1186/s12986-016-0122-x

Gerbe, F., van Es, J. H., Makrini, L., Brulin, B., Mellitzer, G., Robine, S., Romagnolo, B., Shroyer, N. F., Bourgaux, J. F., Pignodel, C., Clevers, H., & Jay, P. (2011). Distinct ATOH1 and Neurog3 requirements define tuft cells as a new secretory cell type in the intestinal epithelium. The Journal of cell biology, 192(5), 767–780. doi: 10.1083/jcb.201010127

Gevers, D., Cohan, F. M., Lawrence, J. G., Spratt, B. G., Coenye, T., Feil, E. J., Stackebrandt, E., Van de Peer, Y., Vandamme, P., Thompson, F. L., & Swings, J. (2005). Opinion: Re-evaluating prokaryotic species. Nature reviews. Microbiology, 3(9), 733–739. doi: 10.1038/nrmicro1236

Gill, S. R., Pop, M., Deboy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M., & Nelson, K. E. (2006). Metagenomic analysis of the human distal gut microbiome. Science (New York, N.Y.), 312(5778), 1355–1359. doi: 10.1126/science.1124234

Gu, S., Chen, D., Zhang, J. N., Lv, X., Wang, K., Duan, L. P., Nie, Y., & Wu, X. L. (2013). Bacterial community mapping of the mouse gastrointestinal tract. PloS one, 8(10), e74957. doi: 10.1371/journal.pone.0074957

Gul Amuk, N., Kurt, G., Karsli, E., Ozcan, S., Acar, M. B., Amuk, M., Lekesizcan, A., & Gurgan, C. A. (2020). Effects of mesenchymal stem cell transfer on orthodontically induced root resorption and orthodontic tooth movement during orthodontic arch expansion protocols: an experimental study in rats. European journal of orthodontics, 42(3), 305–316. doi: 10.1093/ejo/cjz035

Gulhane, M., Murray, L., Lourie, R., Tong, H., Sheng, Y. H., Wang, R., Kang, A., Schreiber, V., Wong, K. Y., Magor, G., Denman, S., Begun, J., Florin, T. H., Perkins, A., Cuív, P. Ó., McGuckin, M. A., & Hasnain, S. Z. (2016). High Fat Diets Induce Colonic Epithelial Cell Stress and Inflammation that is Reversed by IL-22. Scientific reports, 6, 28990. doi: 10.1038/srep28990

Guo, X., Li, J., Tang, R., Zhang, G., Zeng, H., Wood, R. J., & Liu, Z. (2017). High Fat Diet Alters Gut Microbiota and the Expression of Paneth Cell-Antimicrobial Peptides Preceding Changes of Circulating Inflammatory Cytokines. Mediators of inflammation, 2017, 9474896. doi: 10.1155/2017/9474896

Guzman, J. R., Conlin, V. S., & Jobin, C. (2013). Diet, microbiome, and the intestinal epithelium: an essential triumvirate?. BioMed research international, 2013, 425146. doi: 10.1155/2013/425146

Haller, S., & Jasper, H. (2016). You Are What You Eat: Linking High-Fat Diet to Stem Cell Dysfunction and Tumorigenesis. Cell stem cell, 18(5), 564–566. doi: 10.1016/j.stem.2016.04.010

Hansson G. C. (2012). Role of mucus layers in gut infection and inflammation. Current opinion in microbiology, 15(1), 57–62. doi: 10.1016/j.mib.2011.11.002

Hariri, N., & Thibault, L. (2010). High-fat diet-induced obesity in animal models. Nutrition research reviews, 23(2), 270–299. doi: 10.1017/S0954422410000168

Harmon, G. S., Dumlao, D. S., Ng, D. T., Barrett, K. E., Dennis, E. A., Dong, H., & Glass, C. K. (2010). Pharmacological correction of a defect in PPAR-gamma signaling ameliorates disease severity in Cftr-deficient mice. Nature medicine, 16(3), 313–318. doi: 10.1038/nm.2101

Hegardt F. G. (1999). Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase: a control enzyme in ketogenesis. The Biochemical journal, 338 (Pt 3)(Pt 3), 569–582.

Helenius, T. O., Misiorek, J. O., Nyström, J. H., Fortelius, L. E., Habtezion, A., Liao, J., Asghar, M. N., Zhang, H., Azhar, S., Omary, M. B., & Toivola, D. M. (2015). Keratin 8 absence down-regulates colonocyte HMGCS2 and modulates colonic ketogenesis and energy metabolism. Molecular biology of the cell, 26(12), 2298–2310. doi: 10.1091/mbc.E14-02-0736

Hjorth, M. F., Roager, H. M., Larsen, T. M., Poulsen, S. K., Licht, T. R., Bahl, M. I., Zohar, Y., & Astrup, A. (2018). Pre-treatment microbial Prevotella-to-Bacteroides ratio, determines body fat loss success during a 6-month randomized controlled diet intervention. International journal of obesity (2005), 42(3), 580–583. doi: 10.1038/ijo.2017.220

Hollander, D., Vadheim, C. M., Brettholz, E., Petersen, G. M., Delahunty, T., & Rotter, J. I. (1986). Increased intestinal permeability in patients with Crohn's disease and their relatives. A possible etiologic factor. Annals of internal medicine, 105(6), 883–885. doi: 10.7326/0003-4819-105-6-883

Hou, J. K., Abraham, B., & El-Serag, H. (2011). Dietary intake and risk of developing inflammatory bowel disease: a systematic review of the literature. The American journal of gastroenterology, 106(4), 563–573. doi: 10.1038/ajg.2011.44

Human Microbiome Project Consortium (2012). Structure, function and diversity of the healthy human microbiome. Nature, 486(7402), 207–214. doi: 10.1038/nature11234

Hyun, J., Romero, L., Riveron, R., Flores, C., Kanagavelu, S., Chung, K. D., Alonso, A., Sotolongo, J., Ruiz, J., Manukyan, A., Chun, S., Singh, G., Salas, P., Targan, S. R., & Fukata, M. (2015). Human intestinal epithelial cells express interleukin-10 through Toll-like receptor 4-mediated epithelial-macrophage crosstalk. Journal of innate immunity, 7(1), 87–101. doi: 10.1159/000365417

Imaeda, H., Takahashi, K., Fujimoto, T., Kasumi, E., Ban, H., Bamba, S., Sonoda, H., Shimizu, T., Fujiyama, Y., & Andoh, A. (2013). Epithelial expression of interleukin-37b in inflammatory bowel disease. Clinical and experimental immunology, 172(3), 410–416.

James, K. R., Gomes, T., Elmentaite, R., Kumar, N., Gulliver, E. L., King, H. W., Stares, M. D., Bareham, B. R., Ferdinand, J. R., Petrova, V. N., Polański, K., Forster, S. C., Jarvis, L. B., Suchanek, O., Howlett, S., James, L. K., Jones, J. L., Meyer, K. B., Clatworthy, M. R., Saeb-Parsy, K., … Teichmann, S. A. (2020). Distinct microbial and immune niches of the human colon. Nature immunology, 21(3), 343–353.

Jeffery, V., Goldson, A. J., Dainty, J. R., Chieppa, M., & Sobolewski, A. (2017). IL-6 Signaling Regulates Small Intestinal Crypt Homeostasis. Journal of immunology (Baltimore, Md. : 1950), 199(1), 304–311. doi: 10.4049/jimmunol.1600960

Kaiko, G. E., Ryu, S. H., Koues, O. I., Collins, P. L., Solnica-Krezel, L., Pearce, E. J., Pearce, E. L., Oltz, E. M., & Stappenbeck, T. S. (2016). The Colonic Crypt Protects Stem Cells from Microbiota-Derived Metabolites. Cell, 165(7), 1708–1720.

Kappagoda, C. T., Hyson, D. A., & Amsterdam, E. A. (2004). Low-carbohydrate-high-protein diets: is there a place for them in clinical cardiology?. Journal of the American College of Cardiology, 43(5), 725–730. doi. 10.1016/j.jacc.2003.06.022

Katz, K. D., Hollander, D., Vadheim, C. M., McElree, C., Delahunty, T., Dadufalza, V. D., Krugliak, P., & Rotter, J. I. (1989). Intestinal permeability in patients with Crohn's disease and their healthy relatives. Gastroenterology, 97(4), 927–931. doi: 10.1016/0016-5085(89)91499-6

Kennedy, A. R., Pissios, P., Otu, H., Roberson, R., Xue, B., Asakura, K., Furukawa, N., Marino, F. E., Liu, F. F., Kahn, B. B., Libermann, T. A., & Maratos-Flier, E. (2007). A high-fat, ketogenic diet induces a unique metabolic state in mice. American journal of physiology. Endocrinology and metabolism, 292(6)

Khan, S., Waliullah, S., Godfrey, V., Khan, M., Ramachandran, R. A., Cantarel, B. L., Behrendt, C., Peng, L., Hooper, L. V., & Zaki, H. (2020). Dietary simple sugars alter microbial ecology in the gut and promote colitis in mice. Science translational medicine, 12(567), eaay6218. doi: 10.1126/scitranslmed.aay6218

Khosravi, A., & Mazmanian, S. K. (2013). Disruption of the gut microbiome as a risk factor for microbial infections. Current opinion in microbiology, 16(2), 221–227. doi: 10.1016/j.mib.2013.03.009

Kim, B. S., Kim, J. N., & Cerniglia, C. E. (2011). In vitro culture conditions for maintaining a complex population of human gastrointestinal tract microbiota. Journal of biomedicine & biotechnology, 2011, 838040. doi: 10.1155/2011/838040

Kim, J. H., Lee, M., Kim, S. H., Kim, S. R., Lee, B. W., Kang, E. S., Cha, B. S., Cho, J. W., & Lee, Y. H. (2019). Sodium-glucose cotransporter 2 inhibitors regulate ketone body metabolism via inter-organ crosstalk. Diabetes, obesity & metabolism, 21(4), 801–811. doi: 10.1111/dom.13577

Kim, J. T., Li, C., Weiss, H. L., Zhou, Y., Liu, C., Wang, Q., & Evers, B. M. (2019). Regulation of Ketogenic Enzyme HMGCS2 by Wnt/β-catenin/PPARγ Pathway in Intestinal Cells. Cells, 8(9), 1106. doi: 10.3390/cells8091106

Knight-Sepulveda, K., Kais, S., Santaolalla, R., & Abreu, M. T. (2015). Diet and Inflammatory Bowel Disease. Gastroenterology & hepatology, 11(8), 511–520.

Kodukula, K., Faller, D. V., Harpp, D. N., Kanara, I., Pernokas, J., Pernokas, M., Powers, W. R., Soukos, N. S., Steliou, K., & Moos, W. H. (2017). Gut Microbiota and Salivary Diagnostics: The Mouth Is Salivating to Tell Us Something. BioResearch open access, 6(1), 123–132. doi: 10.1089/biores.2017.0020

Koo, B. K., & Clevers, H. (2014). Stem cells marked by the R-spondin receptor LGR5. Gastroenterology, 147(2), 289–302. doi: 10.1053/j.gastro.2014.05.007

Kuang, Z., Wang, Y., Li, Y., Ye, C., Ruhn, K. A., Behrendt, C. L., Olson, E. N., & Hooper, L. V. (2019). The intestinal microbiota programs diurnal rhythms in host metabolism through histone deacetylase 3. Science (New York, N.Y.), 365(6460), 1428–1434. doi: 10.1126/science.aaw3134

Kuzmuk, K. N., Swanson, K. S., Tappenden, K. A., Schook, L. B., & Fahey, G. C., Jr (2005). Diet and age affect intestinal morphology and large bowel fermentative end-product concentrations in senior and young adult dogs. The Journal of nutrition, 135(8), 1940–1945. doi: 10.1093/jn/135.8.1940

Laffin, M., Fedorak, R., Zalasky, A. et al. (2019). A high-sugar diet rapidly enhances susceptibility to colitis via depletion of luminal short-chain fatty acids in mice. Sci Rep **9,** 12294 doi: 10.1038/s41598-019-48749-2

Lagkouvardos, I., Pukall, R., Abt, B., Foesel, B. U., Meier-Kolthoff, J. P., Kumar, N., Bresciani, A., Martínez, I., Just, S., Ziegler, C., Brugiroux, S., Garzetti, D., Wenning, M., Bui, T. P., Wang, J., Hugenholtz, F., Plugge, C. M., Peterson, D. A., Hornef, M. W., Baines, J. F., … Clavel, T. (2016). The Mouse Intestinal Bacterial Collection (miBC) provides host-specific insight into cultured diversity and functional potential of the gut microbiota. Nature microbiology, 1(10), 16131. doi: 10.1038/nmicrobiol.2016.131

Lazar, V., Ditu, L. M., Pircalabioru, G. G., Gheorghe, I., Curutiu, C., Holban, A. M., Picu, A., Petcu, L., & Chifiriuc, M. C. (2018). Aspects of Gut Microbiota and Immune System Interactions in Infectious Diseases, Immunopathology, and Cancer. Frontiers in immunology, 9, 1830. doi: 10.3389/fimmu.2018.01830

Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G., Almeida, M., Arumugam, M., Batto, J. M., Kennedy, S., Leonard, P., Li, J., Burgdorf, K., Grarup, N., Jørgensen, T., Brandslund, I., Nielsen, H. B., Juncker, A. S., Bertalan, M., Levenez, F., … Pedersen, O. (2013). Richness of human gut microbiome correlates with metabolic markers. Nature, 500(7464), 541–546. doi: 10.1038/nature12506

Lee S. H. (2015). Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases. Intestinal research, 13(1), 11–18. doi: 10.5217/ir.2015.13.1.11

Lee, S. H., Kwon, J. E., & Cho, M. L. (2018). Immunological pathogenesis of inflammatory bowel disease. Intestinal research, 16(1), 26–42. doi:10.5217/ir.2018.16.1.26

Lepage, P., Häsler, R., Spehlmann, M. E., Rehman, A., Zvirbliene, A., Begun, A., Ott, S., Kupcinskas, L., Doré, J., Raedler, A., & Schreiber, S. (2011). Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. Gastroenterology, 141(1), 227–236. doi: 10.1053/j.gastro.2011.04.011

Lerman, L. S., Fisher, S. G., Hurley, I., Silverstein, K., and Lumelsky, N. (1984). Sequence-determined DNA separations. Ann. Rev. Biophys. Booeng. 13: 399-423.

Lewis J. D. (2016). The Role of Diet in Inflammatory Bowel Disease. Gastroenterology & hepatology, 12(1), 51–53.

Ley, R. E., Bäckhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., & Gordon, J. I. (2005). Obesity alters gut microbial ecology. Proceedings of the National Academy of Sciences of the United States of America, 102(31), 11070–11075. doi: 10.1073/pnas.0504978102

Lindefeldt, M., Eng, A., Darban, H., Bjerkner, A., Zetterström, C. K., Allander, T., Andersson, B., Borenstein, E., Dahlin, M., & Prast-Nielsen, S. (2019). The ketogenic diet influences taxonomic and functional composition of the gut microbiota in children with severe epilepsy. NPJ biofilms and microbiomes, 5(1), 5. doi: 10.1038/s41522-018-0073-2

Liu, H., Liu, M., Wang, B. et al. (2010). PCR-DGGE analysis of intestinal bacteria and effect of Bacillus spp. on intestinal microbial diversity in kuruma shrimp (Marsupenaeus japonicus). Chin. J. Ocean. Limnol. 28, 808–814 doi: 10.1007/s00343-010-9101-7

Longo, V. D., & Mattson, M. P. (2014). Fasting: molecular mechanisms and clinical applications. Cell metabolism, 19(2), 181–192. doi: 10.1016/j.cmet.2013.12.008

Longo, V.D., Di Tano, M., Mattson, M.P. et al. (2021). Intermittent and periodic fasting, longevity and disease. Nat Aging **1,** 47–59.

Louis, P., & Flint, H. J. (2007). Development of a semiquantitative degenerate real-time pcr-based assay for estimation of numbers of butyryl-coenzyme A

(CoA) CoA transferase genes in complex bacterial samples. Applied and environmental microbiology, 73(6), 2009–2012.

Ludwig D. S. (2020). The Ketogenic Diet: Evidence for Optimism but High-Quality Research Needed. The Journal of nutrition, 150(6), 1354–1359.

Machiels, K., Joossens, M., Sabino, J., De Preter, V., Arijs, I., Eeckhaut, V., Ballet, V., Claes, K., Van Immerseel, F., Verbeke, K., Ferrante, M., Verhaegen, J., Rutgeerts, P., & Vermeire, S. (2014). A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. Gut, 63(8), 1275–1283. doi: 10.1136/gutjnl-2013-304833

Magne, F., Gotteland, M., Gauthier, L., Zazueta, A., Pesoa, S., Navarrete, P., & Balamurugan, R. (2020). The Firmicutes/Bacteroidetes Ratio: A Relevant Marker of Gut Dysbiosis in Obese Patients?. Nutrients, 12(5), 1474. doi: 10.3390/nu12051474

Magro, F., Langner, C., Driessen, A., Ensari, A., Geboes, K., Mantzaris, G. J., Villanacci, V., Becheanu, G., Borralho Nunes, P., Cathomas, G., Fries, W., Jouret-Mourin, A., Mescoli, C., de Petris, G., Rubio, C. A., Shepherd, N. A., Vieth, M., Eliakim, R., European Society of Pathology (ESP), & European Crohn's and Colitis Organisation (ECCO) (2013). European consensus on the histopathology of inflammatory bowel disease. Journal of Crohn's & colitis, 7(10), 827–851. doi: 10.1016/j.crohns.2013.06.001

Mao, J., Hu, X., Xiao, Y., Yang, C., Ding, Y., Hou, N., Wang, J., Cheng, H., & Zhang, X. (2013). Overnutrition stimulates intestinal epithelium proliferation through β-catenin signaling in obese mice. Diabetes, 62(11), 3736–3746. doi: 10.2337/db13-0035

Matikainen-Ankney, B. A., Ali, M. A., Miyazaki, N. L., Fry, S. A., Licholai, J. A., & Kravitz, A. V. (2020). Weight Loss After Obesity is Associated with Increased Food Motivation and Faster Weight Regain in Mice. Obesity (Silver Spring, Md.), 28(5), 851–856. doi: 10.1002/oby.22758

Menni, C., Jackson, M. A., Pallister, T., Steves, C. J., Spector, T. D., & Valdes, A. M. (2017). Gut microbiome diversity and high-fibre intake are related to lower long-term weight gain. International journal of obesity (2005), 41(7), 1099–1105. doi: 10.1038/ijo.2017.66

Mihaylova, M. M., Cheng, C. W., Cao, A. Q., Tripathi, S., Mana, M. D., Bauer-Rowe, K. E., Abu-Remaileh, M., Clavain, L., Erdemir, A., Lewis, C. A., Freinkman, E., Dickey, A. S., La Spada, A. R., Huang, Y., Bell, G. W., Deshpande, V., Carmeliet, P., Katajisto, P., Sabatini, D. M., & Yilmaz, Ö. H. (2018). Fasting Activates Fatty Acid Oxidation to Enhance Intestinal Stem Cell Function during Homeostasis and Aging. Cell stem cell, 22(5), 769–778.e4. doi: 10.1016/j.stem.2018.04.001

Mihaylova, M. M., Sabatini, D. M., & Yilmaz, Ö. H. (2014). Dietary and metabolic control of stem cell function in physiology and cancer. Cell stem cell, 14(3), 292–305. doi: 10.1016/j.stem.2014.02.008

Mowat, A. M., & Agace, W. W. (2014). Regional specialization within the intestinal immune system. Nature reviews. Immunology, 14(10), 667–685. doi: 10.1038/nri3738

Mozaffarian, D., Hao, T., Rimm, E. B., Willett, W. C., & Hu, F. B. (2011). Changes in diet and lifestyle and long-term weight gain in women and men. The New England journal of medicine, 364(25), 2392–2404. doi: 10.1056/NEJMoa1014296

Murakami, M., Tognini, P., Liu, Y., Eckel-Mahan, K. L., Baldi, P., & Sassone-Corsi, P. (2016). Gut microbiota directs PPARγ-driven reprogramming of the liver circadian clock by nutritional challenge. EMBO reports, 17(9), 1292–1303. doi: 10.15252/embr.201642463

Muyzer, G., de Waal, E. C., & Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and environmental microbiology, 59(3), 695–700. doi: 10.1128/aem.59.3.695-700.1993

Müller, M., Hermes, G., Canfora, E. E., Smidt, H., Masclee, A., Zoetendal, E. G., & Blaak, E. E. (2020). Distal colonic transit is linked to gut microbiota diversity and microbial fermentation in humans with slow colonic transit. American journal of physiology. Gastrointestinal and liver physiology, 318(2), G361–G369.

Neurath M. F. (2014). Cytokines in inflammatory bowel disease. Nature reviews. Immunology, 14(5), 329–342. doi: 10.1038/nri3661

Nusrat, A., Turner, J. R., & Madara, J. L. (2000). Molecular physiology and pathophysiology of tight junctions. IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. American journal of physiology. Gastrointestinal and liver physiology, 279(5), G851–G857. doi: 10.1152/ajpgi.2000.279.5.G851

Nystrom, M., & Mutanen, M. (2009). Diet and epigenetics in colon cancer. World journal of gastroenterology, 15(3), 257–263. doi: 10.3748/wjg.15.257

Obanda, D., Page, R., Guice, J., Raggio, A. M., Husseneder, C., Marx, B., Stout, R. W., Welsh, D. A., Taylor, C. M., Luo, M., Blanchard, E. E., Bendiks, Z., Coulon, D., & Keenan, M. J. (2018). CD Obesity-Prone Rats, but not Obesity-Resistant Rats, Robustly Ferment Resistant Starch Without Increased Weight or Fat Accretion. Obesity (Silver Spring, Md.), 26(3), 570–577. doi:10.1002/oby.22120

Ordas, I., Eckmann, L., Talamini, M., and Baumgart., D.C., (2012) Ulcerative colitis. The Lancet, 380 1606-1619.

Ormerod, K.L., Wood, D.L.A., Lachner, N. et al. (2016). Genomic characterization of the uncultured Bacteroidales family S24-7 inhabiting the guts of homeothermic animals. Microbiome **4,** 36 doi: 10.1186/s40168-016-0181-2

Ott, S. J., Musfeldt, M., Wenderoth, D. F., Hampe, J., Brant, O., Fölsch, U. R., Timmis, K. N., & Schreiber, S. (2004). Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. Gut, 53(5), 685–693.

Ozin, Y., Kilic, M. Z., Nadir, I., Cakal, B., Disibeyaz, S., Arhan, M., Dagli, U., Tunc, B., Ulker, A., & Sahin, B. (2009). Clinical features of ulcerative colitis and Crohn's disease in Turkey. Journal of gastrointestinal and liver diseases : JGLD, 18(2), 157–162.

Park, J. H., Kotani, T., Konno, T., Setiawan, J., Kitamura, Y., Imada, S., Usui, Y., Hatano, N., Shinohara, M., Saito, Y., Murata, Y., & Matozaki, T. (2016). Promotion of Intestinal Epithelial Cell Turnover by Commensal Bacteria: Role of Short-Chain Fatty Acids. PloS one, 11(5), e0156334. doi: 10.1371/journal.pone.0156334

Pasolli, E., Asnicar, F., Manara, S., Zolfo, M., Karcher, N., Armanini, F., Beghini, F., Manghi, P., Tett, A., Ghensi, P., Collado, M. C., Rice, B. L., DuLong, C., Morgan, X. C., Golden, C. D., Quince, C., Huttenhower, C., & Segata, N. (2019). Extensive Unexplored Human Microbiome Diversity Revealed by Over 150,000 Genomes from Metagenomes Spanning Age, Geography, and Lifestyle. Cell, 176(3), 649–662.e20.

Perrotta, A. R., Borrelli, G. M., Martins, C. O., Kallas, E. G., Sanabani, S. S., Griffith, L. G., Alm, E. J., & Abrao, M. S. (2020). The Vaginal Microbiome as a Tool to Predict rASRM Stage of Disease in Endometriosis: a Pilot Study. Reproductive sciences (Thousand Oaks, Calif.), 27(4), 1064–1073. doi.10.1007/s43032-019-00113-5

Peterson, L. W., & Artis, D. (2014). Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nature reviews. Immunology, 14(3), 141–153. doi: 10.1038/nri3608

Pinto, A., Bonucci, A., Maggi, E., Corsi, M., & Businaro, R. (2018). Anti-Oxidant and Anti-Inflammatory Activity of Ketogenic Diet: New Perspectives for Neuroprotection in Alzheimer's Disease. Antioxidants (Basel, Switzerland), 7(5), 63.

Potentas, E., Witkowska, A. M., & Zujko, M. E. (2015). Mediterranean diet for breast cancer prevention and treatment in postmenopausal women. Przeglad menopauzalny = Menopause review, 14(4), 247–253. doi: 10.5114/pm.2015.56381

Qi, L., Hu, F. B., & Hu, G. (2008). Genes, environment, and interactions in prevention of type 2 diabetes: a focus on physical activity and lifestyle changes. Current molecular medicine, 8(6), 519–532. doi: 10.2174/156652408785747915

Reikvam, D. H., Erofeev, A., Sandvik, A., Grcic, V., Jahnsen, F. L., Gaustad, P., McCoy, K. D., Macpherson, A. J., Meza-Zepeda, L. A., & Johansen, F. E. (2011). Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression. PloS one, 6(3), e17996.

Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G., Gasbarrini, A., & Mele, M. C. (2019). What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. Microorganisms, 7(1), 14. doi: 10.3390/microorganisms7010014

Rizzello, F., Spisni, E., Giovanardi, E., Imbesi, V., Salice, M., Alvisi, P., Valerii, M. C., & Gionchetti, P. (2019). Implications of the Westernized Diet in the Onset and Progression of IBD. Nutrients, 11(5), 1033. doi: 10.3390/nu11051033

Roushan, N., Ebrahimi Daryani, N., Azizi, Z., Pournaghshband, H., & Niksirat, A. (2019). Differentiation of Crohn's disease and ulcerative colitis using intestinal wall thickness of the colon: A Diagnostic accuracy study of endoscopic ultrasonography. Medical journal of the Islamic Republic of Iran, 33, 57. doi: 10.34171/mjiri.33.57

Sairenji, T., Collins, K. L., & Evans, D. V. (2017). An Update on Inflammatory Bowel Disease. Primary care, 44(4), 673–692. doi: 10.1016/j.pop.2017.07.010

Samarajeewa, A. D., Hammad, A., Masson, L., Khan, I. U., Scroggins, R., & Beaudette, L. A. (2015). Comparative assessment of next-generation sequencing, denaturing gradient gel electrophoresis, clonal restriction fragment length polymorphism and cloning-sequencing as methods for characterizing commercial microbial consortia. Journal of microbiological methods, 108, 103–111. doi: 10.1016/j.mimet.2014.11.013

Sartor R. B. (2008). Microbial influences in inflammatory bowel diseases. Gastroenterology, 134(2), 577–594. doi: 10.1053/j.gastro.2007.11.059

Sato, T., van Es, J. H., Snippert, H. J., Stange, D. E., Vries, R. G., van den Born, M., Barker, N., Shroyer, N. F., van de Wetering, M., & Clevers, H. (2011). Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature, 469(7330), 415–418. doi: 10.1038/nature09637

Schreiber, S., Heinig, T., Thiele, H. G., & Raedler, A. (1995). Immunoregulatory role of interleukin 10 in patients with inflammatory bowel disease. Gastroenterology, 108(5), 1434–1444. doi: 10.1016/0016-5085(95)90692-4

Schwab, M., Reynders, V., Loitsch, S., Steinhilber, D., Stein, J., & Schröder, O. (2007). Involvement of different nuclear hormone receptors in butyrate-mediated inhibition of inducible NF kappa B signalling. Molecular immunology, 44(15), 3625–3632.

Sender, R., Fuchs, S., & Milo, R. (2016). Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLoS biology, 14(8), e1002533. doi: 10.1371/journal.pbio.1002533

Serra, D., Bellido, D., Asins, G., Arias, G., Vilaró, S., & Hegardt, F. G. (1996). The expression of mitochondrial 3-hydroxy-3-methylglutaryl-coenzyme-A synthase in neonatal rat intestine and liver is under transcriptional control. European journal of biochemistry, 237(1), 16–24.

Sheh, A., & Fox, J. G. (2013). The role of the gastrointestinal microbiome in Helicobacter pylori pathogenesis. Gut microbes, 4(6), 505–531. doi: 10.4161/gmic.26205

Shen, L., & Turner, J. R. (2006). Role of epithelial cells in initiation and propagation of intestinal inflammation. Eliminating the static: tight junction dynamics exposed. American journal of physiology. Gastrointestinal and liver physiology, 290(4), G577–G582.

Shi, H., Zhang, B., Abo-Hamzy, T., Nelson, J. W., Ambati, C., Petrosino, J. F., Bryan, R. M., Jr, & Durgan, D. J. (2021). Restructuring the Gut Microbiota by Intermittent Fasting Lowers Blood Pressure. Circulation research, 128(9), 1240–1254.

Shoelson, S. E., Herrero, L., & Naaz, A. (2007). Obesity, inflammation, and insulin resistance. Gastroenterology, 132(6), 2169–2180. doi: 10.1053/j.gastro.2007.03.059

Shouval, D. S., & Rufo, P. A. (2017). The Role of Environmental Factors in the Pathogenesis of Inflammatory Bowel Diseases: A Review. JAMA pediatrics, 171(10), 999–1005. doi: 10.1001/jamapediatrics.2017.2571

Soderholm, A. T., & Pedicord, V. A. (2019). Intestinal epithelial cells: at the interface of the microbiota and mucosal immunity. Immunology, 158(4), 267–280. doi: 10.1111/imm.13117

Spyridopoulou, K., Tiptiri-Kourpeti, A., Lampri, E., Fitsiou, E., Vasileiadis, S., Vamvakias, M., Bardouki, H., Goussia, A., Malamou-Mitsi, V., Panayiotidis, M. I., Galanis, A., Pappa, A., & Chlichlia, K. (2017). Dietary mastic oil extracted from Pistacia lentiscus var. chia suppresses tumor growth in experimental colon cancer models. Scientific reports, 7(1), 3782. doi: 10.1038/s41598-017-03971-8

Stojanov, S., Berlec, A., & Štrukelj, B. (2020). The Influence of Probiotics on the Firmicutes/Bacteroidetes Ratio in the Treatment of Obesity and Inflammatory Bowel disease. Microorganisms, 8(11), 1715. doi: 10.3390/microorganisms8111715

Su, J., Wang, Y., Zhang, X., Ma, M., Xie, Z., Pan, Q., Ma, Z., & Peppelenbosch, M. P. (2021). Remodeling of the gut microbiome during Ramadan-associated intermittent fasting. The American journal of clinical nutrition, 113(5), 1332–1342. doi: 10.1093/ajcn/nqaa388

Su, W., Bush, C. R., Necela, B. M., Calcagno, S. R., Murray, N. R., Fields, A. P., & Thompson, E. A. (2007). Differential expression, distribution, and function

of PPAR-gamma in the proximal and distal colon. Physiological genomics, 30(3), 342–353. doi: 10.1152/physiolgenomics.00042.2007

Suzuki, T., & Hara, H. (2010). Dietary fat and bile juice, but not obesity, are responsible for the increase in small intestinal permeability induced through the suppression of tight junction protein expression in LETO and OLETF rats. Nutrition & metabolism, 7, 19. doi: 10.1186/1743-7075-7-19

Taniguchi, M., Kuda, T., Takei, M. et al. (2020). Effects of fermented Aphanizomenon flos-aquae on the caecal microbiome of mice fed a high-sucrose and low-dietary fibre diet. J Appl Phycol doi: 10.1007/s10811-020-02306-x

Taylor, M. K., Swerdlow, R. H., Burns, J. M., & Sullivan, D. K. (2019). An Experimental Ketogenic Diet for Alzheimer Disease Was Nutritionally Dense and Rich in Vegetables and Avocado. Current developments in nutrition, 3(4), nzz003.

TBSA (2019). Turkey Nutrition and Health Survey https://hsgm.saglik.gov.tr/depo/birimler/saglikli-beslenme-hareketli-hayat-db/Yayinlar/kitaplar/TBSA_RAPOR_KITAP_20.08.pdf

Ting, H. A., & von Moltke, J. (2019). The Immune Function of Tuft Cells at Gut Mucosal Surfaces and Beyond. Journal of immunology (Baltimore, Md. : 1950), 202(5), 1321–1329. doi: 10.4049/jimmunol.1801069

Tokuda, K., Kida, K., Marutani, E., Crimi, E., Bougaki, M., Khatri, A., Kimura, H., & Ichinose, F. (2012). Inhaled hydrogen sulfide prevents endotoxin-induced systemic inflammation and improves survival by altering sulfide metabolism in mice. Antioxidants & redox signaling, 17(1), 11–21. doi:10.1089/ars.2011.4363

Tomasello, G., Tralongo, P., Damiani, P., Sinagra, E., Di Trapani, B., Zeenny, M. N., Hussein, I. H., Jurjus, A., & Leone, A. (2014). Dismicrobism in inflammatory bowel disease and colorectal cancer: changes in response of colocytes. World journal of gastroenterology, 20(48), 18121–18130. doi: 10.3748/wjg.v20.i48.18121

Tourlomousis, P., Kemsley, E. K., Ridgway, K. P., Toscano, M. J., Humphrey, T. J., & Narbad, A. (2010). PCR-denaturing gradient gel electrophoresis of complex microbial communities: a two-step approach to address the effect of gel-to-gel variation and allow valid comparisons across a large dataset. Microbial ecology, 59(4), 776–786. doi: 10.1007/s00248-009-9613-x

Trebicka, J., Bork, P., Krag, A., & Arumugam, M. (2020). Utilizing the gut microbiome in decompensated cirrhosis and acute-on-chronic liver failure. Nature reviews. Gastroenterology & hepatology, 10.1038/s41575-020-00376-3. Advance online publication.

Tremaroli, V., & Bäckhed, F. (2012). Functional interactions between the gut microbiota and host metabolism. Nature, 489(7415), 242–249.

Turnbaugh, P. J., Bäckhed, F., Fulton, L., & Gordon, J. I. (2008). Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell host & microbe, 3(4), 213–223. doi: 10.1016/j.chom.2008.02.015

Turner J. R. (2006). Molecular basis of epithelial barrier regulation: from basic mechanisms to clinical application. The American journal of pathology, 169(6), 1901–1909. doi: 10.2353/ajpath.2006.060681

Turner J. R. (2009). Intestinal mucosal barrier function in health and disease. Nature reviews. Immunology, 9(11), 799–809. doi: 10.1038/nri2653

Van den Bossche, J., Lamers, W. H., Koehler, E. S., Geuns, J. M., Alhonen, L., Uimari, A., Pirnes-Karhu, S., Van Overmeire, E., Morias, Y., Brys, L., Vereecke, L., De Baetselier, P., & Van Ginderachter, J. A. (2012). Pivotal Advance: Arginase-1-independent polyamine production stimulates the expression of IL-4-induced alternatively activated macrophage markers while inhibiting LPS-induced expression of inflammatory genes. Journal of leukocyte biology, 91(5), 685–699. doi: 10.1189/jlb.0911453

Verhoeckx, K., Cotter, P., López-Expósito, I., Kleiveland, C., Lea, T., Mackie, A., Requena, T., Swiatecka, D., & Wichers, H. (Eds.). (2015). The Impact of Food Bioactives on Health: in vitro and ex vivo models. Springer.

Waddell, A., Vallance, J. E., Moore, P. D., Hummel, A. T., Wu, D., Shanmukhappa, S. K., Fei, L., Washington, M. K., Minar, P., Coburn, L. A., Nakae, S., Wilson, K. T., Denson, L. A., Hogan, S. P., & Rosen, M. J. (2015). IL-33 Signaling Protects from Murine Oxazolone Colitis by Supporting Intestinal Epithelial Function. Inflammatory bowel diseases, 21(12), 2737–2746. doi: 10.1097/MIB.0000000000000532

Wan, Y., Wang, F., Yuan, J., Li, J., Jiang, D., Zhang, J., Li, H., Wang, R., Tang, J., Huang, T., Zheng, J., Sinclair, A. J., Mann, J., & Li, D. (2019). Effects of dietary fat on gut microbiota and faecal metabolites, and their relationship with cardiometabolic risk factors: a 6-month randomised controlled-feeding trial. Gut, 68(8), 1417–1429.

Wang, Q., Zhou, Y., Rychahou, P., Fan, T. W., Lane, A. N., Weiss, H. L., & Evers, B. M. (2017). Ketogenesis contributes to intestinal cell differentiation. Cell death and differentiation, 24(3), 458–468. doi: 10.1038/cdd.2016.142

Wang, X., Heazlewood, S. P., Krause, D. O., & Florin, T. H. (2003). Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. Journal of applied microbiology, 95(3), 508–520.

Warden, C. H., & Fisler, J. S. (2008). Comparisons of diets used in animal models of high-fat feeding. Cell metabolism, 7(4), 277.

Xie, G., Zhou, Q., Qiu, C. Z., Dai, W. K., Wang, H. P., Li, Y. H., Liao, J. X., Lu, X. G., Lin, S. F., Ye, J. H., Ma, Z. Y., & Wang, W. J. (2017). Ketogenic diet poses a significant effect on imbalanced gut microbiota in infants with refractory epilepsy. World journal of gastroenterology, 23(33), 6164–6171. doi: 10.3748/wjg.v23.i33.616

Yang, I., Nell, S., & Suerbaum, S. (2013). Survival in hostile territory: the microbiota of the stomach. FEMS microbiology reviews, 37(5), 736–761. doi: 10.1111/1574-6976.12027

Yuan, S., Xie, Y. H., Zhang, H. X., Lian, Z. X., Han, H. B., & Liu, H. (2014). The Application of PCR-DGGE Technique in Sheep Intestinal Microbial Diversity Research. Advanced Materials Research, 884-885, 540–543. doi:10.4028/www.scientific.net/amr.884-885.540

Yuan, T., Wang, J., Chen, L., Shan, J., & Di, L. (2020). Lactobacillus murinus Improved the Bioavailability of Orally Administered Glycyrrhizic Acid in Rats. Frontiers in microbiology, 11, 597. doi: 10.3389/fmicb.2020.00597

Zarrinpar, A., Chaix, A., Xu, Z. Z., Chang, M. W., Marotz, C. A., Saghatelian, A., Knight, R., & Panda, S. (2018). Antibiotic-induced microbiome depletion alters metabolic homeostasis by affecting gut signaling and colonic metabolism. Nature communications, 9(1), 2872.

Zeituni, E. M., Wilson, M. H., Zheng, X., Iglesias, P. A., Sepanski, M. A., Siddiqi, M. A., Anderson, J. L., Zheng, Y., & Farber, S. A. (2016). Endoplasmic Reticulum Lipid Flux Influences Enterocyte Nuclear Morphology and Lipid-dependent Transcriptional Responses. The Journal of biological chemistry, 291(45), 23804–23816. doi: 10.1074/jbc.M116.749358

Zhang, J. M., & An, J. (2007). Cytokines, inflammation, and pain. International anesthesiology clinics, 45(2), 27–37. doi: 10.1097/AIA.0b013e318034194e

Zhao, M., Jiang, Z., Cai, H., Li, Y., Mo, Q., Deng, L., Zhong, H., Liu, T., Zhang, H., Kang, J. X., & Feng, F. (2020). Modulation of the Gut Microbiota during High-Dose Glycerol Monolaurate-Mediated Amelioration of Obesity in Mice Fed a High-Fat Diet. mBio, 11(2), e00190-20.

Zheng, D., Liwinski, T., & Elinav, E. (2020). Interaction between microbiota and immunity in health and disease. Cell research, 30(6), 492–506. doi: 10.1038/s41422-020-0332-7

Zoetendal, Erwin G et al. “The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates.” The ISME journal vol. 6,7 (2012): 1415-26. doi: 10.1038/ismej.2011.212

APPENDICES

A. Laboratory Equipment

Autoclave	Nüve, SteamArt
Balances	Precisa, XB220A
DGGE system	BioRad, DCode Universal Mutation System
Electrophoresis system	BioRad, mini sub cell GT
Spectrophotometers	Thermo Scientific, Nanodrop 2000
Gel documentation system	BioRad Gel Doc Xr
Incubator	Memmert, INE 400
Laminar flow	Delta, DLT
Magnetic stirrer, heater	Heidolph MR hei-standard
Microwave oven	Samsung, ME731K
Thermal Cycler	Thermo Fisher Scientific, SimpliAmp™
Thermal Cycler	BioRad, CFX96 Real-Time System
Power supply	BioRad, PowerPac Basic
Power supply	BioRad, PowerPac Universal
Pipettes	Thermo Scientific, Finnpipette; 2.5 µl, 10 µl, 200 µl, 1000 µl
Vortex	VELP Scientifica
Refrigerators	Vestel, -20^{o}C and Panasonic VIP Plus -80^{o}C
Water Bath	Microtest, MSB
Centrifuges	Thermo Scientific MicroCL-17R, CL10
pH meter	Metter Toledo MP220

B. DGGE Sequences

GCGTATAATTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTCTTAG
TCAGGTACCGTCATTTTCTTCCCTGCTGATAGAGCTTTACATACCGAAAT
ACTTCTTCACTCACGCGGCGTCGCTGGATCAGGGTTCCCCCCATTGTCC
AATATTCCCCACTGCTGCCTCCCGTAGGAAG

TTTTATTACCGCGGCTGCTGGCATTATAACCGCGGCAGCCGGCATGTAG
TTAGCCGGGGCTTCGTAGTCAGGTACCGTCATTTTCTTCCCTGCAAAAA
GAGCTTTACATTCCGAAACCATTTTTCACCCCAGCGGGGTCTCTTCCTCC
GGGTTTCCCCCCTTGTGCAATATTCCCCACTGCTGACTCCATTAAGAACT
GCTGCCTCCCGTAGGA

TCATTTTCTACCCTGCTGATAGAGCTTTACGTACACGAGATACTTCTATC
ACTCACGCGGCGTCGCTGCATCAGGGTTTCCTTTTGGGAGTT

CAGACCCGGGGAGCTTAATTACCGCGGCTGCTGGCACGTAGTTAGCCG
GGGCTTCTTAGTCAGGTACCGTCATTTTCTTCCCTGCTGATAGAGCTTTA
CAGTACCGAGATACTTCTTCACTCACGCGGCGTCGCTGCATCAGGGTTT
CCCAAGGTGGGCCT

ATGCAGCGCGCGCGTGAGGAAGAAGGTTTTCGGATTGTAAACCTCTTTT
GGCAGGGAAGAAAATGACGGTACCTGAAGAATAAGCCCCGGCTAACTC
CGTGCCAGCTGCCGCGGTTATATGCCAGCAGCCGCGGTAATA

AGCCGAGGGAACTGTTCATTACCGCGGCTGCTGGCACGTATTTAGCCGG
AGCTTCTTACTCAGGTACCGTCAATTTCTTCCCTGCTGATAGAGCTTTAC
ATACCGAAATACTTCTTCGCTCACGCGGCGTCGCTGCATCAGGGTTTCC
CCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAAAGATTCCCTACTG
CTGCCTCCCGTAGG

AGGTACCGTCATTTGATTAGCTGCCGCTCTGACTTTCGTTCTTCAATACC
TCTTCACTTATACGATGTCAAAACCTTCTGGTTTCCCCCATTGTGCAATA

TTCCCACCTGCTGCCACCGCCAAGAATTCCCTACTGCTGCCTCCCGTAG
GA

TATTACCGCGGCTGCTGGCACGTTATTATCCCCCCGGCTGCGGGCAGGT
ACCGTCCCGGGGTTTGCTATTCAGGCTCCGTCACTTTCTTCCCTGCCGAT
AGAGCTTTACATATCCGAAATACtTTCTACACTCACCCATG-
CGTCAAACCTATCATCAC

CCGCGGCTGCTGGCACGTATTTAGCCGGTGCTTTTTCTTCAGGTACCGTC
ATTCTTCTTCCCTGCTGACAGAGCTTTACATACCGAAATACTTCCTCGCT
CACGCGGCGTCGCTGCATCA

ACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTCTTAGTCAGGTACCG
TCATTTTCTTCCCTGCTGATAGAGCTTTACATACCGAAATACTTCCTCAC
TCACGCGGCGTCGCTGCATCAG

CCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTCTTGGTCAGGTACCGT
CATTTTCTTCCCTGCTGATAGAGCTTTACATACCGAAATACTTCCTCACT
CACGCGGC GTCGCTGCATCAGGGT

GCTGGCACGTAGTTAGCCGGGGCTTTTTAGTCAGGTACCGTCTTTTTTCT
TCCCTGCTGATAGAGCTTTACATACCGAAATACTTCTTCACTCACGCGG
CGTCGCTGC ATCAG

CCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTAGTCAGGTACCGT
CATTTTCTTCCCTGCTGAAAGAGCTTTACATACCGAAATACCTTCTTCAC
TCACGCGGCGTCGCTGCCTTCGCAGG

GAAACCGCGGGTCCTGGCACGTAGTTAGCCGGGGCTTCCTAGTCAGGT
ACCGTCATTTTCTTCCGTGAAGAAAGAGCTTTACCTACACGAAATAATT
TTATCAACTCCCGAGGGGTGTTATGCAGCACA

CCGCGGCTGCTGGCACGTAGTTAGCCGTCCCTTTCTGGGTAGTTACCGT
CACTTGATGAGCTTTCCACTCTCACCAACGTTCTTCTCTACCAACAGAGT
TTTACGATCCGAAAACCTTCTTCACTCACGCGGCGCGCTCGGTC

ATGCAGCGACGCCGCGTGAGTGAGAGTTTTCGGATCGTAAAGCTCTGTT
GGCAGGGAAGAACATGAGTGTGCCTGAATAGCAAGCCCCGGGTAGGTA
CCTGCCCGCAGCCGCGGGGATAATAACGTGCCGCCGCCCGCGGTATA

GTCGCGTGAGTGAGACGGTCTTCGGGGTGGTAACTCTGTTGGTAGGGA
AGAAAGTTGGTGAGAGTGTGGGCTTTGATGTGCCGTATCCTACCCGATC
GGCCCGTCTAATGCCTGCCCCGCGGTCTAAT

ATAACCGCGGCTGCTGGCACGGAGTTAGCCGGGGCTTATTCGTCAGATA
CCGTCATTTTCTTCCCTCACAAAAGAAGTTTACAATCCGAAAACCTTCA
TCCTCCACGCGGCGTTGCTGCTTCAGGGTTTCCCCCATTGAGCAATATTC
CCTACTGCTGCCTCCC

CCGACGTCCGCGTGAGTGATAGACATGCTCCTCTGGGTTGTAAGCTTCT
TTTATATGGAAATAAAACAGGGTATGCGTACCCTCTTGTCTGTACCATA
TGAATAAGGATCGGCTGGCTATAATGCCAGCAGCCGCGGTAATA

CCGCGGCTGCTGGCACGTAGCTAGCCGGGGCTTGTTAGTCAGGTACCGT
CATTTTCTTCCCTGCCGACAGAGCTTTACATACCGAAATACTTCCTCACT
CACGCGGCGTCACTGCATCATGGTTTCCCCCATTGTGCAATATTCCCCA
CTGCTGCCTCCCGTAAGA

AATAACCGCGGCGGCGGGCAGGTATTAACCCGTGGCTTTCGGGTAGGA
ACCCGCCGAAACGTGAACAGTCACTCTCACGCACTTTCTTCTCTAACAA
CAGAGTTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCCA
TCCCGCTTTCCCCCCTTGGGGAAGAATCCCTACTGCTGCCTCCCGTAGG
G

TATTACCGCGGCGGCTGGCATTGAGTTAGCCGATCCTTATTCATAAGGT
ACATGCAAAGCCGTACACGTACCGCACTTTATTCCCTTATCAAAGACGT
TTACAATTCATAAATCCATCATCCTTCACCCCCCTTGCCTGGTTCAGGCT
TCCGCCCATTGACCAATA

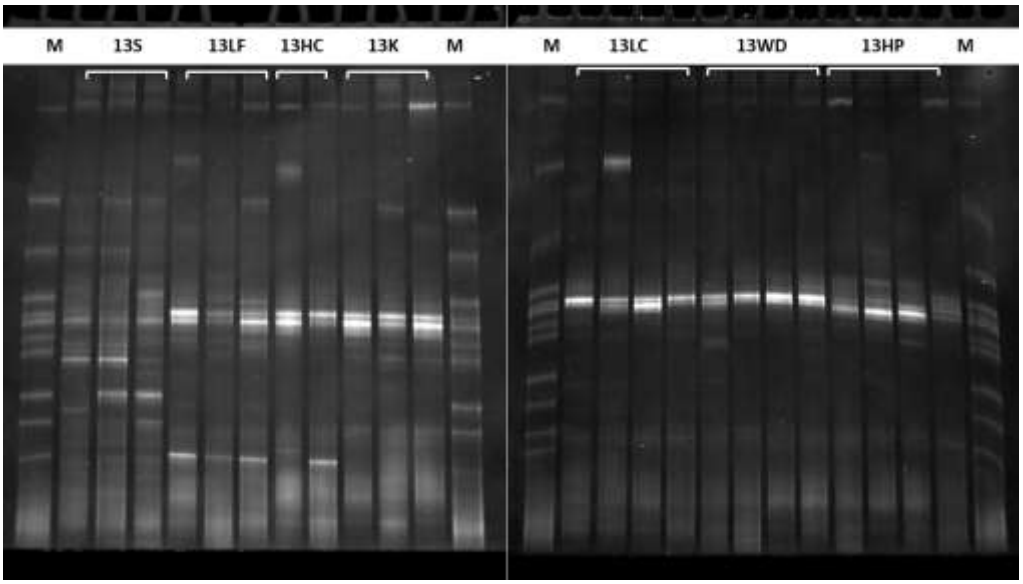
GCTGGCACGTAATTAGCCGTCCCGTTGTGGGTAGTTACCGTCACGGGAT
GAGCTTTCCACTCTCACCAACGTTCTTCTCTACCAACAGAGTTTTACGAT

CCGAAAACCTTCTTCACTCACGCGGCGTTGCTCGGTCAGACTTGCGTCC
ATTGCCAAGGATTCCCTACTGCTGCCTCCCGTAG

GGAGCACGCCGCGTGGGTGAAGAAGGTCTTCGGATCGTAAAACCCTGT
TGTTAGAGAAGAAAGTGCGTGAGAGTAACTGTTCACGTTTCGACGGTTT
CTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC

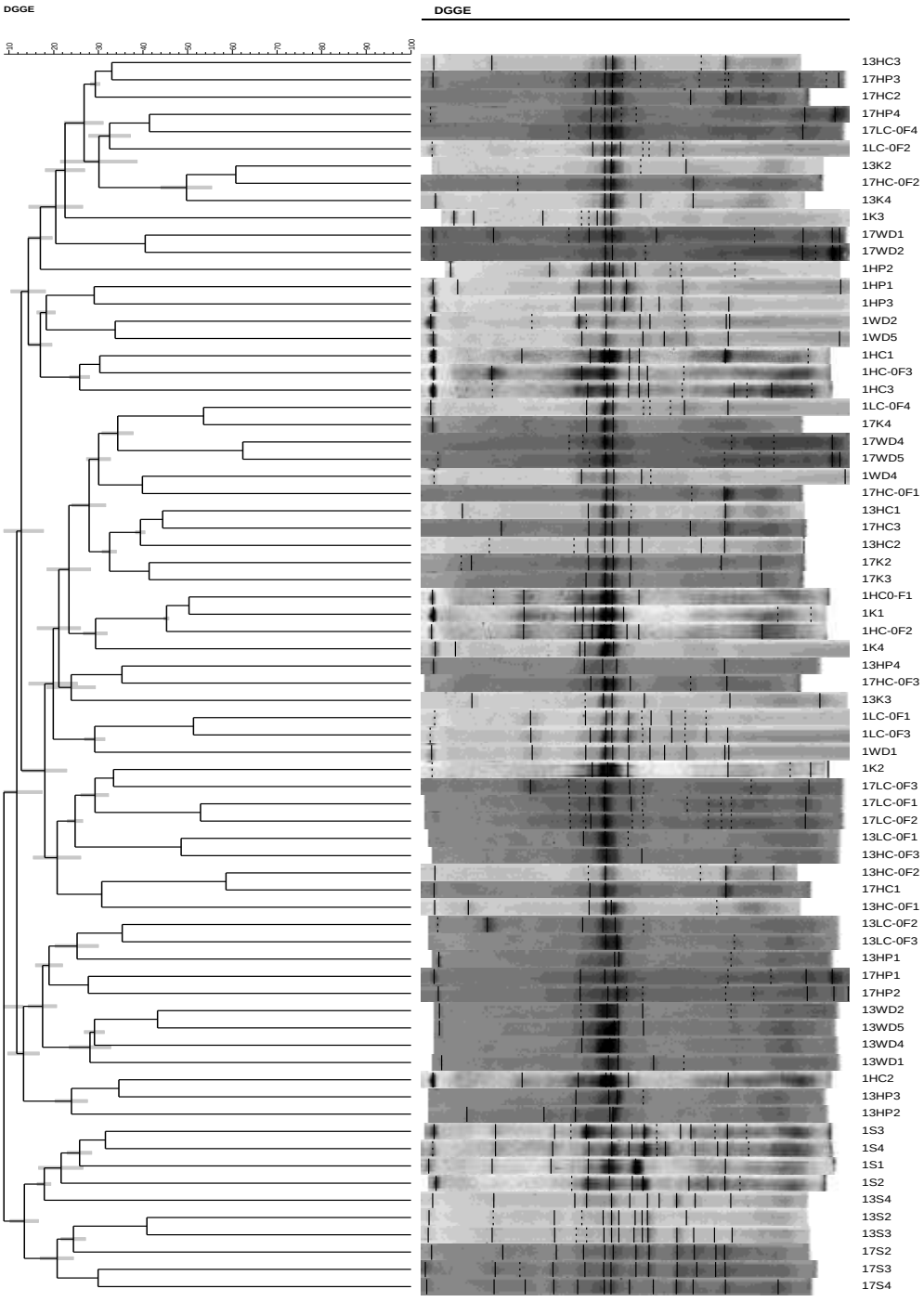
CGTGAGTGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTTGCCGGGGAAA
AAGGCAGCGTCATGAAATGGGAGCTGACTGATGGTGCCCCGCCAGAAA
CTCACGGCATAATACGTGCCAGCAGTTGATGATGCTGCCTTTTTTCCCC
GGCATCATAGCTTTACCACCCGAAGGCCTTCTTCACTCACGCCGTTGCG
CTATATCCCGCACGGCCCCATCGACTAAGATTCCCTACTGCTG

C. DGGE Profile



DGGE gel profile of GC357f/518r PCR products. DGGE gel profile of the 13th week stool samples. (M: Marker, S: Standard chow diet, LC: Low Carbohydrate diet, HC: High Carbohydrate diet, K: Ketogenic diet).

D. DGGE Dendogram



Similarity (Pearson correlation coefficient- UPGAMA) of the DGGE banding patterns of 1th, 13th and 17th week stool samples. (S: Standard chow diet, LC: Low Carbohydrate diet, HC: High Carbohydrate diet, K: Ketogenic diet, WD: Western diet, HP: High protein diet)

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Ulutaş, Mehmet Sefa Ulutaş
Nationality: Turkish (TC)
Date and Place of Birth: 17 December 1988, Samsun
Marital Status: Married
Phone: +90 5059890404
email: sefaulutas@gmail.com
Address: Siirt University, Faculty of Science and Literature, 56100, Siirt

EDUCATION

Degree	Institution	Year of Graduation
MS	ITU Molecular Biology and Genetic & Biotechnology	2013
BS	ITU Molecular Biology and Genetic	2011
High School	Patnos Anatolian High School	2006

WORK EXPERIENCE

Year	Place	Enrollment
2020-Present	Siirt University	Research Assistant
2019-2020	Massachusetts Institute of Technology (MIT)	Visiting Researcher
2014-2019	Middle East Technical University	Research Assistant
2013-2014	Siirt University	Research Assistant
2011-2013	Istanbul Technical University	Research Assistant
2011	Siirt University	Research Assistant

FOREIGN LANGUAGES

Advanced English

PUBLICATIONS

1. Fincan, S. A., Özdemir, S., Karakaya, A., Enez, B., Mustafov, S. D., **Ulutaş, M. S.**, & Şen, F. (2021). Purification and characterization of thermostable α-amylase produced from Bacillus licheniformis So-B3 and its potential in hydrolyzing raw starch. *Life sciences*, *264*, 118639.

2. Cheng C.W., Biton M, Haber A.L., … **Ulutas M.S**, et al. (2019) Ketone Body Signaling Mediates Intestinal Stem Cell Homeostasis and Adaptation to Diet. *Cell*. 2019;178(5):1115-1131.e15.

3. Özdemir S., Okumuş, V., **Ulutaş M. S**., Dündar A., Akarsubaşı A. T., Dumonted S., (2016) Production and characterization of thermostable α-amylase from thermophilic *Anoxybacillus flavithermus* sp. nov. SO-19, Starch, 68: 1244-1253

4. Özdemir S., Okumuş, V., **Ulutaş M. S**., Dündar A., Akarsubaşı A. T., Dumonted S. (2015) Isolation of a Novel Thermophilic Anoxybacillus flavithermus SO- 13, Production, Characterization and Industrial Applications of its Thermostable α-Amylase, Journal of Bioprocessing & Biotechniques, 5:3-9.

5. Bermek H, Catal T, Akan S.S, **Ulutaş M.S.**, Kumru M, Özgüven M, Liu H, Özçelik B, Akarsubaşı A.T (2014) Olive mill wastewater treatment in single-chamber air-cathode microbial fuel cells, World J Microbiol Biotechnol. 1177-85.

6. Özdemir S., Matpan F., Okumuş, V., Dündar A., **Ulutaş M.S**., Kumru M., (2012), Isolation of a thermophilic Anocybacillus flavithermus sp. Nov. and production of thermostable α-amylase under solid-state fermentation (SSF), Ann Microbiol 62: 1367-1375.