EFFECTS OF MATERNAL METABOLIC DISORDERS ON OFFSPRING ANTIMICROBIAL PEPTIDES EXPRESSION: CHANGE IN ANTIMICROBIAL DEFENSE SYSTEM

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ABSTRACT

EFFECTS OF MATERNAL METABOLIC DISORDERS ON OFFSPRING ANTIMICROBIAL PEPTIDES EXPRESSION: CHANGE IN ANTIMICROBIAL DEFENSE SYSTEM

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Birth is a traumatic and stressful event for all living organisms. Neonate meets a world full of microbes and has to adapt the new external environment. Throughout all developmental periods and adulthood stages, balanced microbiota crucial for the health of offspring. Antimicrobial peptides (AMP) have enormous impacts on the establishment of the microbiome and determining the specific composition of the bacterial community as well as its spatial organization. This thesis investigated the effects of maternal metabolic disorders on antimicrobial protein (AMP) expression, which is crucial for establishment of gastrointestinal system (GIS) probiotic composition and mucin expression on offspring intestinal cells. As Using whole-genome microarray analysis, changes in AMP expressions were detected and also ceratin gene expressions were validated by RT-PCR. Besides, changes in probiotic bacterial species populations were determined in those regions. Our results might reflect how maternal metabolic disorders fluctuate offspring mainly mucosal immunology parameters for the future microbiome establishment. Finally, tight junction proteins of intestinal cells were investigated to understand the pathogenic bacterial translocations that might be essential for chronic inflammation and obesity tendency of offspring.

Keywords: Anti-microbial peptides, chronic inflammation, obesity

ÖZ

ANNENİN METABOLİK HASTALIKLARININ YAVRU ANTİMİKROBİYAL PEPTİTLERİN İFADESİNE ETKİSİ: ANTİMİKROBİYAL SAVUNMA SİSTEMİNDEKİ DEĞİŞİKLİKLER

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Doğum tüm canlı organizmalar için travmatik ve stresli bir olaydır. Yeni doğan yavru, mikroplarla dolu dünyayla tanışır ve yeni dış çevreye uyum sağlamak zorundadır. Tüm gelişim dönemleri ve yetişkinlik aşamasında, dengeli mikrobiyota yavruların sağlığı için çok önemlidir. Antimikrobiyal peptidler (AMP) mikrobiyom yerleşmesinin yanı sıra bu bölgeye yerleşebilecek bakterilere karar vererek bakteri topluluğunun organizasyonu üzerinde etkileri vardır. Bu tezde, maternal metabolik bozuklukların; yavru bağırsak hücreleri tarafından ifade edilen müsin ve gastrointestinal sistem (GIS) probiyotik bileşimi için önemli olan AMP ekspresyonu üzerindeki etkileri araştırılmıştır. Tüm genom mikrodizi analizi kullanılarak AMP ekspresyonlarında değişiklikler saptanmıştır ve seçilen belli genlerin ifadeleri RT-PCR ile doğrulanmıştır. Ayrıca bu bölgelerde belirli probiyotik bakteri türleri popülasyonlarında gerçekleşen değişimler belirlenmiştir. Sonuçlarımız, maternal metabolik bozukluklarla beraber mukozal immünolojide gerçekleşen değişikliklerin yavru gelişim sırasında yerleşmesi beklenen mikrobiyomu nasıl etkileyebileceğini yansıtabilir. Son olarak, bağırsak hücrelerinin sıkı bağ proteinlerinin analizi sonucunda azalan sıkı bağ proteinleri patojenik bakteriyel translokasyonlarına neden olarak gelecekte yavru için kronik inflamasyon ve obezite oluşturabilme potansiyelini taşıyabilir.

Anahtar Kelimeler: antimikrobiyal peptit, obezite, kronik inflamasyon

To my family

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LIST OF ABBREVIATIONS

AJs Adherens junctions

AMP Antimicrobial peptides

APCs Antigen-presenting cells

Ccf Commensal colonizing factors

CLDN Claudin

DAMPs Damage associated patterns

DC Dendritic cells

DM Diabetes melitus

GDM Gestational diabetes mellitus

GI Gastrointestinal

GIS Gastrointestinal system

IBD Inflammatory bowel disease

IEC Intestinal epithelial cells

IRF-3 Interferon regulatory factor 3

JAM Junctional adhesion molecule

LPS Lipopolysaccharide

MAPK Mitogenactivated protein kinase

MLC Myosin light chain

NF-kB Nuclear factor kappalightchain-enhancer of activated B cells

NK Natural killer cells

NOD2 Nucleotide-binding oligomerization domain 2

PAMPs Pathogen-associated molecular patterns

PGDM Pre-gestational diabetes mellitus

PRR pattern recognition receptors

STZ Streptozocin

T1DM Type 1 Diabetes melitus

T2DM Type 2 Diabetes melitus

TJs Tight junctions

TLR Toll like receptors

TNF Tumor necrosis factor

CHAPTER 1

INTRODUCTION

1.1 Obesity and Relationship with Diabetes

Obesity is a multi-factorial chronic disease. With respect to etiology, it can be divided into 3 groups: syndromic, monogenic and common. The common obesity category constitutes %95 of obese people population[1]. After the disruption between energy intake vs. expenditure, it is generated and increases the possibility of many other diseases like Type 2 diabetes, hypertension, cardiovascular disease[2].

As a metabolic disorder, Diabetes Mellitus (DM) is a chronic multifactorial disorder and changes cellular carbohydrate, protein, and fat metabolism and changes cellular functionalities. Generally classified in two conditions: Type1 and Type 2 with respect to causative or initiative condition[3]. Type 1 DM (T1DM) is an autoimmune disorder. Individuals who are susceptible to genetic alterations, infectious agents, and certain environmental factors might trigger immune response via altered β-cells or against molecules in β-cells that are similar to pathogenic proteins. These changes initiate the auto-destruction of β-Langerhans islet cells of the pancreas[4].

Type 2 DM (T2DM) has different etiology and pathophysiological conditions when compared to T1DM. T1DM patients generally tend to have not obesity and frequently subjected to diabetes ketoacidosis. As for a general explanation for T2DM, it can be described with a combination of low insulin generation from β-cells of the pancreas and peripheral insulin resistance. Both peripheral insulin resistance and pancreatic β-cell dysfunction initiate T2DM to develop[5].

Insulin resistance leads to deficiencies of glucose transport into the muscle cells fatty acids level increase in the plasma, fat breakdown, and hepatic glucose production increases[6].

Obesity, and along with disruption of metabolic homeostasis, can initiate insulin resistance and generate prediabetic condition early, but not DM directly. However, continuous metabolic disruption causes dysfunction of pancreatic β-cells. It carries prediabetic condition to diabetic condition in which the number of β-cells decreases and an insufficient amount of insulin secretion occurs with developing stages of DM[7]. As an observable condition, abnormal glucose tolerance is observed with increment of postprandial blood glucose levels[8]. Besides, because of the failure of hepatic gluconeogenesis, fasting hyperglycemia may develop. In such condition, both macrovascular and microvascular complications start to appear similar to T1DM. These vascular changes initiate both cellular and organ level dysfunctionalities[9]

1.1.1 Maternal Metabolic Condition and Effects on Offspring

Statistical tests have found that the prevalence of obesity influences all age ranges including childbearing age, and this incidence varies significantly with age[10]. During pregnancy, T2 DM mediated obesity, and obesity has a significant effect on not only mother, but also offspring health during pregnancy. Increases in asthma prevalence, stroke, thrombotic complications, maternity complications, postpartum hemorrhage, and even caesarean section for mothers with obesity were observed[11].

Although the lethal functions of obesity for mother and offspring during gestation are currently understood, the impact of maternal obesity on the subsequent life of both mother and offspring is being studied under investigation. Studies about obese mothers demonstrated that with the occurrence of preeclampsia and gestational diabetes during pregnancy, the likelihood of diabetes progression and cardiovascular disease rises significantly after childbirth. On the other hand, studies also have shown

that developmentally impaired offspring during birth, and later obesogenic characteristics may evolve along with other metabolic disorder characteristics, cardiovascular disorders, cognitive and behavioral dysfunctions[12].

1.1.2 Diabetes and Pregnancy

Maternal diabetes can be investigated in two main categories: pre-gestational diabetes mellitus (PGDM) and gestational diabetes mellitus (GDM). PGDM particularly covers the cases when diabetes diagnosis given to women before they ever became pregnant. GDM covers the instances where diabetes diagnosis is not given before having pregnancy, but under pregnancy, condition develops diabetes[13].

1.1.3 Animal Models for Maternal Obesity and Type2 Diabetes Studies

For obesity and diabetes studies, animal models are generated to produce common conditions comparable to human obesity to examine obesity and/or the impact of maternal obesity on offspring health[14].

Vertebrates have robust balance systems for energy and are accepted as suitable animal models. Research conducted on both maternal obesity and diabetes and their effect on offspring is usually carried out with this animal models[14].

In vertebrate studies about obesity, disorders are typically defined by the use of pellets with increased high fat and/or carbohydrate component. Some model animals, however, can withstand consumption of such pellets, and this causes some experimental difficulties. An alternative diet form called cafeteria diet (CAF) has evolved to address such challenges, including daily junk food intake with high fat/energy. CAF diet can initiate long-lasting neuronal changes in certain brain regions where metabolism, appetite and satiety are regulated. Generally, this form of diet carries various types of processed foods such as snacks, chips, cookies, desserts, chocolates were preferred (Table 1)[15].

CAF studies also found that this diet not only generate obesity condition but also generate more spontaneous like diabetes condition too other than using chemotoxic agent streptozocin (STZ). It is also assumed that the cafeteria diet could be more suitable for diabetes, obesity, and metabolic disorder studies than usual high-fat and/or carbohydrate diets[15].

1.2 Microbiota

As Hippocrates mentioned, “death sits in the bowels” and “bad digestion is the root of all evil” in 400 (B.C.); the crucial role of the intestine for human health recognized even in ancient times. After several decades of research on gastrointestinal living organisms, the general intention and approach were on pathogens and how they generate disease conditions. However, with the recent recognition of the beneficial effect of commensal microbes on health, most studies focused on the interaction between gastrointestinal microbiota and mammalian host[16].

Almost all multicellular organisms have interactions with surrounding microbes. As a human beings, we harbor a vast number of unicellular eukaryotes, archaea, bacteria, and also viruses. All of those microorganisms that live in a hierarchical manner and harmony within hosts are called the normal flora or microbiota[17].

Fifty bacterial phyla had been described for human gut microbiota. Predominantly strict anaerobes constitute than facultative anaerobes and aerobes by two to three times more. Fusobacteria, Bacteroidetes, Verrucomicrobia, Proteobacteria, Actinobacteria, Firmicutes, and Cyanobacteria phyles. Although occurrence of species present in the gut varies widely between study groups, it can be estimated as a composition of over 35,000 bacterial species[16].

Mammalian are born with mother-offspring transferred bacteria, which develop with host-microbial interaction and continues till from birth to death. It is thought that these transitions might affect their life long journey together[18].

With recent studies, it is estimated that the number of bacterial cells in the host is almost similar to the number of cells in the human[1920]. Also, with corrected studies, the total weight of the bacteria living in/on our body is predicted to be 200 gr. Gene number of the microbiota in gene content passes our genes by more than 100 fold, and that is why it is called the second human genome[21]. In world wide studies like human intestinal tract (MetaHIT), Asian Gut, and Human Microbiome Project, the purpose was to identify and characterize the human microbiome to understand communication between these two phyla. These projects have shown that microbiota is highly diverse than predicted previously, and a large persantages of these variaties uncharacterized. For these studies, GIT is preferred because of populated by more than 500 bacterial species and being mostly colonized organ. Early colonizers are facultative anaerobes and by low diversity and dominance by Proteobacteria and Actinobacteria phyla at neonatal microbiota for initiation of the colonization point.

After birth, gut microbiota is a very complex living ecosystem for the host and co-evolved during development and immense effects on physiology[22]. That is why it has been postulated with different axes in the body in which gut microbiome has a role in every axis for the host: gut-liver axis, gut-immune system axis, gut-heart axis, gut-muscle axis, gut-brain axis, and even gut-renal axis. With recent developments, the gut microbiota was considered an organ and thought as the second brain of the human, the second liver, and the the second genome.[23242526]

In general approach; the effects of gut microbiota on the host can be categorized into two: beneficial effects (via healthy gut microbiota) and the harmful effects (via dysbiosis). A deeper understanding of balanced gut microbiota may be the first step towards a better understanding the significant impact that gut microbiota has on host health[27].

1.2.1 Healthy gut microbiota

In the literature, worldwide, there is no consensus about a definition of a 'healthy gut' yet. Functions of the gut microbiota are heavily conserved between individuals, whereas a particular mixture of bacterial species distinguishes the gut microbiota of each organism due to inter-individual and intra-individual differences[28].

Besides; The structure of microbiota is very complex and differs considerably between people and even within them[29]. Noteworthy, it was recognized that each organism is colonized by more than 1,000 species that come from just a few phyla. In contrast, nearly all the studies agree on the two leading bacterial phyla, Firmicutes and Bacteroidetes[2830].

Having shown the high heterogeneity of the stable gut microbiota[29] (Figure 1), the next step will be to consider the physiological factors that lead to programming this natural variance, as well as the factors that might theoretically interrupt it and drive it to dysbiosis.

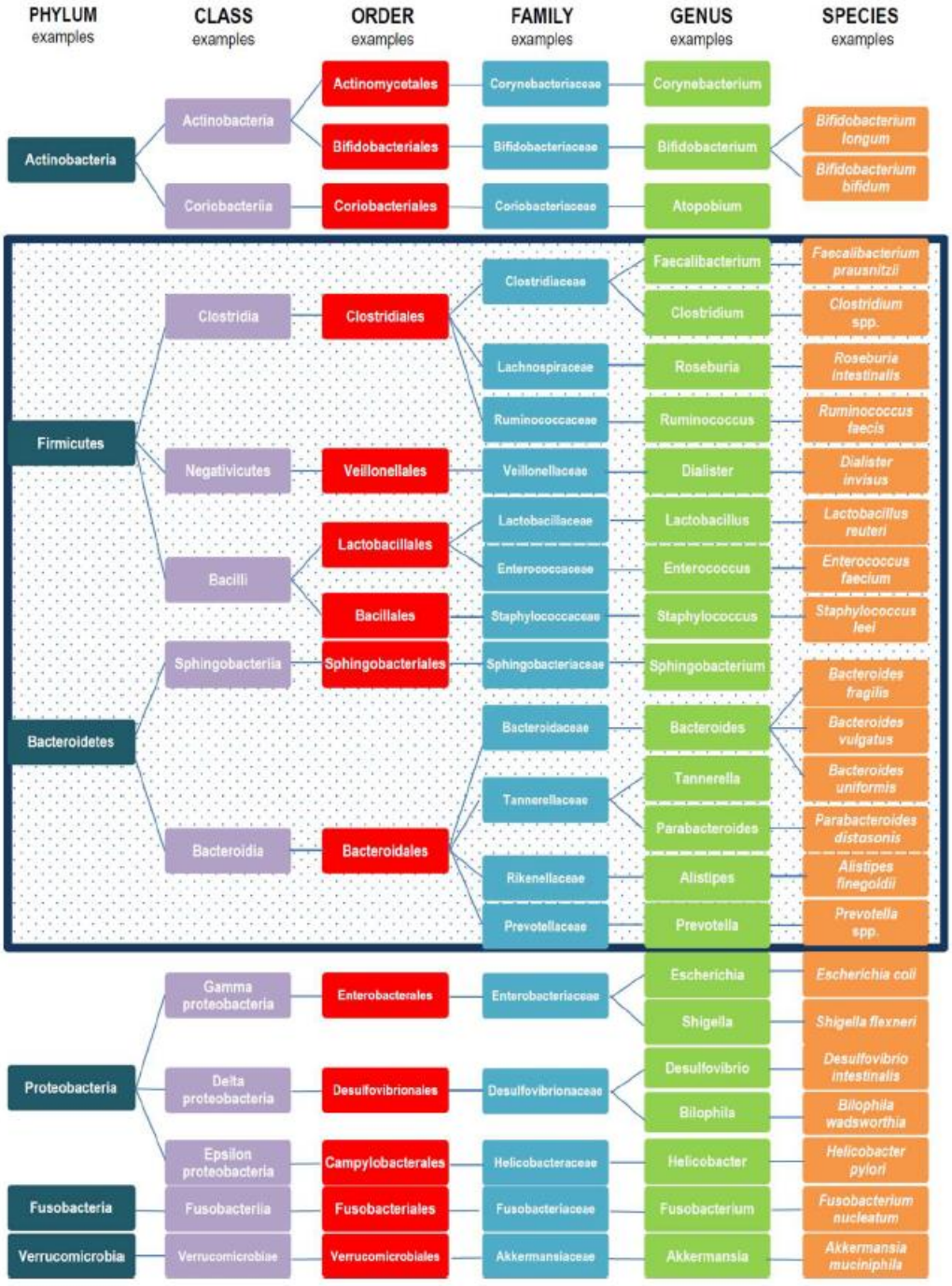

Figure 1: Examples of taxonomic gut microbiota composition (Adapted from Rinninella et al. 2018)

1.2.2 Extrinsic host factors

After birth via natural vaginal delivery, offsprings are mainly colonized by microbiome similar to their mothers. On the other hand, for gut microbiome for infants born via cesarean section, more susceptible inhabit with commensal skin bacteria[31]. Studies about comparing these two conditions have found that the microbial diversity of cesarean section born children were significantly diminished[32].

On the other hand, interestingly, the long-term effect of the delivery method have not supported by later on set adult studies and it is not clear yet[28]. At this point, host-directed forces might have a huge role during developmental periods.

In general, terms, when we investigate maternal microbial fluctuations during pregnancy, we can realize that gut microbiota mothers changes from the first to third trimester. At 1st-trimester, the microbiota is shown to be similar to nonpregnant but towards to 3th-trimester there is less Faecalibacterium, and more Proteobacteria and Actinobacteria and there is general decreased richness is observed, which is heavily correlated with inflammation and loss of energy. At this point again, interestingly composition of the offspring's microbiota similar to the maternal composition of first trimester[33]. These result might make us focus on host-directed forces for colonization and communication between microbial component.

1.2.2.1 Method of infant feeding

When we investigate Mother milk, it has oligosaccharides, indigestible sugars that are unique and consumed by the infant's gut microbiota like Bifidobacterium and Bacteroides. Crucially, while breastfeeding, the type and amount of the breast milk carbohydrate content might change and it can be reflected in the gut microbiota composition[34].

Generally, we can consider breast milk as a prebiotic because; Oligosaccharides of breast milk contribute to the establishment of specific gut microbiota and very important for synbiosis process. It includes 102 to 104 per ml for humans, which means at least 105 bacteria per day[35]. In addition to these, mother milk also accommodates immunological factors like Lactoferrin and IgA and also antimicrobial peptides which shapes colonization of the commensal bacteria[36]**.**

Lifestyle-related factors

The long-term effects of diet have been investigated for many study groups, and diet can be considered a well-known factor associated with the composition of gut microbiota. For example, diets includes fruit and fiber are generally associated with higher diversity of microbiota and a dominance of Prevotella over Bacteroides. For western diets, with high fat and/or sugar and low amounts of fiber, resulting in diminished Firmicutes and elevated enteric pathogens[37].

Studies have revealed a significant association between gut microbiota composition and diets[38]. Besides, different groups have demonstrated the implicance of short-term diet on the gut microbiota composition[39].

However, complex results have also been observed: diet was associated with the gut microbiota trace; however, it can explain roughly 6% of the variations[40].

As for exercise, there are several reports, mainly in rodents, investigating the impact of exercise on the intestinal microbiota composition. Human studies apparently focus more on comparing athletes to controls. For example, at one analysis, it is found that athletes have a more complex gut microbiota composition relative to the control group, which may partly be attributed to their specific diet (high protein intake).[41]

In addition, studies about professional athletes with sedentary controls revealed that SCFA produced by microbiota enhanced in athletes, indicating the importance of exercise at metagenomics and metabolomics levels in addition to compositional level[42].

1.2.2.2 Environmental factors

Geography: there are a few reports focused on the variations in the composition of intestinal microbiota depending on geographic location. It is found that there are substantial variations in the microbial makeup of people residing in the US (metropolitan areas) relative to people living in the Venezuelan Amazons and rural Malawian populations [43].

More experiments have also shown that people that live in western countries inherit distinct microbiota when compared to those living in underdeveloped countries. Those variations may be explained by variations in genetic history and lifestyle, including nutrition and grooming[44]. Moreover, a study on 1020 healthy residents from 23 populations finds regional latitude contributing to the concentration of Firmicutes (positive) and Bacteroidetes (negative)[45].

1.2.2.3 Having Siblings

There are several reports that have measured baby gut microbiota with and without older siblings. Statements in this area are also tentative and inconsistent, i.e., although one study recorded a decline in α-diversity measures in infants with older siblings[46], such indices were seen to rise in another study[47].

1.2.2.4 Pet owning

There is no consensus. While studies could not find a huge effect on infants' gut microbiota composition for pet owners [47], one research has shown that infants grown in homes with pets have raised a diversity of gut microbiota [46].

1.2.2.5 Microbial factors

Though it may look very significant, so far, less knowledge is known about the role of microbial factors in host-microbial composition stability. Studies identified a genetic region, namely commensal colonizing factors (ccf), that could have a role. This region harbors genes encoding for use with polysaccharides and shown to be preserved among various species of the genus Bacteroides. Deletion of the genes mapped in the murine model to the ccf loci resulted in defective colonization, and after an induced microbiome disturbance, these genes were found to be required for re-colonization of Bacteroides. In fact, multiple independent experiments reported a distinction for the wild-type bacteria 's physical colonization position relative to the harboring ccf-mutant species as that only wild-type bacteria may live inside the intestinal crypts. Interestingly, it has been shown that crypt-associated species can survive in the presence of antibiotic treatment (with the potential to repopulate the GI tract later), which indicates the significance of this locus for the survival and stability for Bacteroides in the gut microbiota[48].

1.2.3 Intrinsic host factors

1.2.3.1.1 Age

Before birth, after, and shortly after childbirth, a low diversity microbiota from mother, food, and atmosphere colonizes the infant's GI tract[4948]. When the solid food begins, the gut is slowly occupied by more complex microbiota, and when formed (from about 18 months to 3 years old), this compositional configuration stays highly robust (although one study has shown that the gut microbiota of adolescents varies from that of adults[50]. Diversity diminishes again in the elderly.

Such age ranges have been found to be influenced by improvements in the ratio of 0.4, 10.9, and 0.6 for children, adolescents, and elderly respondents between firmicutes and bacteroidetes, respectively[51]. However, another fascinating research carried out on three classes of people aged 99 to 104 years (centenarians), 63 to 76 years, and 25 to 40 years, showed that the gut microbiota altered dramatically only in centenarians, but the composition was very identical in young adults and seventy-year-olds[52].

In addition, age was reported as one of the 18 non-redundant variables correlated with microbiota composition resulting from one of the most detailed analyzes performed to date, and this finding was verified by another significant general population-based field analysis[53]. Finally, age was discovered to be responsible for approximately 5 percent of all differences in a crucial analysis exploring the various genetic and non-genetic influences involved in defining gut microbiota function[40].

1.2.3.1.2 Gender

While gender was generally considered among the covariates in microbiota studies for years, there was no reliable scientific background to this. In 2006, a gender influence was identified only for a particular bacterial community in a survey of respondents from four countries in France, Germany, Italy, and Sweden[54]. In addition to ethnicity and age, gender was found to be associated with functional microbial richness. Gender was also found to be responsible for approximately 2 percent of the variability in the makeup of gut microbiota[4055].

1.2.3.1.3 Fecal chromogranin A (CgA)

CgA is also found to be adversely correlated with gut microbiota. By neurons, CgA generally secreted when there is a stress condition related to the endocrine system and immune cells. I is known as an indicator of neuroendocrine activation.

At one study, with 126 host intrinsic and extrinsic factors, CgA has shown a crucial association, covering several taxa that abundances account for more than 50 percent composition. However, CgA was also found to be adversely correlated with the sum of fruit and vegetable consumption in the sample population, so any inference should be drawn with caution[56].

1.2.3.1.4 Genes

Twin experiments indicated host-directed genetics might have crucial role in developing the intestinal microbiota composition[57]. Since then, further hereditary tests, and gene research in both murine and human have endorsed this theory. Selected candidate gene studies are summarized in Table 1. GWAS studies have utilized good sample sizes; unfortunately, most of the signals were only identified in one analysis, and so little overlaps are observable between the findings (the best finding we have so far is the signal linked to the LCT gene, which has continuously been shown to be correlated with Bifidobacterium)[58].

Table 1: Microbiota candidate gene studies

Gene name	Gene function
Nucleotide-binding, oligomerization domain 2 (NOD2)	Mediates the host response to the bacterial peptidoglycan and is implicated in susceptibility to Crohn's disease.
Fucosyltransferase 2 (FUT2)	Is responsible for the presence of ABO histo-blood group antigens found on the GI mucosa and secretions
Human leukocyte antigen (HLA)-DQ	Recognizes and presents foreign antigens to the immune cells and its mutations predisposed the carrier to the Coeliac disease (CoD)
Immunity-related GTPase M (IRGM)	Involved in the regulation of autophagy
lactase (LCT)	Translates to the lactase (an enzyme for hydrolyzing lactose in the GI tract)

Finding has been related to the strong specificity of the phenotype. This implies that several different genes and loci are involved in the development of the phenotype, and each locus contributes to a very limited fraction of the variations that make it very complex for the detection or repeat in various studies[59].

Also, where the impact sizes are very low, variations in technique and study methods used in the various experiments may easily overshadow the underlying effects. In studies, it was realized that persons from specific microbiota sample cohorts, mainly clustered by the studies which showed that any variation in the test methods like primer choice for 16S tests, DNA extract techniques, sequencing, and bioinformatics methods might introduce systemic biases into these tests[60,61]

1.2.3.2 Beneficial effects of the gut microbiota for host health

Parts of the gastrointestinal microbiota are harmless and helpful for the host organism. That microbiota has communication with the host and affects it in various ways. Possibly the most recognized impact of gut microbiota is their role on the digestive compartment because most of the fibers cannot be digested by the enzymes intestine, in comparison microbiota, that has a remarkably rich source of enzymes can achieve digestion[62].

Limited-digestible carbohydrate digestion achieved by carbohydrate-active enzyme (CAZymes) fermentation, produced by gut microbiota, resulting in generation of energy with ATP and SCFAs as the end products of this fermentation process. Beneficial effects SCFAs have been shown to host body[63].

As an example; acetate, propionate, butyrate, isobutyrate, 2-methyl propionate, valerate, isovalerate, and hexanoate are the most common SCFAs. SCFAs are the critical source of energy for colonocytes and prevent colorectal cancer[64].

Faecalibacterium, roseburia, et F. Bacteria named SCFA producers include prausnitzii. Gut microbiota also interacts extensively with the host. It plays an essential role in regulating the metabolism of glucose and cholesterol, i.e., through

various mechanisms, it reduces the plasma levels of glucose and cholesterol and thus could promote cardiovascular health[65,66,67].

Another significant role of intestinal microbiota on host physiology is related to its impacts on the GI tract's functional framework through its effects on tissue regeneration, intestinal barrier integrity, and GI vascular system morphogenesis[68]. Gut microbiota also possesses the exciting ability to regulate host immune homeostasis. From germ-free studies, the importance of gut microbiota in host immune system development is identified by study groups [69].

As it is known, innate and adaptive immunity develops with gut microbiota, and these species have huge function in the maturation of lymphoid lung-associated tissue. Besides, certain bacterias may defend the host from enteropathogenic microbes and eventually sustain a balanced immune system/function[30]. Healthy microbiota is also crucial for homeostasis of some other tissues, of which bone mass homeostasis is notable through control of osteoclastogenesis[70]. Another essential function the microbiota performs for the host is the development of vitamin B12, vitamin K, folate, biotin, thiamine, and riboflavin, mainly achieved by bifidobacterium. They also promote the ingestion of both dietary and fat-soluble vitamins through their influence on bile acids[66]. Bile acid signaling is a mechanism by which the gut-liver axis functions and the gut microbiota plays a crucial role in the host's bile acid pool homeostasis through its numerous bile salt-based enzymes[71]. The essential everyday microbiota task is to detoxify toxic bioactive compounds in the host body. It has been recognized that more than 40 bioactive molecules require direct modification by the microbiome in the body, including xenobiotics[72].

Moreover, the microbiota has enormous impacts on the metabolism of drugs. Perhaps significant explanations for the reported individual variations in reaction to drug therapy, which remind treatments toward to customized medicine. It has also been shown that healthy microbiota associates with several host pathways like the endocannabinoid system[7374].

1.2.4 Dysbiosis induced effects

Given the enormous contact between host and gut microbes ,and also the essential roles these species perform, it is apparent that disruption of this relationship, recognized as dysbiosis, will result in a multitude of pathological conditions/diseases in the host. A literature review reveals that dysbiosis is correlated with a growing array of illnesses that can be categorized as 'extra-intestinal' and 'gastrointestinal' conditions in two separate groups: extra-intestinal diseases, gastrointestinal disorders[75].

For extra-intestinal diseases, studies have shown that factors underlie all of those illnesses have a relationship with dysbiosis. Pathological bowel permeability and microbial impact on epigenetic regulators are among the pathways that could be implicated in many of them. Related diseases are: allergy, asthma, atherosclerosis, dermatitis, autism, cardiovascular disorders, cystic fibrosis, depression, diabetes mellitus, neurological disorders, obesity, Parkinson's, psoriatic arthritis, rheumatoid arthritis [76,77,78].

As for gastrointestinal disorders, it has been recognized that several GI diseases are correlated with an imbalanced partnership between intestinal microbiota and host. Among the significant ones: fatty liver disease, celiac disease, colon diseases, colorectal cancer, IBD, IBS, cirrhosis, necrotizing enterocolitis[79].

1.2.5 Dysbiosis

Antibiotics, xenometabolites, and others: A few hundred drugs have so far have shown to perform a substrate function for the metabolism cycle of microbial enzymes. All those xenometabolites that could attack both host cells and microbes might theoretically alter the composition of the microbial. Effects of antibiotics on short-term and long-term on alteration of gut microbiota have been studied for years. It should, therefore, be remembered that specific bacteria respond differently to

different antimicrobial treatment[80]. Besides, antibiotics and other xenobiotics can also affect the gene expression of gut microbiota[81].

Alcohol dependency can be correlated with altered structure and function of the intestinal microbiota. In addition, it has been shown that alcohol usage can enhance the Gram-negative bacteria, that can lead to the increase of endotoxin and acetaldehyde. In this case, acetaldehyde may improve intestinal permeability through specific alterations in close junctions and result in further endotoxin absorption. This alcohol-induced endotoxin could then be passed to the liver and circulation, leading to inflammation in many other organs[82].

Stress: Although most of the research involved in the study of the gut-brain axis is interested in the impact of microbes on stress and regulating it by bacteria-containing items such as probiotics, there is proof of a negative influence stress on the makeup of the gut microbiota from several studies [83,84,85].

1.3 Species commonly found in intestinal microbiota according to the taxonomic classification

Seven phyla (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, Verromicrobia, and Cyanobacteria-like) a phylum while being on Bacteria Domain (Euryarchaeota) is located in Archaea Domain[86]

Filum Firmicutes: generally consists of Gram-positive bacteria, and also certain types of gram-negative bacteria that have lipopolysaccharide[86].

Family Clostridiaceae (Clostridial Cluster I): None of the bacteria in this group use O2 as an electron acceptor for ATP production. While these species inhibit their reproduction in the presence of oxygen, they can start anaerobic reproduction. These bacteria are chemolithotrophic and organoheterotrofic. Among them, there are saccholytic species, proteolytic, peptidolytic, lipolytic species, and also species that break down organic salts[87].

A typical feature of these bacteria is from substrate substances forms various concentrations of acetic acid, lactic acid, ethanol, butyric acid, propanol and butanol. Their Deoxyribonucleic acid (DNA) G + C content is between 22-53%. Within this group; Spore forming Clostridium botulinum, Clostridium perfringens, and Clostridium tetani species are found. Except for the clostridia genus; Also, Eubacterium and Sarcina genus are also located in Cluster I[87].

Family Lachnospiraceae: In this family that forms the Clostridial XIV group include; Anaerostipes, Blautia, Butyrivibrio, Catonella, Clostridium, Coprococcus, Dorea, Eubacterium, Lachnospira, Johnsonella, Roseburia, Ruminococcus, ve Shuttleworthia species of the genus. These bacteria are all essential anaerobes and polysaccharylitic produce butyrate. DNA G + C content is between 29-51%[88].

Anaerostipes spp, as the main products of glucose, produce butyrate, acetate, and lactate and break up the acetate. Blautia species, on the other hand, after glucose metabolism produce acetate, ethanol, hydrogen, lactate, and succinate. Species belonging to the genus Clostridium, belonging to the Lachnospiraceae family (C. bolteae, C. clostridioforme, C. hathewayi, C. indolis, C. jejuense, C. nexile, C. saccharolyticum, C. scindens, C. sphenoides, C. symbiosum ve C. xylanolyticum) as breaking down different types of carbohydrates they form acetate, lactate, propionate, succinate, butyrate, ethanol, CO^2 and H^2. Dorea spp can form ethanol, format, acetate, CO^2 and H2 from glucose metabolism but they cannot create butyrate. In the genus Eubacterium (E. cellulosolvens, E. eligens, E. hallii, E. ramulus, E. rectale ve E. ventriosum) produce lactate as the main product of glucose metabolism. E. ramulus, by breaking down the flavon ring form acid and hydroxyphenyl probionic acid and hydroxy phenylacetic. Lachnospira species are primarily ferment pectin. Roseburia species, on the other hand, produce butyrates from carbohydrates and short chain fatty acids. Species belonging to the genus Ruminococcus (R. gnavus, R. lactaris, R. obeum, R. torques) produce lactic acid and acetic acid[86].

Family Ruminococcaceae (Clostridial clusters III and IV): Clostridial cluster III, Acetivibrio and some Clostridium species do not exist as a common group of bacteria in human intestinal microbiota[86].

Clostridial cluster IV (Clostridium leptum group) includes genus Anaerofilum, Anaerotruncus, Butyricoccus, Clostridium, Faecalibacterium, Flavonifractor, Pseudoflavonifractor, Ruminococcus, ve Subdoligranulum species. They are polysaccharidic commonly produce butyrate and mandatory anaerobes. DNA G + C contents are 27 to 60%. Anaerofilum type bacteria break down monosaccharides such as glaucous and xylose, and they form lactate, acetate, ethanol, and CO^2 as fermentation products. Anaerotruncus spp produces acetic acid and butyric acid from glucose metabolism. Species belonging to the genus Clostridium (C. leptum, C. methylpentosum, C. sporosphaeroides, and C. viride) have effects on different carbohydrates, and as a final product, they produce acetate, propionate, butyrate, propanol, CO^2, and H^2. Species belonging to the genus Eubacterium (E. desmolans and E. Siraeum) produce lactate, butyrate, ethanol, and / or H2. Faecalibacterium spp can form D-lactate, format, and butyrate and can break acetate. Species belonging to the genus Ruminococcus (R. albus, R. bromii, R. cellulosi, R. callidus and R. flavefaciens) as product of glucose metabolism form acetate, format, succinate ethanol, CO^2 and H^2 [86].

Family Veillonellaceae: Members of this family are Gram negative. In intestinal microbiota widely Genus Anaeroglobus, Dialister, Megasphaera, Mitsuokella, Selenomonas, and Veillonella can be observed. These bacteria can only ferment galactose and mannose and they produce acetate, propionate, isobutyrate, butyrate and isovalerate as products[89].

Family Peptostreptococcaceae (Clostridial cluster XI, Clostridium lituseburense grup): Within this group Genus Clostridium, Eubacterium, Filifactor, and Peptostreptococcus bacteria are included. The most important genus is Clostridium and includes C. bartlettii, C. bifermentans, Includes C. difficile, C. irregulare, C. lituseburense, and C. sordellii species. This group are saccharolitic and produces

acetate, butyrate, valerate, isovalerate from glucose metabolism. In addition, to a lesser extent produces propionate, ethanol, proponol, isobutanol and H^2 [86].

Family Eubacterium (Clostridial cluster XV, Eubacterium limosum group): In this group, Acetobacterium, Alkalibacter, Anaerofustis, Eubacterium, and Pseudoramibacter genus are located. The species belonging to the genus Eubacterium; E. aggregans, E. barkeri, E. callenderi, and E. coprostanoligenes. produce Butyrate, formate, lactate and / or hydrogen from glucose metabolism[86].

Family Erysipelotrichaceae (Clostridial cluster XVI, Clostridial cluster XVIII Clostridium ramosum group): This group includes the genus Clostridium, Erysipelothrix, Eubacterium, and Holdemania. They perform carbohydrate fermentation and can produce butyrate, lactate, acetate, CO^2, and H^2. For Clostridial cluster XVIII, Clostridium cocleatum, Clostridium ramosum, Clos- tridium saccharogumia, and Clostridium spiroforme species include. These bacteria form acetate, formate, lactate, and succinate[86].

Class Basilli: The most important team of this class is Lactobacillales. Includes Enterococcaceae, Lactobacillaceae, Leuconostocaceae, Streptococcaceae families, which are defined as Lactic acid bacteria. These are Gram-positive, non-spore-forming facultative anaerobic bacteria, DNA G + C is less than 50%, and forms to lactic with fermentation. This group of bacteria is often found in foods and beverages. A group Lactobacillaceae, which includes Lactobacillus and Pediococcus species. Lactobacillus spp. are mandatory saccharolytic. with glucose fermentation forms lactic acid, often, the end product was acetic acid in anaerobic environments. For Pediococcus spp, it can use different substrates, and the end product is usually lactic acid. The Bacillales team also includes the Bacillaceae and Staphylococcoccaceae families. Although a small part of Bacillus spp is mandatory anaerobic, most of them make aerobic or facultative anaerobic respiration. They are spore bacteria, and they reduce nitrate to nitrite. Exotoxin production is observed in pathogenic species[90].

Filum Bacteriodetes: Gram-negative, DNA G + C content is 28-61%, and the most important genus is Bacteroides[86].

Family Bacteroidaceae: Bacteroides spp are saccharilitic and have enzymes that break down complex sugars and polysaccharides to simple compounds that the host can use. Metabolic end products are acetate and succinate[91].

Family Prevotellaceae, Porphyromonadaceae ve Rikenellaceae: The species in Prevotellaceae and Porhyromonadaceae are mostly in the oral microbiota. In this group. Alistipes and Rikenella spp belonging to the family Rikenellaceae are found in the gastrointestinal tract. Alistipes spp is saccharilitic, and the main end product is succinic acid. Rikenella spp, on the other hand, are weaker sacrolytic bacteria and form acetate as the final product[86].

Phylum Actinobacteria: The vast majority of members are Gram-positive and have high DNA G + C (60-64%) content[86].

Family Bifidobacteriaceae: The most crucial type of intestinal microbiota for a human can be said Bifidobacterium spp. Generally found species; B. adolescentis, B. angulatum, B. bifidum, B. breve, B. catenulatum, B. longum, and B. Pseudocatenulatum. These bacteria can use many polysaccharides as substrates and form acetate and lactate as end products. They are also commonly found in the mouth microbiota[92].

Family Coriobacteriaceae: formed wit reclassification of some species of the genus Eubacterium. Species of this group in the gastrointestinal tract Collinsella, Coriobacterium, and Eggerthella spp. Usually, the end product of glucose metabolism they form acetate, lactate, and H^2 [86].

Phylum Euryarchaeota: This group of bacteria in the Archaea domain is a mandatory anaerobe and they are responsible for the formation of methane. This group of bacteria, in the human gastrointestinal tract They metabolize major fermentation products such as alcohol, KZYA, CO^2, and H^2. This type of bacteria are tiny in microbiota[93].

Phylum Fusobacteria: Important families in this phylum are Fusobacteriaceae. Peptone and glucose energy source and creates butyric acid as the final product. Fusobacterium nucleatum and Fusobacterium necrophorum in this group are also responsible for endotoxin formation[86].

Phylum Verrumicrobia: In fact, the bacteria contained in this phylum are usually marine bacteria. But in recent years, human intestine of Akkermansia muciniphila species in studies on microbiota reveals that it is an essential bacteria in the flora. It is c chemoorganotrophic bacteria and can use mucin as a source of carbon, energy, and nitrogen. Also forms sulfate from mucin fermentation[94].

Phylum Proteobacteria: Class Delta Proteobacteria involves Desulfovibrio spp (D. desulfuricans subsp. desulfuricans, D. fairfieldensis, belonging to the family D. piger, and D. vulgaris) and Bilophila spp which are belonging to the family Desulfovibrionaceae which produces H^2S in the gastrointestinal tract of intestinal microbiota Desulfovibrio spp (D. desulfuricans subsp. desulfuricans, D. Fairfieldensis.These bacteria that reduce sulfate are essential anaerobic bacteria. They can use many different substrates for sulfate reduction reactions. These bacteria accomplihes oxidation of sulfur compounds SO^4 and SO^3, mostly found in foods and beverages as preservatives. Bilophila spp nonsaccorilitic bacteria and acetate are their main end product[95].

Class Gamma Proteobacteria: In this group, Cronobacter, Enterobacter, Escherichia, Klebsiella, Salmonella, Shigella, and Yersinia species are found and belongs to Enterobacteriaceae family. This group members are generally facultative anaerobic bacteria and are usually pathogens[86].

Class Epsilon Proteobacteria: Campylobacter spp and Helicobacter spp species are also included in this group[86].

1.4 From Microbiome to Intestinal Integrity and Mucosal Immunology

1.4.1 Intestine

Gastrointestinal (GI) is among the body's external barrier provides the largest environment. Small intestine for humans; has 6-7 m, and the colon has 1.5 m with wider in diameter. The surface of the intestine is approximately equivalent 32 m2 and every day, is exposed to 1 or 2 kg of nutrient, process 45 tons of nutrients from beginning to death. In the small intestine, time of passage took 5 h and 20 h in the colon. Every 3 to 4 days, the epithelium tissue of GI is regenerated completely[96].

The small intestine can be investigated in three parts: duodenum, jejunum, and ileum. Absorption of nutrients and control of the entry of harmful contents is acquired at the luminal surface. As for the mucosal surface, the small intestine has villi and extends to the lumen and increases the area. Villi is not observed in the cecum and the colon. For colon, it starts right after the small intestine at the cecum, followed by the ascending colon, then lastly, the rectum that terminates at the anus[96].

1.4.1.1 Intestinal epithelial cells

Epithelial cells, forms not only physical barrier but also a functional immunological barrier. Crypt stem cells give rise to several mature intestinal epithelial cells (IEC), such as goblet cells, microfold cells (M cells), enterocytes, Paneth cells, and enteroendocrine cells[97].

Enterocytes regulates the absorption of nutrient for circulation. The goblet cells produce the mucus layer that generates certain type of protection as inhibiting bacteria into or towards to epithelial cells. The Paneth cells are especially found in the ileum distal part and provide host defense against microbes and function like neutrophils. If they are exposed to bacteria or bacterial antigens, or even constitutively secretes several antimicrobial peptides into the crypt lumen. Also, M

cells are seen at organized lymphoid tissue like Peyer's patches in the small intestine. M cells transport antigens from the lumen to dendritic cells (DC) and lymphocytes. In this way, the adaptive immune system is informed[96].

Protective gel-like molecules called mucus and overlay the epithelium secreted by goblet cells consist of two layers; outer and inner. Bacterias are usually found in the outer part and could not penetrate. The most important thing for this protection mechanism is that purity of the inner mucus layer is relies on amount and variety of antimicrobial peptides. If there is dysregulation of antimicrobial peptide expression; penetration of bacteria into the epithelial surface can be possible and generate many physiological dysfunctions and cause susceptibility to colitis and colorectal cancer.[98]

1.4.1.2 Pattern recognition receptors

The epithelial cells have huge roles for innate immunity; these cells express different pattern recognition receptors (PRR): Nucleotide-binding oligomerization domain 2 (NOD2) and Toll-like receptors (TLR). NOD2 is mainly expressed by epithelial cells in the ileum ex. the Paneth cells [99]

In fact, TLR expression distribution can change along the intestine, as TLR2 is found generally in the proximal part of the colon and declines distally down the colon. For CD14 and TLR4, amount of expression by epithelial cells in the colon is high than in the small intestine. However, the expression of dysregulated TLR4 and CD14 in intestinal can generate activation of the mucosal innate immune system and might cause inflammatory bowel disease (IBD) pathogenesis.[100]

After PRR binding, secretion of chemokines, pro-inflammatory cytokines (interleukin (IL)-1, IL-6, interferon (IFN) gamma, tumor necrosis factor (TNF), and AMPS; (HD)-5 and HD-6 by Paneth cells triggered[96].

1.4.1.3 Epithelial integrity

A selective physical barrier is formed by epithelium as limiting the permeation of proinflammatory signals and at the same time, absorbing water and nutrients both the paracellular passage of molecules and transcellular way. Also, as mentioned, epithelial cells are also responsible for immunity: ex. PRR and secretions of antimicrobial peptides[97].

Cell junctions have a crucial role in forming physical barriers: tight junctions (TJs), adherens junctions (AJs), and desmosomes. TJ complexes regulate paracellular permeability, which forms a continuous band that encircles the epithelial cells and forms intercellular interactions[101].

AJs bind cells by bridging neighboring cells' cytoskeletons by interactions with cadherin (transmembrane), catenin (intracellular construction), and the cytoskeleton. Cadherin-catenin interactions generate a powerful adhesive impact between cells in addition forms to cell polarity, regulates proliferation, and migration[102].

1.4.1.4 Tight junctions and their molecular composition

TJs are composed of transmembrane proteins, and intracellular proteins, and these proteins are highly dynamic. They form highly selective or semipermeable for ions solutes, water for their size, and charge. It also inhibits pathogens, toxins, and antigens as pro-inflammatory agents. There are two distinct TJ paracellular pathways; a CLDN dependent restrictive porous path for the flow of tiny and ionic molecules and an occludin-dependent wide channel pathway responsible for the flow of macromolecules[103].

As forming homophilic and heterophilic interactions, occludin, claudin, and junctional adhesion molecule (JAM) transmembrane proteins form the barrier through intersecting points in intercellular space[104]. Intracellular domains interact with intracellular proteins, thereby anchoring to the actomyosin ring (a protein

complex made up of actin and myosin molecules). Myosin light chain (MLC) phosphorylation/dephosphorylation, and the intrinsic cloudin structure are important physiological features that control paracellular permeability. It is crucial that occludin amount contributes to the number of strands in the paracellular space[101105].

MLC behavior is regulated for the contraction of the actomyosin chain. Indeed, MLC phosphorylation by MLC kinase induces actomyosin ring contraction, thus as opening the paracellular pathway and permeability might be increased in this way.

For the IEC cell membrane, there are two domains; apical and basolateral domains. With certain physiological circumstances, TJs serve as a barrier to prevent the proteins from intermingling from the apical portion of the cell to the basolateral section, while preserving cell polarity[106].

1.4.1.4.1 Occludin

It is a transmembrane protein consist of two extracellular and intracellular domains. The intracellular part interacts with ZO1 and connects occludin to the cytoskeleton. Occludine deletion leads to the enhanced flux of macromolecules in the paracellular system and triggers an inflammatory reaction. On the other hand, the deletion of occludin only had a marginal impact on smaller molecular fluxes. Several researches reported reduced production of intestinal occludin in many clinical disorders, including IBD, CC, and GVHD.

1.4.1.4.2 The claudin family

Claudin (CLDN) are transmembrane structures that has two extracellular loops forming homophilic and heterophilic communication with adherent epithelial cells. Claudins can be investigated in two groups: barrier-forming (tight epithelia) and channel-forming (leaky epithelia). For leaky epithelium, we can say that the paracellular pathway is much ion conducive than the transcellular way. As for

channel-forming ones, they exhibit different selectivity types, i.e., for cations, for anions, or for water[107].

Overexpression studies of CLDN2 have shown that increased paracellular permeability as a way of enhancing the pore numbers without changing the selectivity of the charge; on the other hand, overexpression of CLDN4 affects the charge selectivity without changing the pore numbers. Using knockout mouse models, a tightness is shown in different epithelia for several claudins (e.g., CLDN1 and CLDN4)[97].

The tightness of the IECs is affected by the combination and numbers of the CLDN connected at the same strand. Also, changes in number of TJ correlated with an enhenced transepithelial electrical resistance value and declined permeability; however, for certainity, it is difficult predict from the total amounts of CLDNs expressed by the cells to number of TJ strands[104].

Inflammation affects the CLDN protein expressions, as seen in inflammation in the intestine like IBD and collagenous colitis. Colonic tissue from IBD patients with active disease, but not in those with inactive it was shown that levels of CLDN1 and CLDN2 proteins increased. In contrast, there is no correlation with CLDN4 - expression with disease condition[108].

1.4.1.4.3 Junctional adhesion molecule

JAMs are transmembrane proteins that support to epithelial integrity via intercellular interactions as spanning across the cell membrane. Studies conducted on JAM-A-deficient epithelial cells have shown that enhanced permeability. Besides, it is demonstrated that JAM-A has role for the regulation of intestinal permeability to both smaller and larger molecules. Increased levels of B and T lymphocytes as well as polymorphonuclear leukocytes (PMN) are observed in JAM-A knockout mice studies. Moreover, in IBD, decreased colonic expression of JAM-A has been observed[109,104].

1.4.1.4.4 Intracellular molecules

The transmembrane proteins' intracellular domains associate with intracellular scaffolds proteins like ZO and interact with actomyosin ring. Interactions between transmembrane protein and actomyosin ring form cytoskeletal control of epithelial integrity. Consequently, the actomyosin ring's contraction and tension acquired by MLC activity result in increased paracellular permeability[101].

Intracellular TJ proteins are classified roughly into two groups: PDZ domain-containing and non-PDZ domain-containing proteins. These non-PDZ proteins are mainly involved in signaling modulation and are not explicitly related to the essential structural elements. However, many intracellular TJ-related proteins have one or multiple PDZ domains[110].

ZO-1 was the first identified intracellular TJ specific protein. It has three PDZ domains, an SH3 domain, and also guanylate kinase domain. Also, it is a scaffolding protein for membrane-associated guanylate kinase family. ZO-1 interacts with CLDNs and occludin like TJ-related transmembrane proteins. For ZO-1,the carboxyl-terminal region interacts with the actomyosin ring. Besides, ZO-1 has a huge role as being at the center of a protein interaction network. Interaction between the ZO-1 and the CLDNs will play a significant role in assembling the TJ strands[111].

PARD3 and the membrane-associated guanylate kinase inverted proteins can be given as an example of other intracellular scaffolding proteins. Those proteins interact with intracellular signaling proteins like protein kinases and PTEN and, as this way, have a role in the regulate cell polarization and Akt signaling[112].

1.4.1.5 Disrupted barrier function and disease development

Disruption vascular barrier is associated with increased permeation of certain molecules, and initiates activation of the immune system, intestinal inflammation, and tissue specific damage. The dysfunctional barrier integrity can generate several

inflammatory conditions, like IBD, colitis, GVHD, and celiac disease, obesity, and also diabetes[113114]. However, whether the inflammatory process is a consequence of the enhanced permeability or whether the increased permeability is a consequence of the inflammatory process remains unclear [108].

In certain studies, inflammatory cytokines changed the expression of TJ-related proteins and epithelial integrity. Studies have also demonstrated roles of non-coding RNAs in regulating and maintaining TJ structures also[115].

1.5 Host-Microbe Interactions

We are released from the womb's safe atmosphere into a planet loaded with microbes, and interactions with them can be either transiently or permanently. In response to different host-microbiome and microbiome interactions at epithelial niches, the sub-populations of microbes are formed. The impact that the microbiome exerts on us depends on the adaptation approach the microbes can acquire. We co-exist in a natural environment in a mutualistic and advantageous interaction, where the microbiota will extend our genome and provide us with inherent plasticity and characteristics that have not developed in humans. The microbiome, for example, gives us several vitamins and essential amino acids. It also enables metabolization of harmful xenobiotics and digests potentially unavailable polysaccharides into useable short-chain fatty acids. Thus, the microbiome makes us avoid pathogens by strengthening our immune response and creating an inhospitable, energetically arid environment for pathogen invasions. In the case of pathogens, though, the microbes can prefer a colonization strategy that is harmful to us and may contribute to illness. The overall result on how we get influenced by pathogenic invasion is heavily contingent on our immune status[116].

A weakened immunity response is a prime target for pathogens, while hyperactivation of immunity causes sepsis or severe influenza may also damage health. In addition, microbial influences can also determine the outcome of microbial

invasion, which can be explained by the virulence factors for pathogens. These factors help the microbe adapt to the host environment, Inhibit host defenses, and encourage host transmission. Furthermore, genetic or environmental features for the host such as nutrition, exercise, or antibiotic usage may disrupt the fragile host-microbe balance[117].

It may contribute to altered microbiome composition with consequences for several diseases. For example, mice that are defective in a microbial sensors of innate immunity (Toll-like receptor 5) exhibit deviations in gut microflora composition and metabolic syndrome symptoms. Ironically, these effects are transferable from those knockout animals to wild-type germ-free animals by inoculating them with the altered gut flora[118].

In contrast, gut microflora transfer from lean human donors to patients with metabolic syndrome can lead to several symptoms being alleviated[119]. IBD and diabetes are often linked with microbiota dysregulation, which usually due to immunological or dietary changes. This also reinforces the belief that preserving an optimum microbiota contributes significantly to well-being and protecting against infection[120].

1.5.1 Innate Immunity

Without clinical symptoms, microbiota regulation, and the pathogens access inhibition acquired in normal conditions. But in other conditions, defense response is required. Acute inflammation condition; the organism responds to stimuli from pathogens and/or harmful endogenous factors (disease or tissue injury), with strong immune activation. The non-specific, or inherent, protection has no previous knowledge of the danger to work; instead, it reacts to large groups of threats in a pre-programmed manner. The innate responses are fast-acting relative to the adaptive, or antigen-specific reaction assembled days later (1.5 h for recruiting neutrophils to the site). Lagging time behind a replicating microbe for innate responses might take 20

minutes or less generation duration. During this time, defense mechanisms are already present at the infection site to maintain the defenses and support the infiltration of immune cells. Normally, without the requisition of the innate effector cells or the adaptive immune response, these protections will clear an infection as evolutionary immune system factors[121].

The innate immune system is an evolved ancient defensive mechanism, and one of the evolutionary reasons is for the battle between host and microbe for the same resources. Unicellular species have already evolved defensive mechanisms for sensing and fending off infectious pathogens. However, pathways for separating self from non-self emerged with the emergence of multicellular species and their related microbiome's inherent immunity. Distinguishing commensal microbes from pathogens and having the ability to check healthy host cells from a damaged one became also important[122].

An efficient immune system is might be the most important thing for the existence of a multicellular organism in order to deal with successful community formation ability of competitive microbes and their ability to evolve. It might be reason evolutionarily for the main contributor to insect, fungal, and invertebrate immune response and serves as the first line of protection in vertebrates[123].

1.5.1.1 Innate immune system function

With the help of infiltrating or resident cells like monocytes, macrophages, and neutrophils (phagocytic cells), and also epithelial innate immune response operates Chemical, anatomical, microbiological, mechanical, and immunological factors form a barrier to infection at this host-microbial interface[116].

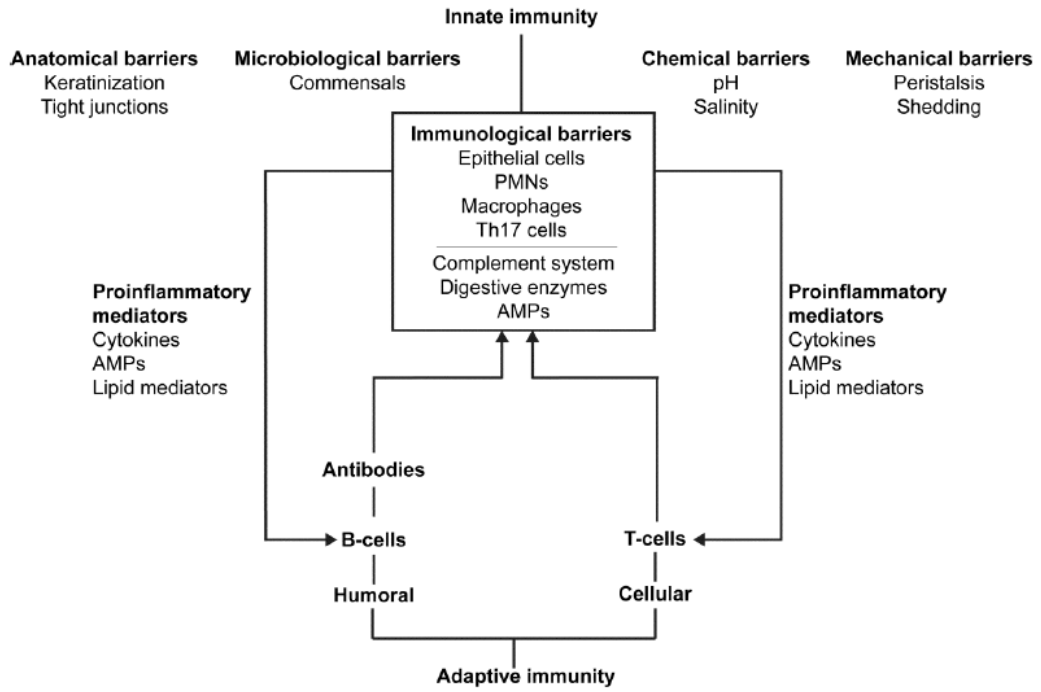

Figure 2: Mammalian barrier defenses. In order to achieve microbial clearance, several barrier functions has roles and cooperate. AMPs act explicitly as antibiotic and indirectly as modulate the immune response to recruit and stimulate innate and adaptive immune cells (Adapted from Cederlund, 2012).

The anatomical barrier to infections is acquired by mechanisms via tight junctions and keratinization and which compartmentalize sensitive tissues in efficiently sealed epithelial layers. The mechanical barrier is acquired by shedding of cells, cilia movement, and peristalsis, which expels unwanted colonizers. Secretion of mucus, saliva, and tears containing substances inhibiting the proliferation and spread of microbes provides the chemical barrier. The microbiological barrier consists of the bacteria that already occupy our bodies, creating an inhospitable atmosphere for microbial invasions[116].

Normally pathogens can not infiltrate the epithelial lining's integrated defenses, since these cells have an essential intrinsic immunological shield via the production of AMPs. However, pathogen virulence factors or tissue damage may allow microbes to penetrate the epithelial barriers. In that situation, the microbes will face resident neutrophils, macrophages, dendritic cells, or complement system molecules

that may neutralize the microorganism. And if the microbe evades, more innate immune responses are generated via pattern recognition receptors expressed on epithelial cells, macrophages, and dendritic cells[123].

Activating pattern recognition receptors or signals from compromised cells may result in a cascade of responses that eventually contribute to immune gene activation, antimicrobial peptide secretion, and proinflammatory cytokine activation. It would result in inflammation, mobilization, and release of neutrophils accompanied by a fast flood of circulating macrophages and lymphocytes, phagocyting the pathogen. In fact, phagocytic cells may also express antimicrobial molecules for the extracellular killing of pathogens in combination with epithelial cells of the infected region. Eventually, inputs from the innate immune system can activate further adaptive immune responses. While the innate immune system's responses are encoded as germline and pre-programmed, their cells can still guide and customize the adaptive immune responses to large groups of pathogens through the secretion of different cytokines[123].

1.5.1.2 Cells of innate immunity

1.5.1.2.1 Epithelial Cells with Immunological Roles

The epithelial cells have important contributional roles on the immune protection and control of the microbiota through the antimicrobial secretion components, including AMPs and mucins, but not exclusively immune cells. Furthermore, epithelial cells function as sentinel cells by expressing receptors for the identification of surface and cytoplasmic patterns. The epithelial cells can also serve as barrier protective orchestrators. This is accomplished by integrating epithelial cells with antigen-presenting cells (APCs) or lymphocytes accompanied by cytokine secretion, by recruiting cells from both the innate and adaptive immune systems[124,125,126].

1.5.1.2.2 Granulocytes

Three types of granulocytes: neutrophils, eosinophils, and basophils. The most concentrated of leucocytes in peripheral blood are polymorphonuclear neutrophils (PMNs), makes up 40%-70% of all leukocytes. The granules carry components of microbicides like antimicrobials, Polypeptides, proteases, and reactive oxygen-generating enzymes[127].

Chemotactic cues from the epithelia and native macrophages attract the PMNs to an infection location. Extravasate from the blood to site by microvessel endothelium in inflamed tissues. They are also the first immune cell to enter an infection site. PMNs spill their granules into a distinct order during the process. PMNs also have phagocytic abilities. As this way, engulf and degrade opsonized and non-opsonized microorganisms by both oxygen-dependent or independent mechanisms in phagolysosomes[127,128].

Oxygen-independent pathways digestive enzymes and/or antimicrobial polypeptides are generally used. Besides, PMNs can also undergo NETosis: releasing extracellular neutrophil traps, or NETs consisting of nuclear or mitochondrial chromatin and DNA laden with microbicidal molecules capable of catching and destroying extracellular pathogens[129].

1.5.1.2.3 Macrophages

Macrophages can frequently found in connective tissues, liver, heart, and scalp, and are generally initial immune cells to experience an infecting microbe. In addition, macrophages circulate, which can migrate to an infection site. Like another phagocytes, the macrophage surface includes receptors that detect microbes or cell debris[126].

Unlike the PMNs, macrophages function like phagocytes engulfing and digesting cell debris or pathogens in their phagolysosomes. They also orchestrate the

innate and adaptive immune responses through the presenting of antigens, activates lymphocyte, and other immune cells through the release of cytokines and inflammatory lipid mediators[123].

1.5.1.2.4 T-helper 17 cells

Th17 cells are a subset of T-helper cells, which produce the transcription factor ROR-gamma (retinoic acid receptor (RAR)-related orphan receptor-gamma) and IL17 and IL22 secretions[130]. Different form Th1 and Th2 cells, and are essential mucosal innate immunity orchestrators along with macrophages. They recruit neutrophils and induce epithelial expression of AMP[131].

Innate defense against infection is aided by additional cells also. These encompass the cytotoxic natural killer cells (NK) that function on virally infected or tumor cells, and mast cells, which have roles in inflammatory reactions. The antigen-presenting dendritic cells functions as sentinel cells, molecular sampling of infection-related molecules at the site of infection by pattern recognition receptor. On recognition of molecules, dendritic cells migrate to the lymph nodes to present adaptive immune cells (B- and T-cells)[123].

1.5.1.2.5 Receptors of innate immunity

Pattern recognition receptors (PRRs) recognize pathogenic or damage-associated molecular signals. PRRs covers also Toll-like receptors (TLR) (found in the surface endosome and lysosomal) and NOD-like receptors (NLRs))found in cytosol). Pathogen-associated molecular patterns (PAMPs) are also recognized by PRRs, generally structurally conserved molecular sets that are distinct and usually important to the particular microbes. Lipopolysaccharide, lipoteichoic acid, unmethylated CpG DNA, double-stranded RNA, peptidoglycan, and specific structural motifs specific for microbes subtypes are all example PAMPs and all of

them recognized by PRRs. In addition, PRRs may also identify molecular damage associated patterns (DAMPs) or alarmins (ex. high-mobility protein B1 (HMGB1), self-DNA, calprotectin, and heat shock proteins) which are warning signals for aberrant tissue conditions in the host by detecting different [132,133].

PAMPs and DAMPs triggers PRRs and activates proinflammatory mitogen-activated protein kinase (MAPK) signaling and activate nuclear factor kappa light chain-enhancer of activated B cells (NF-kB) or interferon regulatory factor 3 (IRF-3). It will result in enhanced chemokine and cytokine production that can trigger immune cells, eliciting systemic responses like a fever. Also, receptor activation can result in the release of microbicidal compounds, such as reactive oxygen species and AMPs. As expected, PRRs are essential to recognizing signatures coming from pathogens or damaged tissue, and hence the innate immune response. PRR mutations result in hyporesponsiveness against a wide variety of pathogens with enhanced vulnerability to infection. In one study, it is shown that NOD-2 mutations reduce defensin production and are correlated with Crohn's disease[134–136].

1.5.1.3 Endogenous regulators of innate immunity

Most molecules have a role in the regulation of the innate immune response. Cytokines comprise one source, a heterogeneous collection of proteins produced and secreted by many forms of cells. Cytokines have roles both systemically and locally inflammatory responses. They act on receptors, usually G-coupled with proteins, and trigger different responses in the intended cells (ex. chemotaxis or proliferation). Based on the sort of immune reaction they generate, cytokines may be subdivided into two groups. Type 1 cytokines are related to innate immune processes, mediating inflammatory responses, and are typically generated in response to pathogenic stimuli by phagocytes, dendritic cells, endothelial or epithelial cells. Type 2 cytokines are primarily produced in response to specific antigenic stimuli by T-cells and NK-cells, and primarily invoke humoral immune responses. Alarmins are another category of molecules that can trigger both the innate and adaptive immune

systems in response to damage or infection. HMGB1 and AMPs like defensins, cathelicidins, and azurocidin can be given as an example[137,138].

Hormones can serve as immunomodulators, too. Many inflammatory disorders correlate with puberty-present inherent immunity. Include lupus erythematosus, rheumatoid arthritis, the syndrome of Sjögren, and spondylitis ankylosing. Many hormones found to modulate the immune response include insulin-like growth factor-1, prolactin, thyroid hormones, and anti-inflammatory glucocorticoids[139,140].

1.5.2 Adaptive Immunity

The continuous evolution and proliferation of pathogens make multicellular organisms to evolve from the innate immune systems to additional adaptive immune responses. Because adaptive immune evolved from the innate immune system and is dependent on several innate immune functioning and their cells. The adaptive immune system generally relies on the innate immunity to recognize and hold attacking pathogens until 3-5 days after infection while triggering and running the adaptive immune system. The adaptive immune system relies primarily on the B- and T-cells, whose recruitment and selective expansion are guided by antigen-presenting cells like macrophages and dendritic cells[141].

The type of microbial recognition receptors are an essential distinction between the innate and adaptive immunity. The innate system uses on a germline encoding receptors with broad specificities. On the other hand, the adaptive immune system produces a significant number of antigen receptors adapted to the identification of extremely specific epitopes[123].

Adaptive immune receptors are quickly modified by somatic mutation to the rapidly emerging pathogens. The adaptive immune receptor gene fragments are permutated through gene splicing known as somatic recombination. Such recombination produces a large and complex immune cell community of clonally

distributed immune receptors. This group of cells is then purged through the method of clonal deletion from the cells containing auto-reactive receptors. The remaining immune cells are released into circulation and clonally enlarged in reaction to the identification of antigen. Additionally, identification of an antigen may contribute to the development of memory cells, which grant an immunological memory of past infections. In addition, these cells can build both a quicker and better response in the event of re-infection[116].

1.6 Antimicrobial Peptides

Antimicrobial peptides and proteins are gene-encoded crucial effector and modulatory molecules of innate immunity. They are found at all developmental periods; in the cells and tissues of the uterus, fetus, and the neonate. The presence of all developmental periods implicates their crucial role in immunity during pregnancy and in early development[142].

These molecules are also almost found in all living organisms, including bacteria, vertabrate, plants, and invertebrates. Such a way of defense is one of the ancient ways of controlling or killing unwanted microbes before adaptive immunity evolved. To date, more than 2000 unique AMPs have been described and are placed in the database of antimicrobial peptides[143].

1.6.1 Structure of AMPs

Members of AMP family peptides are various molecules that exhibit significant variability in the composition of amino acids, physical structure, and distribution of tissue between species. The structure and distribution heterogeneity of interspecies is likely to indicate the differential competitive pressures that the host puts on microbes by the host. Combinations of AMPs jointly display a wide variety of behavior against bacteria, fungi, protozoa, and other viruses. On the other hand, if

we focus on any one defined AMP, it can be realized a microbicidal activity to subsets of microbes more specific[144].

For their structural characteristics, AMPs range in length between 5-60 amino acid residues and generally ~30 long residues[145]. They are produced by proteolytic cleavage with or without antimicrobial activity from larger precursor proteins[146]. AMPs bear a high proportion of residues of cationic amino acids interspersed with hydrophobic residues and follow a cationic secondary amphipathic structure[147]. Notably, observations of anionic AMPs, e.g., dermcidin, are often suspected to be involved in the membrane by receptor contacts, ionic bridges, or internal targets[148].

Based on their primary and secondary structures; cationic AMPs may be subdivided. The prevalent secondary structures of AMPs are antiparallel beta-sheets, alpha helically folded peptides, and also peptides enriched in particular amino acids such as arginine, glycine, proline, or phenylalanininine. AMPs' cationic effects also increases their solubility in aqueous fluids as enhancing their bioavailability[116].

1.6.2 Expression and regulation AMPs

AMPs are produced constitutively (ex. by epithelial) or regulated when epithelial or immune cells are subjected to bacteria. As for granulocytes, AMPs are contained in ready-to-secrete intracellular granules in response to stimuli[149]. The distribution and expression rates of AMPs inside a given tissue are extremely niche specific[150]. As an example; human alpha-defensins are expressed primarily in PMNs or Paneth cells, but the beta-defensins are in epithelial cells[151].

Generally, secreation of AMPs are not independent, but instead as a mixture of multiple tissues- and context-dependent co-regulated or co-located AMPs, working in conjunction to achieve optimum microbial killing or shaping establishment of commercial certain species[149].

Many conditions such as microbial elements, pro-inflammatory triggers, hypoxia, or tissue damage may cause the release of AMPs or are either constitutively

secreted. In addition, sodium butyrate and vitamin D may either promote or inhibit the production and release of AMPs, some of which might be suitable for potential medicinal usage[152].

1.6.3 Mode of microbicidal action

Specific AMPs kill microorganisms through a variety of mechanisms. Four kinds of action can be mentioned here (Figure 3): First one, AMPs impede the biosynthesis of certain intracellular or extracellular biomolecules like: lipoteichoic acid, nucleic acids, and glycopeptides, then block cell wall synthesis and contribute to metabolism abnormalities that eventually cause the death of microorganisms. The second way, AMPs improve the K^+ efflux in cytoplasm and mitochondria and the measurement of cellular Ca_2^+. Accumulation of Ca_2^+ results in disruption of the Ca_2^+ homeostasis, followed by the generation of ROS. Disruption of homeostasis and ROS production in Ca_2^+ contributes to apoptosis in cells. Third way, by disrupting ATP synthase activities or blocking the electron transfer chain, AMPs make ATP synthesis inhibition. Carbon metabolism is also disrupted.

In addition, AMPs reduce ATP-dependent enzyme function by direct contact with ATP. The cellular ATP-dependent processes are either disrupted by energy metabolism or damage to enzyme activity. As the fourth way, AMPs interface explicitly with microbial membranes, the interaction causes fluctuation of membranes and broad transmembrane pores, or induces depolarization of membranes by killing the membrane capacity. Furthermore, AMPs may associate with essential membrane proteins, such as certain proteins and transporters from outside participants. Both AMPs-membrane interactions induce membrane instability and ultimately contribute to cell death. It is suggested through the electrostatic attraction of AMPs to the electronegative components of the microbial surface. Due to anionic molecules, and/or phospholipids (ex. LPS, teichoic or lipoteichoic acid), microbial membrane is generally negatively charged. That is compared to the eukaryotic cell membranes, with the addition of zwitterionic

phospholipids and cholesterol bearing a more balanced charging condition. When integrated into the lipid bilayer after an AMP has been electrostatically attracted to the membrane lipids, destabilizing the membrane of the microorganism. Ionic gradient and osmotic potential of membrane decreases, as this way, impede the respiration of the microbe. There are currently four mechanisms suggested for the antimicrobial activity of AMPs that rely on the membrane. The carpet model, barrel-stave model, toroidal pore model, and aggregate model (Figure 3)[147,146,153,154].

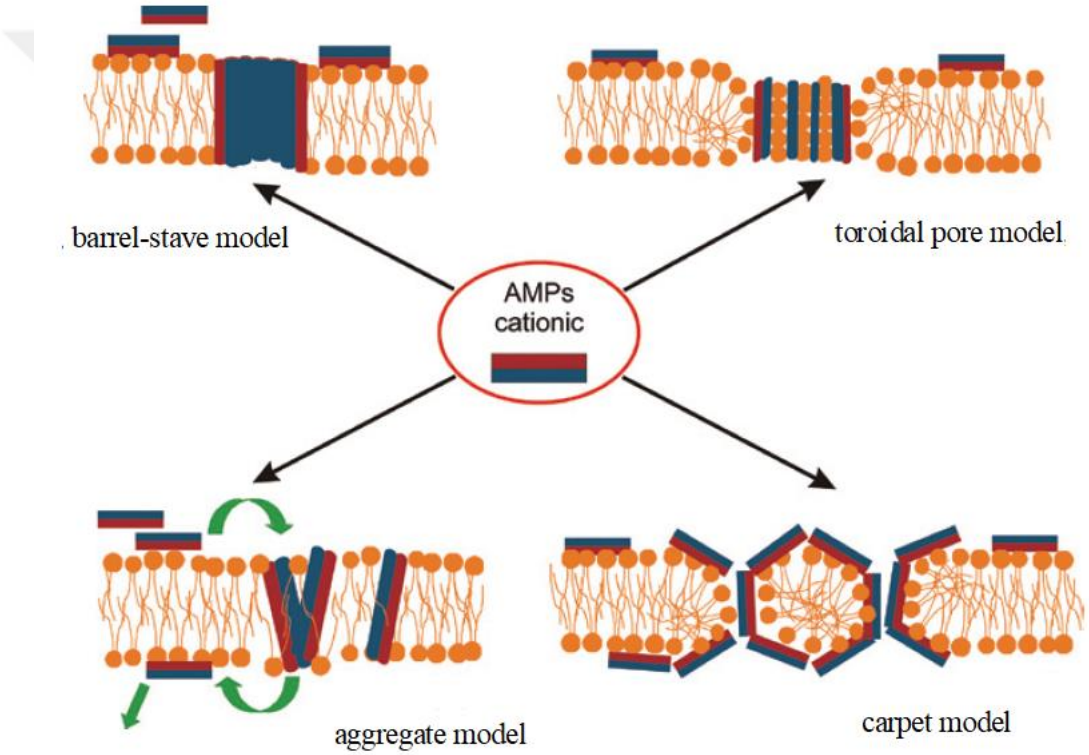

Figure 3: Four basic models of AMPs interaction with a microbial membrane

1.6.4 Additional activities of AMPs

AMPs have been the principal mode of protection against pathogens throughout evolutionary history. Nevertheless, AMPs have taken on new functions in the immune system, with the growing importance of immune defense. Earlier

studies have shown that AMPs can directly microbicidal and deletions of specific one enhances infection susceptibility[155].

With recent studies, however, suggest that AMPs should be seen as more broad-based, rather than host defense; there are findings that the antimicrobial actions of a variety of AMPs are either blocked at physiological concentrations of salts, divalent cations, and serum lipoproteins, but these peptides can still be known to be active in vivo in inhibiting microbial infections. The reason might be because AMPs are not typically secreted as separate bodies; there would be a synergistic impact on the microbicidal activity. Besides, the deletion of one AMP causes impaired synergism and, as a result, increased susceptibility to infection[156].

AMPs are not only simple antimicrobials but modulates both adaptive and innate immune cells. Several AMPs have been shown to act as pro-inflammatory response modulators or cell differentiation and proliferation modulators for macrophage and leukocyte. The can modulate the expression of chemokines, reactive oxygen species, and reactive nitrogen species. Other AMPs were shown as chemoattractants for immune cells, cytokine expression inducers, or T- and dendritic cell response regulators[157–161].

It has also been shown that AMPs, by either binding to key inborn receptor ligands, inhibiting receptor-mediated signaling, or directly interacting with its cognate receptor, attenuate tissue and context-dependent proinflammatory responses. Also, with different study groups, it has been shown that AMPs can stimulate angiogenesis and also can enhance wound healing and also controlling symbiotic bacterial population[162].

1.6.5 One more additional activity: controlling the symbiotic bacterial population

For well over three decades, antimicrobial peptides (AMPs) have been identified with their ability to eliminate pathogens or essential mediators of the innate

immune response in organisms. In addition, with recent studies, it is found that AMPs also have a role during symbiotic bacterial communication as a controlling way of communication by hosts. Interestingly, AMPs target the microbiome and have a crucial biological role as not disturbing microbial symbiont species but making them in check. AMPs and the adaptations of symbionts to are an evolutionary careful balance. As this way, establishment of the host-microbe homeostasis is formed[163].

For long time explorations on host-microbiome, we don't have effective specific targets on specific individual species. However, AMPs secreted from epithelial cells of intestine not only have protection role on the host against pathogens but also shape and control the composition of the intestinal microbiota. With different study groups, it is realized that one of the possible central roles for AMPs might be determining the composition of microbiome[144].

From animal studies, we know that expression intensity of α-defensin family AMPs dramatically affects the composition of the community. Studies in this field also have found that, interestingly, commensal bacteria were resistant inflammation-associated AMPs. It is identified that mechanism for AMPs' resistance gained via lipopolysaccharide (LPS) modification in the phylum Bacteroidetes[162].

AMP family member proteins account for specific bacterial communities and shape species-specific communities. For example, AMP C16G2 was able to selectively target for pathogen Streptococcus mutants in vitro oral condition. In addition, one research group has discovered R-Spondin 1 molecule that induces intestinal stem cells to change into Paneth cells to secrete a variety of AMPs, which has specific antimicrobial activities against pathological species and do not affect on symbiotic ones[164].

Generally, AMPs are not effective in each one of them only. A combination of AMPs and their harmony determines the elemental integrity of the bacterial species and their organization. There are exciting conditions observed in the symbiosis of legume plants with nitrogen-fixing rhizobium bacteria, in which AMPs

forces the bacteria into a terminally segregated state and manipulates the symbiont physiology to optimize the gain to the host. These peptides are used as instruments for enslaving symbionts, controlling their reproduction and also keeping them in check for their metabolic activity for nitrogen fixation[144,163].

In addition, at insects and protists: for symbiotic nutritional interactions, AMPs can promote metabolite movement through the symbiont via membrane permeabilization and generate usage of molecules from symbionts[144,163].

1.6.6 Neonatal Host Defence

Neonate meets a new environment after birth: from the highly sterile womb environment to the world of a fully microbial environment. Not only the environment but also the sterile source of nourishment is changed from sterile umbilical cord to a non-sterile from breast milk. At the beginning and following months, the neonatal adaptive immune system is very naive. While time passing, the adaptive immune system maturates, but before this process, neonates rely on, with the exemption of maternal immunoglobulins, mostly on innate immune factors, e.g., complement system, NK-cells, phagocytes, AMPs, APCs for the defense against infections[165,166,167–169].

At birth, the vernix caseosa covers neonate that includes AMPs and microbicidal lipids that protect the skin from unwarranted colonization. In addition, neonatal skin starts to increase the expression of AMPs[170,171]. Innate immunity of the gut are getting stronger in the first weeks of life to adapt and develop immune functioning. It is found that the concentration of LL-37 is higher in neonatal feces compared to fetal stools. In another study, it is observed that similarly high level of LL-37 mCRAMP in the gut epithelia of neonatal mice during the first 2 weeks postpartum[172,173].

CHAPTER 2

MATERIALS AND METHOD

2.1 Animals and diet

A total of 20 female Wistar rats (5 weeks old) were purchased from Kobay A.Ş (Ankara, Turkey). Control group (n=10) maintained on ad libitum standard chow (SC) diet. Cafeteria diet (CAF) group (n=10) fed with junk human foods varied daily with ad libitum SC diets. For CAF diet; crackers, cookies, chips, etc. were provided (Table 2)[174]. Food consumption and weight changes were checked daily for 8 months (Figure 4); the weight of the control group was approximately 310g±10, and the CAF group was 570g±10 (****p<0.0001, Figure 5).

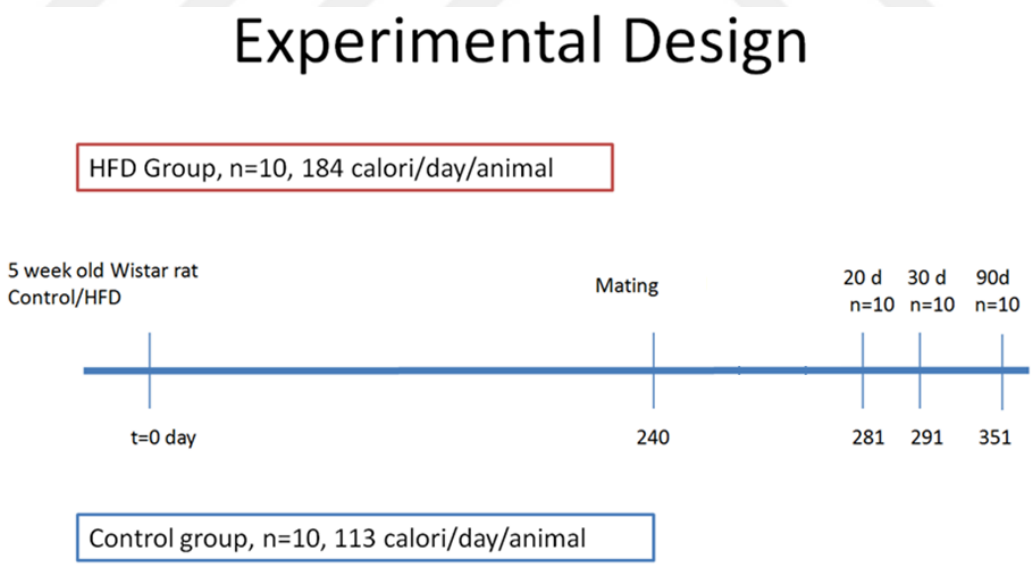

Figure 4: Average calorie intake and experimental design for the CAF and control groups (n=10 rat/group). For each group, n=10 offspring were sacrificed at different ages (20th, 30 th and 90 th days).

Table 2: **The ingredients of the cafeteria diet**

Energy and Food Ingredients (100 g)	Total kcal	Total fat g	Total Carbohydrate g	Protein g	Sugar g
Control Diet					
SC 7001 (Harlen)	382	4	54	25	0
CAF Diet					
Crackers					
Çay Keyfi (Eti)	462	20.4	67.8	5.8	28.5
Cookies					
Hoşbeş (Eti)	493	24.5	63.9	7.6	28.4
Hanımeller (Ülker)	427	18.1	62.1	3.9	25.0
Cereals					
Nesquik mısır gevreği (Nestle)	372	4.1	76.1	7.6	30.7
Chips					
Lays Wavy (Frito-Lay)	536	36	54	7	0
Lays Klasik (Frito-Lay)	529	33	51	7.0	0
Doritos (Frito-Lay)	491	24.5	60.5	7.2	2.3

Until weaning, all groups were continued on their selected diet. At weaning day all experimental groups' mothers were returned back to SC diet. After 3 the weeks of gestation, from each mother 8 to 12 offspring were born approximately.

2.2 Sample collection

From each groups of offspring, rats were sacrificed at 20th (n=10) , 30th (n=10) and 90th (n=10) days. Number of sacrificed offspring and the time table were shown in the experimental desing Figure 4. Plasma was obtained by blood centrifugation (2000X *g*, 4°C, 15 min). Intestines were collected and cleaned from stool and stored at −80°C for further analysis. All animal work was approved by Middle East Technical University Animal Ethics Committee, Ankara, Turkey (Approval No: 03.24.2014/08).

2.3 Maternal Glucose Tolerance Test

The rats were placed in different cages with water but without food for at least 16 hours. Then, first glucose levels measured at time via Accu-Chek Compact glucometer (Roche Diagnostics, USA). Then, after 30% dextrose solution administration (ip, 2 gr/kg bodyweight) glucose levels were measured futher at 5., 10., 15., 30., 60., and 120. min. (Figure 6).

2.4 RNA isolation

2.4.1 Intestinal Tissue Investigation

From colonic tissue (<30 mg) total RNA were isolated with RNeasy Plus Mini Kit (Qiagen, USA). For purifying RNA from tissues rich in RNases, 10 µl β-mercaptoethanol (β-ME) was added. Manufacturer's instructions were followed in all steps. DNA elimination acquired with gDNA eliminator spin column. RNA was eluted with 40 µl nuclease free water. The concentrations of the RNA samples were detected via NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For microarray and RT-PCR analysis total RNA was used.

2.5 Microarray procedure

Transcriptome analyses were performed from intestinal tissues of the rats in experimet and control groups (n =5) by SurePrint G3 Rat Gene Expression 4x44K (Agilent, Santa Clara, CA, USA) and the results were registered in GEO with the platform.

Microarray data were analyzed by using BRB ArrayTools 4.4.0 (http:// linus.ncbi.nih.gov/BRB-ArrayTools.html). Data were quantile normalized and log-transformed, threshold the intensity at the minimum value if the intensity is below the minimum 10 and log intensity variations *p*-value>0.05 were applied.

Differentially expressed genes between groups were selected according to *p*-value ($p<0.05$) and fold change 2.

2.6 cDNA synthesis

According to the manufacturer's instructions, cDNA was synthesized with 100 ng RNA via Qiagen RT2 First Strand cDNA Synthesis Kit (Qiagen, USA, cat. No. 330529). Default cycling conditions were used. Quantification was performed in a real time PCR instrument, the Rotorgene Q thermocycler coupled to a fluorescence detector (Qiagen, USA).

2.7 Stool Analysis

The stool samples were collected from intestinal cecum region and stored at –80 C until analysis. Bifidobacterium spp, Bacteroides fragilis, Lactobacillus spp, Akkermansia mucinophilia Faecalibacterium prausnitzi ve Enterobacteriaceae were studied quanitatively by real-time PCR.

Preperation of the standards:

In this study, Bifidobacterium brevis ATCC 15700, Bacteroides fragilis ATCC 25285, Lactobacillus acidophilus ATCC 4356, Akkermansia muciniphila ATCC BAA-835 and Faecalibacterium prausnitzii ATCC 27766 from the American Type Standard Culture Collections (ATCC) were used (Table 3). The primary sequences specific to 16S rRNA of the bacteria were used and at least three standards were implemented to obtain the standard curve. The numbers of copies of the bacterium in the clinical samples were determined from the drawn standard curves.

DNA extraction:

The DNA was isolated from the stool samples using an extraction kit designed specially for stool samples (QIAamp DNA Stool Mini Kit, Qiagen, Hilden,

Germany) following the extraction protocol of the isolation kit (Protocol: Using Stool Tubes for Isolation of DNA from Stool for Human DNA Analysis).

Table 3: Primers belonging to standard strains, target region volume and PCR temperature

Microorganisms	Primer name	Primer (5'=>3')	Target Region	Annealing temperature (°C)	Referen
Bifidobacterium spp.	g-Bifid-F	CTCCTGGAAACGGGTGG	550	55	[8]
Bifidobacterium spp.	g-Bifid-R	GGTGTTCTTCCCGATATCTACA			
Bacteroides fragilis group	g-Bfra-F	ATAGCCTTTCGAAAGRAAGAT	495	50	[8]
Bacteroides fragilis group	g-Bfra-R	CCAGTATCAACTGCAATTTTA	495	50	
Lactobacillus spp.	Lact-F	AGCAGTAGGGAATCTTCCA	341	50	[9]
Lactobacillus spp.	Lact-R	CACCGCTACACATGGAG	341	50	[9]
Akkermansia mucinophilia	AM-1-F	CAGCACGTGAAGGTGGGGAC	327	60	[10]
Akkermansia mucinophilia	AM-2-R	CCTTGCGGTTGGCTTCAGAT	327	60	[10]
Faecalibacterium prausnitzii	Fprau223-F	GATGGCCTCGCGTCCGATTAG	199	58	[11]
Faecalibacterium prausnitzii	Fprau223-R	CCGAAGACCTTCTTCCTCC	199	58	[11]

2.8 Real Time-PCR(RT-PCR) Analysis

Total RNA was extracted from islets by total RNA isolation kit (5 Prime) according to the manufacturer's instructions. The amount of RNA in all groups was equalized and then cDNA was synthesized according to the cDNA synthesis kit (Qiagen, USA) protocol. RT-PCR kit (Qiagen, USA) was used for the gene expression analysis. RT-PCR mix was added to the cDNA samples. PCR tubes were placed in the instrument (Rotor-Gene Q, Qiagen, USA) and RT-PCR was done at 40 cycles under: 15 s at 94°C, 30 s at 61°C and 30 s at 72°C. Data was analyzed using

the $2^{-\Delta\Delta CT}$ method and MIQE guidelines were fallowed[175].

2.9 Western Blot Analysis

Proteins were isolated from intestinal ileum regions via Ripa buffer (Santa cruz, USA). The protein content of the homogenates was measured via Bradford assay[176]. For Western blot analysis[177], occludin (Proteintech, 13409-1-AP), ZO-1 (Invitrogen, #PA5-28858) and Mucin (abcam, ab11197) levels were used to evaluate the structural integrity of intestinal tissue. Sample buffer and reducing agent were added to each sample according to the amount of protein to be loaded and then protein samples were heated in at 95°C for 7 minutes. Proteins (25 μg) were loaded to the Mini-Protean TGX stain free gels (Biorad, USA) and run at 150 V for 1 hour. Transfer to PVDF membrane was performed at 25 V, 2.5 A for 8 minutes. Nonspecific binding was blocked in blocking buffer (5% BSA (w/v) 1% TBST) at room temperature for 1 hour. The membrane washed three times with TBST for 10 minutes before incubating primary antibody at 4°C for 12-16 hours on a shaker. Then the membrane was incubated with a secondary antibody after washing steps four times with TBST for 5 minutes[177]. For the imaging, the luminol A and B solutions were mixed in a 1:1 ratio, then the mixture was added to the membrane and after 1 minute, the image was acquired. Protein expression changes was determined using the Image Lab. (Software version 5.2.1.) program and the results were evaluated statistically.

2.10 Statistical analysis

The RT- PCR was done with 3 technical replicates. The fold changes for each group were calculated via $2^{-\Delta\Delta Ct}$, and statistical differences were evaluated with the student's t-test (GraphPad Prism 6).

Western blot Images were quantified with the ImageJ program and statistical analysis was performed with the the Student's t-test (GraphPad Prism 6). Data were considered as significant with *p<0.05 and as highly significant with**p<0.01.

CHAPTER 3

RESULTS

3.1 Maternal Cafeteria Diet, and Diet-Induced Obesity and Diabetes

Statistically, significant weight changes were acquired between the control and CAF group mothers after 8 months of cafeteria diet before gestation (Figure 5). Average weight of the CAF group was 522.9g±27.10 and the average weight of the control group was 326.6g±11.23 (p****< 0.0001) (Figure 5). Since the CAF group was 1.39X heavier than the control group, the CAF group was obese.

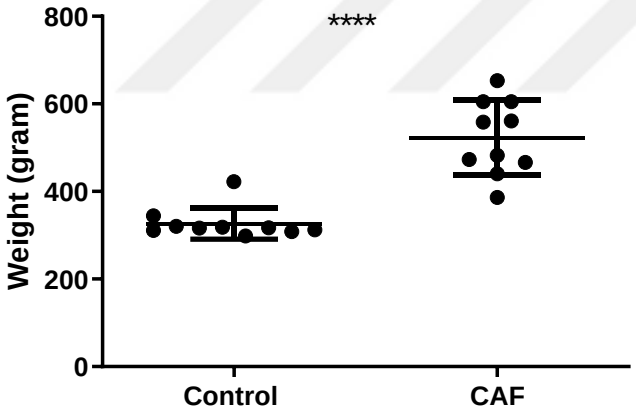

Figure 5 : Weight(g) changes before gestation for mothers (n=10rat/group). The average weight of the control group was 310g and the CAF group was 570g (****p<0.0001) (Teker, H. T., 2014).

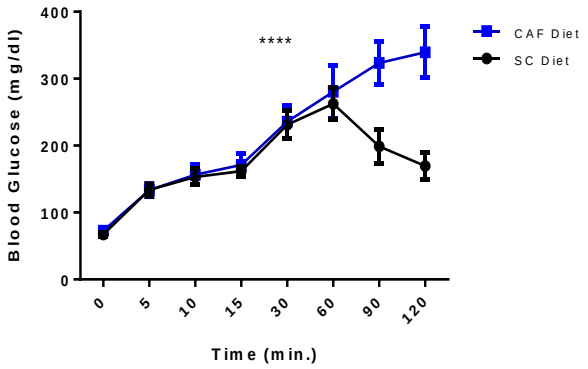

Figure 6 : The control and CAF group mother's glucose tolerance test. In the CAF group, glucose was not cleared from blood after 120 min. (****p< 0.0001 from the control (Two-way ANOVA) (Teker, H. T., 2014).

3.2 Offspring Metabolic Parameters

Throughout the experiments, it was observed that a number of death of offspring at the day of birth from the diabetic mothers which may be because of high plasma insulin and high blood glucose level (data is not shown).

Offspring blood glucose levels and plasma insülin levels were investigated in order to test metabolic conditions of offspring. At day 20 lower blood glucose levels were detected at the CAF diet group (control: 144.3 ± 6.303, CAF: 100.4 ± 7.646). At 30 day, on the other hand, the blood glucose levels became higher when compared to the control group (control: 153.4 ± 2.243, CAF: 164.9 ± 2.687). At 90 day, there were no significant changes between the groups(control: 139.7 ± 0.8300, CAF: 141.3 ± 0.7133) (Figure 7).

Offspring plasma insulin levels have shown that statistically significant changes were observed at 20 (control: 0.4214 ± 0.08935, CAF: 0.7464 ± 0.09430), 30 days (control: 153.4 ± 2.243, CAF: 164.9 ± 2.687). Offspring of maternally diabetic rats had higher insulin levels at their plasma, but towards to adulthood at 90 these values were normalized (control: 139.7 ± 0.8300, CAF: 141.3 ± 0.7133) (Figure 8).

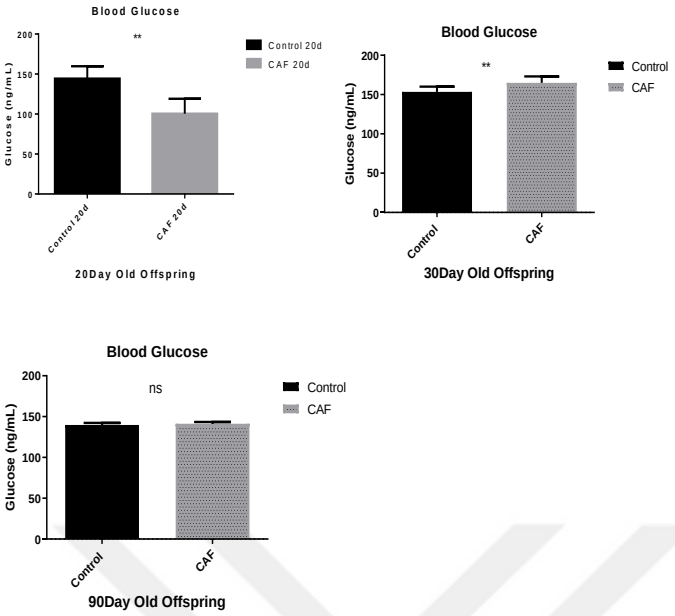

Figure 7: Blood glucose levels for 20, 30 and 90 day old offsprings from both control and the CAF group mothers. Blood glucose levels decreased at 20 day old CAF group offsring. Blood glucose levels increased at 30 day old CAF group offsringcompared to control group. At 90 day old blood glucose levels normalized.

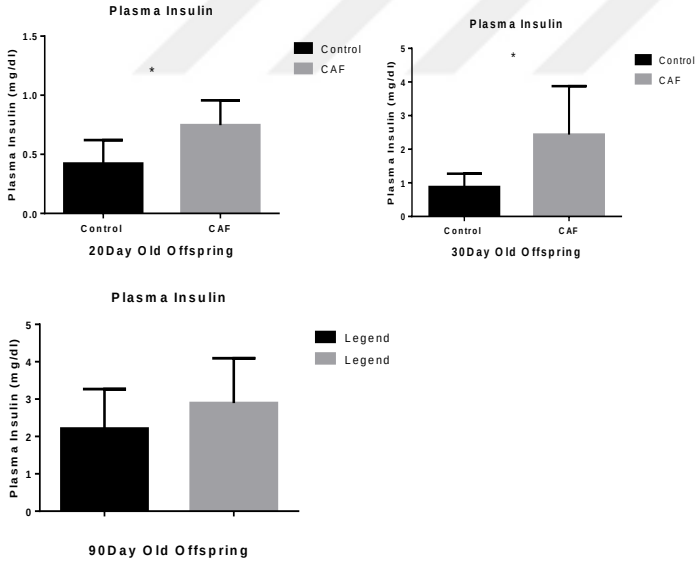

Figure 8: Plasma insulin levels for 20, 30 and 90 day old offsprings from both control and CAF groups mother. Plazma insulin levels incresed both at 20 and 30 day old CAF group offspring compared to the control group. At 90 day old plasma insulin levels statistically not significant.

3.3 Significantly Altered AMPs from Microarray Analysis

In order to investigate maternal effects of metabolic dysfunctions on offspring AMPs expression, ileum regions of 20 day, 30 day and 90 day-old offspring intestine investigated initially via whole genome expression analysis and significant AMP expressions (.2-fold change and FDR,0.05) were selected. AMP genes that were significant (.2-fold change and FDR,0.05) at both 20 day and 30 day old offspring are selected are shown at Table 4.

Our results have shown that general tendency to downregulation of AMP genes for 20 and 30 day old offspring and normalization observed at 90 day old offspring. At day 20 more than 2-fold change expressional down-regulation observed for; defensin family member of defensin RatNP-3 precursor, defensin beta 27, defensin alpha-like 1,defensin NP-4 precursor,defensin beta 43,defensin beta 51,defensin beta 52,defensin alpha 24,defensin alpha 8,defensin alpha 9 were all down-regulated more than.

For reg family members, regenerating islet-derived 3 beta, regenerating islet-derived 3 gamma,regenerating islet-derived 1 alpha were more than 2-fold change down regulated at 20 day old offspring. For C-type lectin domain family members; C-type lectin domain family 2 member D-like 1,C-type lectin domain family 2 member D2,C-type lectin domain family 2 member G, were also down regulated more than 2-fold change at 20 day old offspring. Only C-type lectin domain family 1 member A 2-fold up regulated at 20 day old offspring. At day 30 lower expression levels of defensin family member defensin RatNP-3 precursor,defensin alpha-like 1,defensin NP-4 precursor,defensin beta 43, defensin alpha 8, defensin alpha 9,defensin alpha 10, defensin alpha 7 were down regulated more than 2-fold change. However, defensin beta 22, defensin beta 52 and regenerating islet-derived 3 beta were upregulated more than 2-fold change.

Table 4: **Fold change of the probe sets encoding AMP genes that were significant (.2-fold change) in one of the performed comparative analyses with control groups**

20 Day Old					
UniqueID	GeneSymbol	GeneName	P-value	FDR	Log2FC
A_44_P203401	RatNP-3b	defensin RatNP-3 precursor	0,001	0,023	-3,06
A_64_P021728	Defb27	defensin beta 27	0,014	0,059	-1,03
A_64_P031394	Defal1	defensin alpha-like 1	0,001	0,025	-2,18
A_64_P032504	Np4	defensin NP-4 precursor	0,018	0,068	-2,74
A_64_P058953	Defb43	defensin beta 43	0,001	0,019	-2,25
A_64_P075562	Defb51	defensin beta 51	0,004	0,038	-1,18
A_64_P105933	Defb52	defensin beta 52	0,005	0,040	-2,00
A_64_P124105	Defa24	defensin alpha 24	0,002	0,029	-1,25
A_64_P131573	Defa8	defensin alpha 8	0,002	0,029	-2,56
A_64_P131577	Defa9	defensin alpha 9	0,009	0,048	-1,79
A_44_P271658	Reg3b	regenerating islet-derived 3 beta	0,020	0,072	-3,74
A_44_P273839	Reg3g	regenerating islet-derived 3 gamma	0,004	0,036	-4,80
A_64_P124535	Reg1a	regenerating islet-derived 1 alpha	0,005	0,039	-3,92
A_64_P057924	Clec2dl1	C-type lectin domain family 2 member D-like 1	0,007	0,046	-1,15
A_64_P069365	Clec2d2	C-type lectin domain family 2 member D2	0,003	0,032	-1,25
A_64_P138179	Clec2g	C-type lectin domain family 2, member G	0,004	0,036	-1,22
A_64_P141175	Clec1a	C-type lectin domain family 1, member A	0,002	0,027	1,66
30 Day Old					
UniqueID	GeneSymbol	GeneName	P-value	FDR	Log2FC
A_43_P15602	Defb22	defensin beta 22	0,003	0,158	1,38
A_44_P203401	RatNP-3b	defensin RatNP-3 precursor	0,013	0,199	-1,03
A_64_P031394	Defal1	defensin alpha-like 1	0,003	0,158	-1,15
A_64_P032504	Np4	defensin NP-4 precursor	0,003	0,156	-1,43
A_64_P058953	Defb43	defensin beta 43	0,004	0,163	-1,09
A_64_P105933	Defb52	defensin beta 52	0,000	0,119	3,27
A_64_P131573	Defa8	defensin alpha 8	0,002	0,156	-1,06
A_64_P131577	Defa9	defensin alpha 9	0,004	0,160	-1,06
A_44_P271658	Reg3b	regenerating islet-derived 3 beta	0,006	0,173	1,08
90 Day Old					
		There is no significant change			

3.4 Microarray validation using RT-PCR

For microarray validation, a set of 7 genes were selected with respect to significant changes observed at microarray analysis. Selected genes were analyzed with RT-PCR. The obtained fold changes were compared with the datasets (Table 4). Majority of the genes (5 out of 7) were consistent both in array and PCR. The two genes remaining (Clec2g, Muc1,) detected opposite direction; they were down-regulated in the microarray dataset, but up-regulated in the RT-PCR dataset.

Our results have shown that for C type lectin family members; Reg3b and Reg3g have significantly decreased at 20 day and normalized at 30 day and Towards to adulthood significantly higher expressions at 90 day old (Figure 9, 10). For clec2g decrease at 20 and 30 day, but significantly higher expressions at 90 day old observed (Figure 15).

For defensins: Defb52 have significantly decreased at 20 day and significantly fluctuated and increased at 30 day. They were however normalized at 90 day (Figure 11). Defb43 was significantly higher at both 20 and 30 day old offsprings, bu normalized at 90 day (Figure 12). Defb1 have significantly decreased at 20 day and significantly increased at 30 day. And contioned significantly higher expressions at 90 day old (Figure 13). Defa8 have significantly decreased at 20 day and 30 day, but at 90 day expression level was significantly higher than control groups (Figure 14).

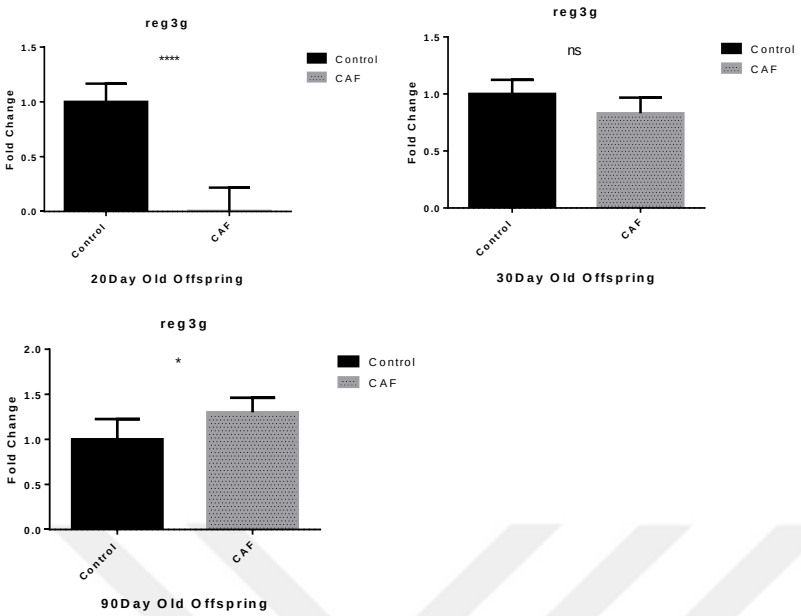

Figure 9: Relative expression of reg3g by RT-PCR. reg3g expression levels were significantly decreased for CAF offsprings at 20 day. At 30 day old offspring there were no statistically significant changes. At 90 day old offsprings reg3g expression levels were significantly increased for CAF diet offspring vs. the controls.

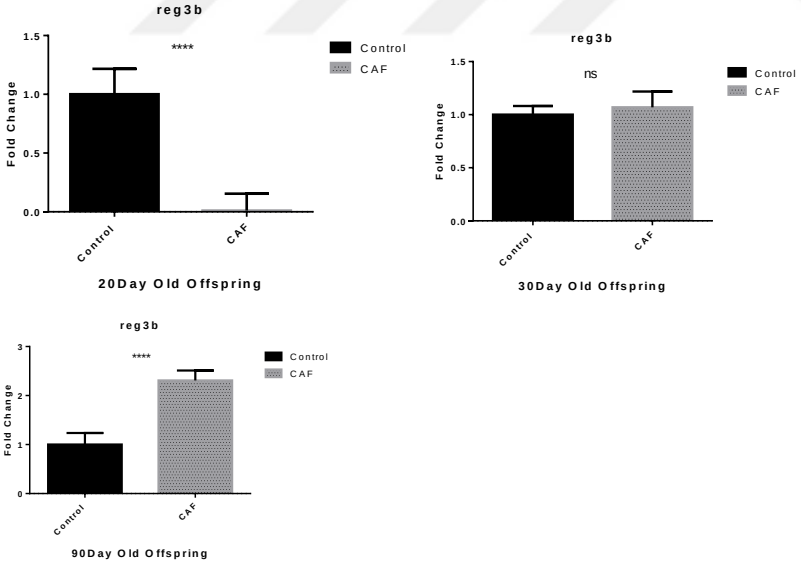

Figure 10: Relative expression of reg3b by RT-PCR. reg3b expression levels significantly decreased for CAF diet offsprings at 20 day. At 30 day old offsprings there is not statistically significant changes observed. At 90 day old offsprings reg3b expression levels were significantly increased for CAF offspring compare to the control group.

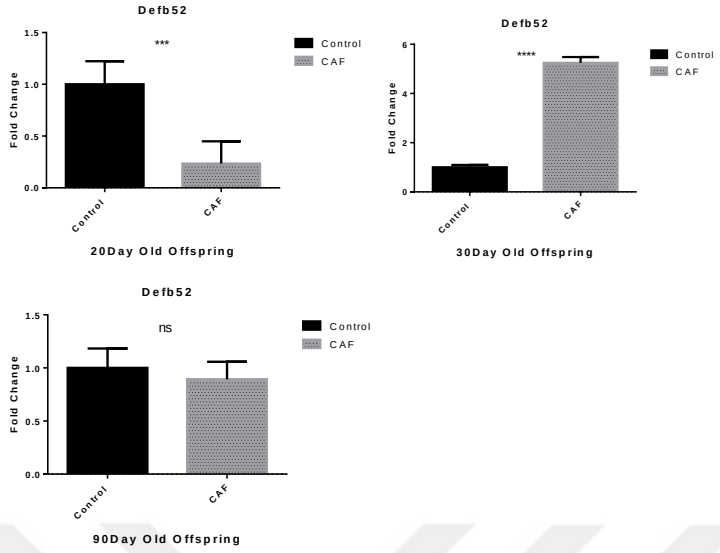

Figure 11: Relative expression of Defb52 by RT-PCR. Defb52 expression levels significantly decreased for CAF diet offsprings at 20 day. At 30 day old offsprings Defb52 expression levels significantly increased for CAF diet offsprings. At 90 day old offspring there was no statistically significant changes.

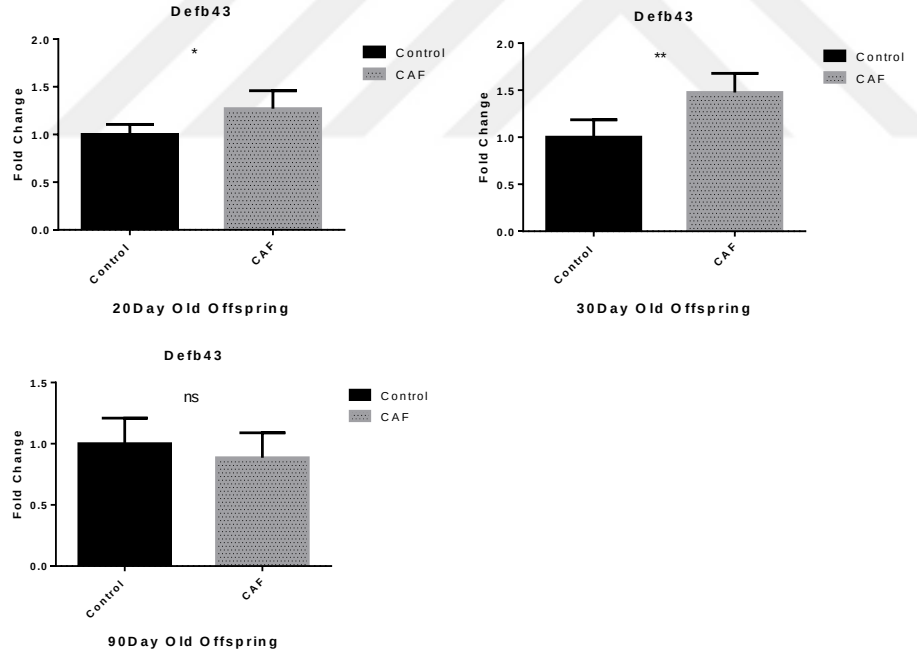

Figure 12: Relative expression of Defb43 by RT-PCR. Defb43 expression levels were significantly increased for CAF offspring both at 20 and 30 day old for the CAF group. At 90 day old offspring there were no statistical significant changes were detected.

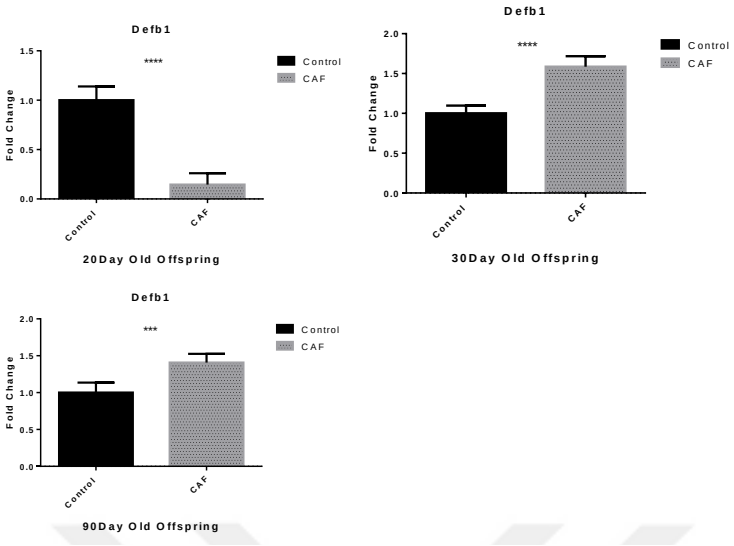

Figure 13: Relative expression of Defb1 by RT-PCR. Defb1 expression levels were significantly decreased for the CAF offspring at 20 day. At both 30 and 90 day old offsprings Defb1 expression levels were significantly increased for CAF offspring vs. controls.

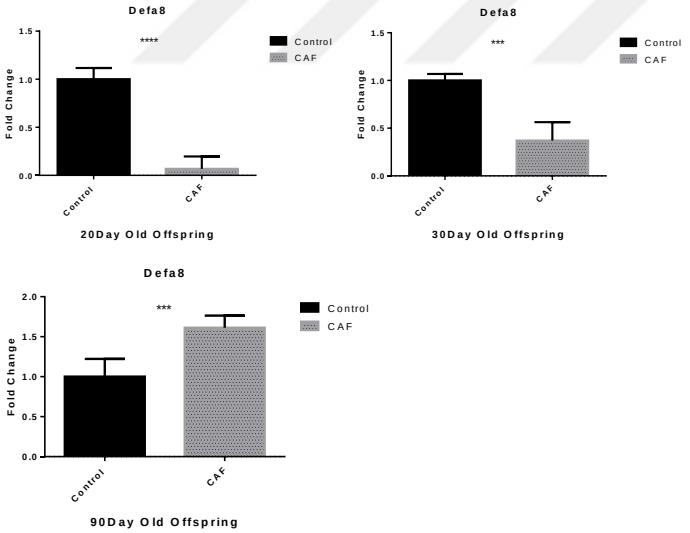

Figure 14: Relative expression of Defa8 by RT-PCR. Defa8 expression levels were significantly decreased for the CAF group at 20 and 30 day. Ninty day old offsprings Defa8 expression levels were significantly increased for the CAF group vs. the control offspring.

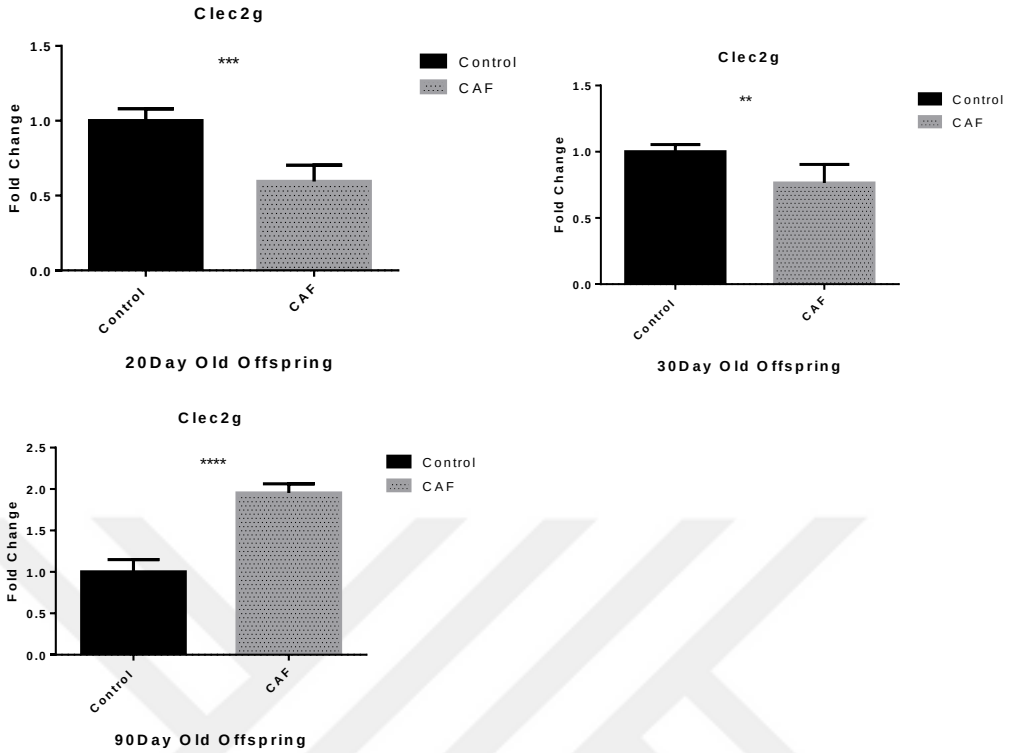

Figure 15: Relative expression of Clec2g by RT-PCR. Clec2g expression levels were significantly decreased for the CAF offspring at 20 and 30 day. Ninty day old offsprings Clec2g expression levels were significantly increased for the CAF group compare to the control group.

3.5 Quantitative Comparison of Important Bacterial Microbiota Species

The quantification of bacteria in stool was represented under log10/g logarithmic scale. The average values of the bacterium at log10/g stool are presented in Figure 16-21. The number of B. fragilis and Lactobacillus spp were increased at 20d and 90d rats (p=0.002 and 0.003, respectively) and A. mucinophilia were increased at 20d and 30d rats (p=0.015 and 0.025, respectively) and mother rats in CAF group (p=0.025) when compared with controls. Faecalibacterium prausnitzi were increased at 20d rats (p=0.008) and did not change at other rats and Bifidobacterium spp. were increased in mother rats (p=0.01) of the CAF group.

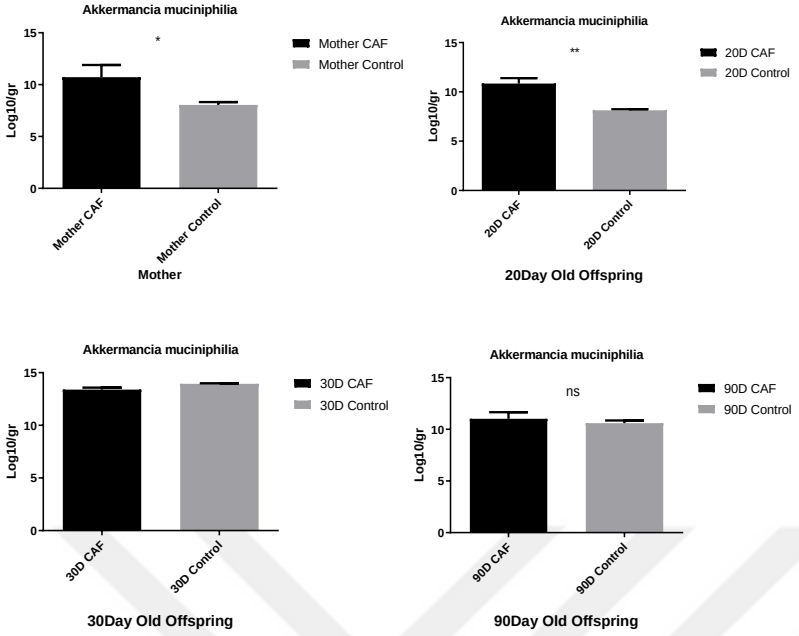

Figure 16: Akkermansia mucinophilia quantification on mother and 20, 30 and 90 day old offspring: Relative amount of bacteria in stool was represented under log10/g logarithmic scale.

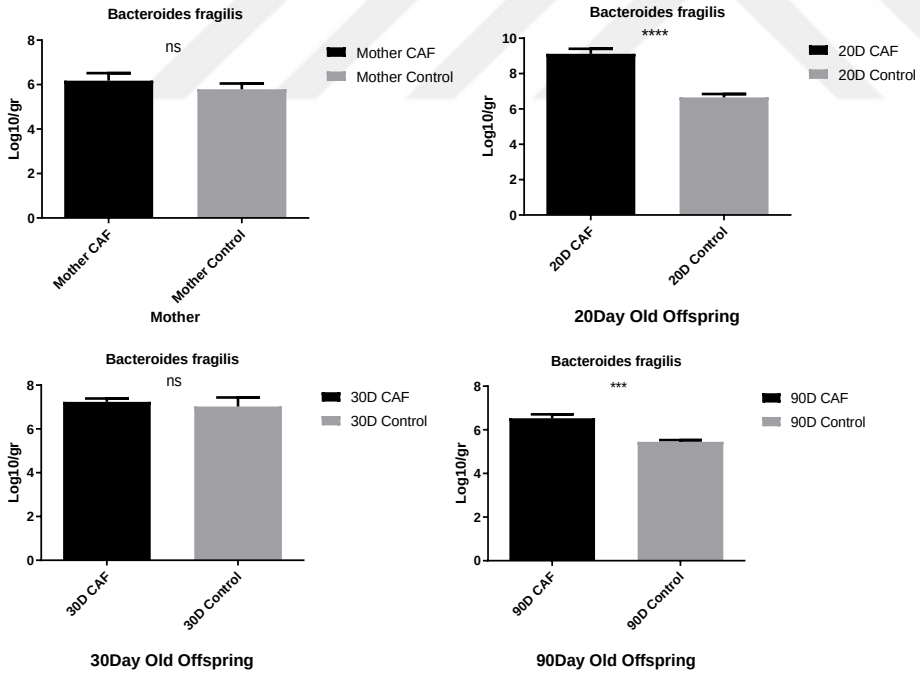

Figure 17: Bacteroides fragilis quantification on mother and 20, 30 and 90 day old offspring: Relative amount of bacteria in stool was represented under log10/g logarithmic scale.

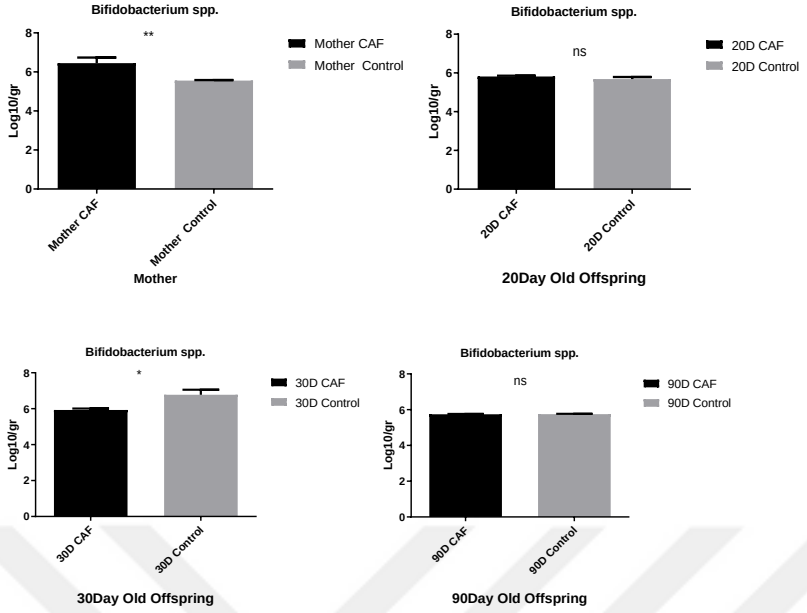

Figure 18: Bifidobacterium spp.quantification on mother and 20, 30 and 90 day old offspring: Relative amount of bacteria in stool was represented under log10/g logarithmic scale.

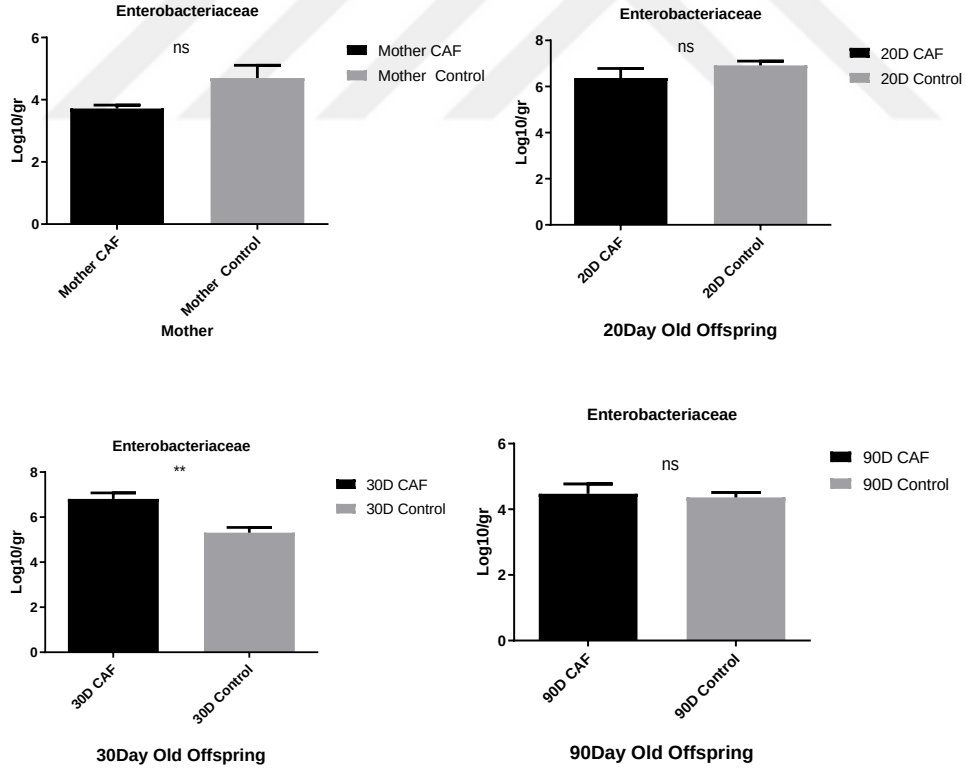

Figure 19: Enterobacteriaceae quantification on mother and 20, 30 and 90 day old offspring: Relative amount of bacteria in stool was represented under log10/g logarithmic scale.

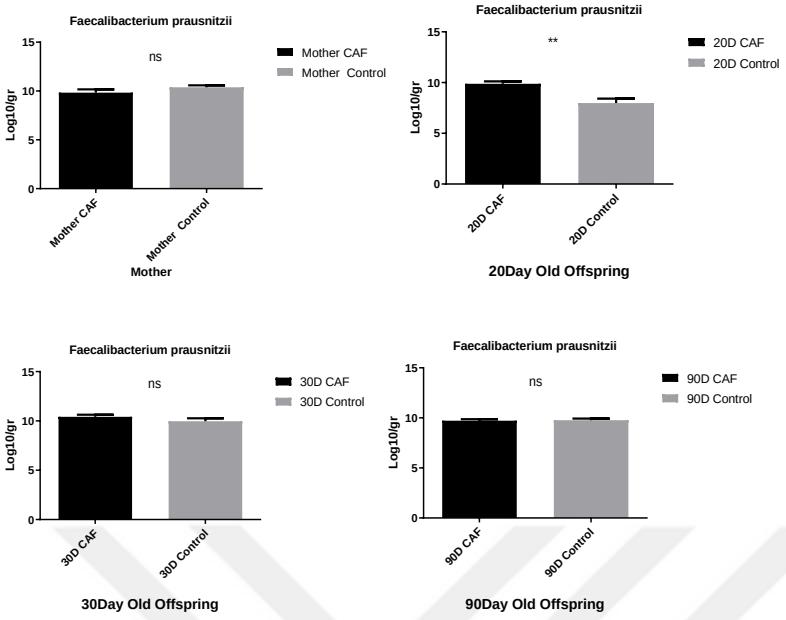

Figure 20: Faecalibacterium prausnitzii quantification on mother and 20, 30 and 90 day old offspring: Relative amount of bacteria in stool was represented under log10/g logarithmic scale.

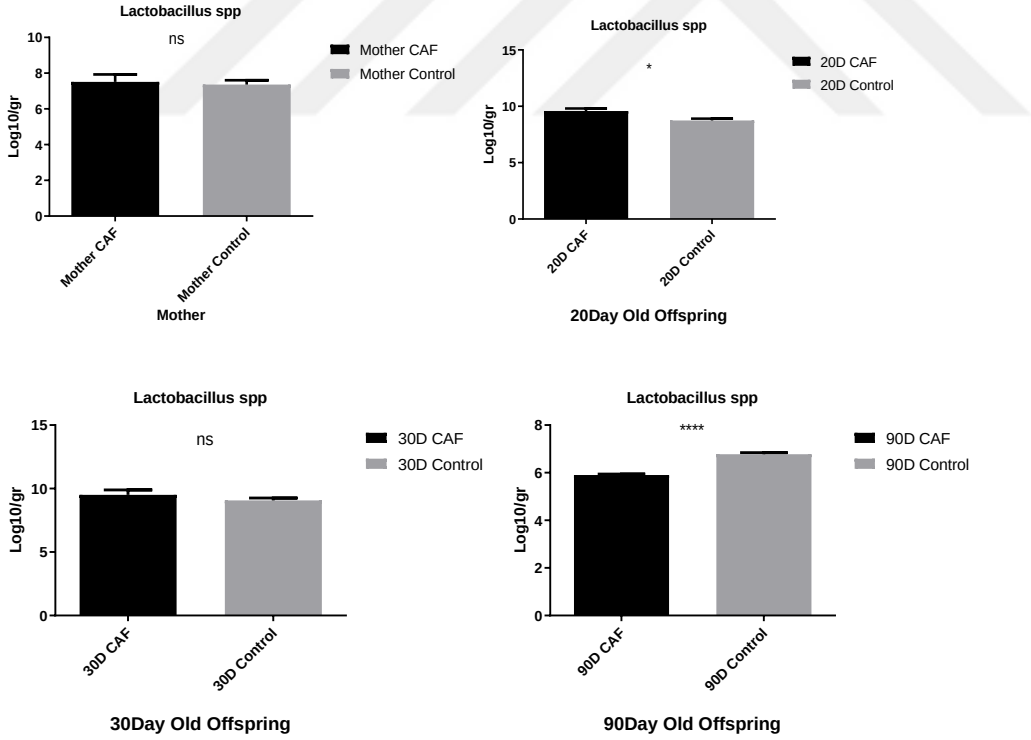

Figure 21: Lactobacillus quantification on mother and 20, 30 and 90 day old offspring: Relative amount of bacteria in stool was represented under log10/g logarithmic scale.

3.6 Intestinal Barrier Investigation

3.6.1 Tight Junction Investigation

As for our intestinal barrier investigation, we investigated occludin and zone-occludin at protein level from the intestinal ileum region. Our results have shown that there were statistically significant declined for occludin levels at 20 day (Figure 22) and 30 day (Figure 23) and then normalization at 90 day old offspring (Figure 24) in the CAF group vs the controls. On the otherhand, zone-occludin (ZO) levels were only decreased statisticaly significant at 30 day old offspring of the CAF group vs. the controls (Figure 26). As seen in Figure 25 and 27, there were not any significant changes in ZO levels in 20 day and 90 day old offspring in the CAF group vs the control group, respectively.

3.6.1.1 Occludin

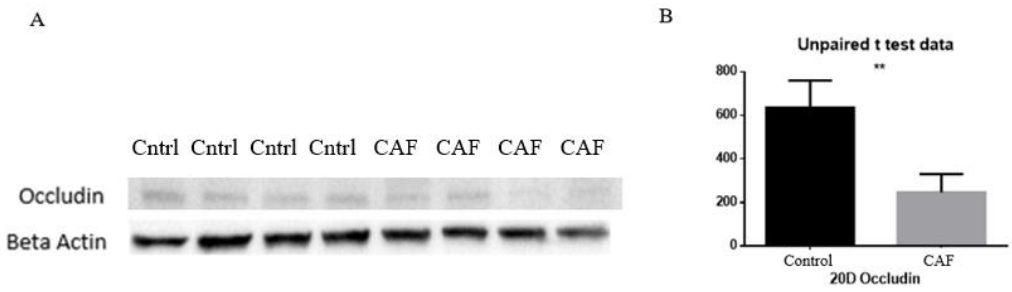

Figure 22: Western blot analyses of occludin at 20 day old offspring.A) Blots were probed with anti-occludin and anti-β-actin. Cntrl: controls/4 animal samples CAF: cafeteria diet group/4 rat samples B) Quantitative analysis of band intensity, which were normalized to β-actin expression. ** $p<0.001$. Experiments were repeated 3 times, n=3.

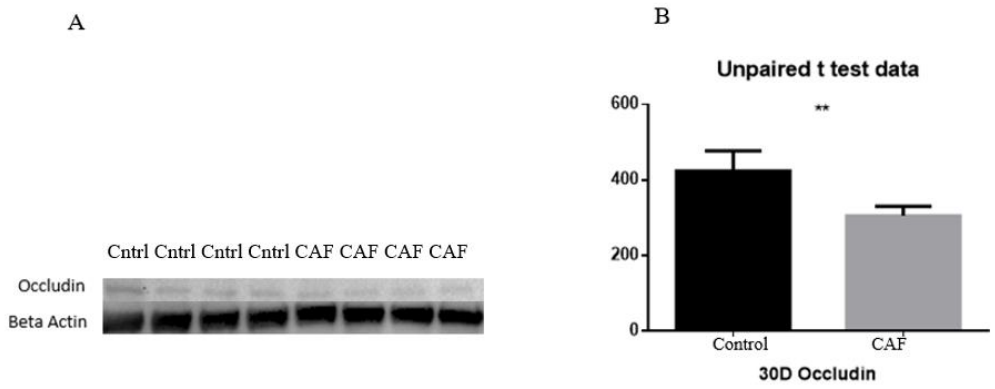

Figure 23: Western blot analyses of occludin at 30 day old offspring. A) Blots were probed with anti-occludin and anti-β-actin. Cntrl: controls/4 rat samples, CAF: cafeteria diet group/4 rat samples b) Quantitative analysis of band intensity, which were normalized to β-actin expression. ***p*<0.001. Experiments were repeated 3 times, n=3.

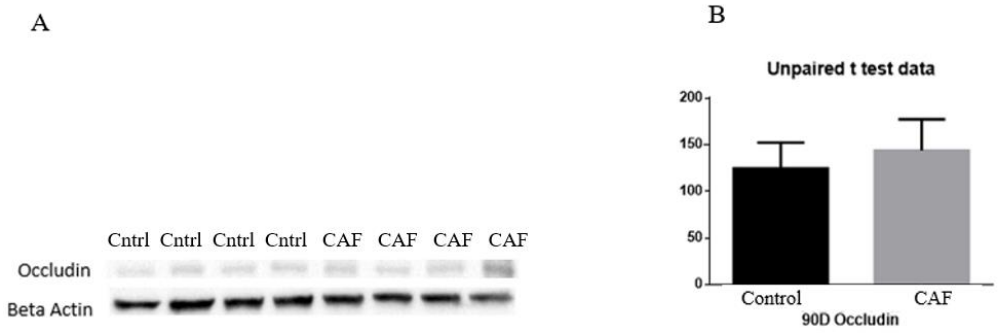

Figure 24: Western blot analyses of occludin at 90 day old offspring. A) Blots were probed with anti-occludin and anti-β-actin. Cntrl: controls/4 animals samples, CAF: cafeteria diet group/ 4 animals samples B) Quantitative analysis of band intensity, which were normalized to β-actin expression. Experiments were repeated 3 times, n=3.

3.6.1.2 Zone-Occludin

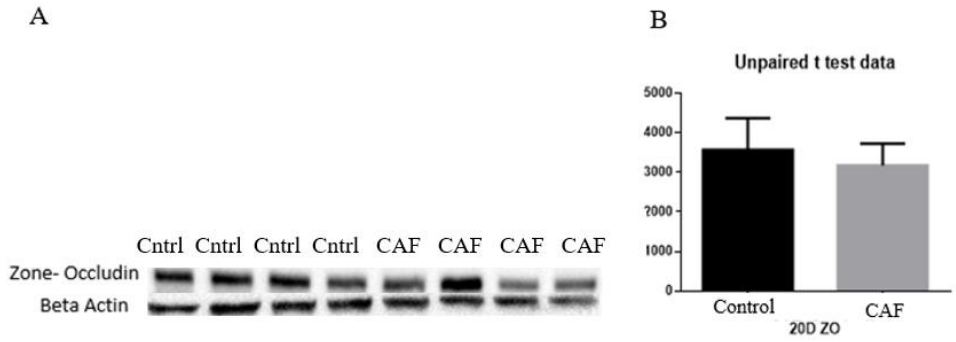

Figure 25: Western blot analyses of Zone-Occludin (ZO) at 20 day old offspring. A) Blots were probed with anti-ZO and anti-β-actin. Cntrl: controls/4 rat samples, CAF: cafeteria diet group/4 rat samples b) Quantitative analysis of band intensity, which were normalized to β-actin expression. Experiments were repeated 3 times, n=3.

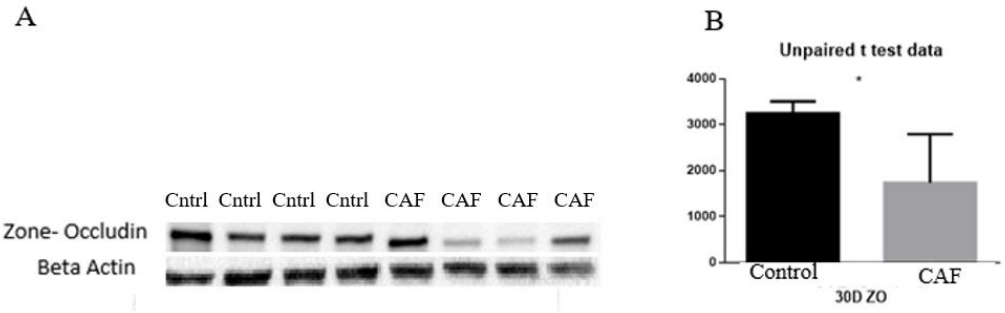

Figure 26: Western blot analyses of Zone-Occludin (ZO) 30 day old offspring. A) Blots were probed with anti-ZO and anti-β-actin. Cntrl: controls/4 rat samples, CAF: cafeteria diet group/4 rat samples b) Quantitative analysis of band intensity, which were normalized to β-actin expression. **p* <0.05. Experiments were repeated 3 times, n=3.

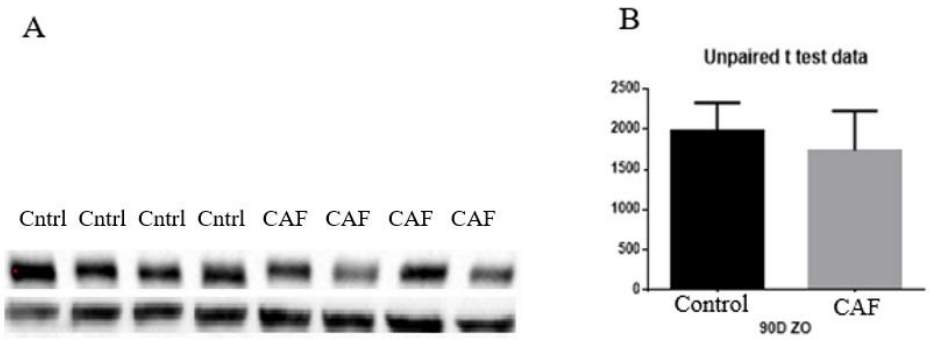

Figure 27: Western blot analyses of Zone-Occludin (ZO) at 90 day old offspring. A) Blots were probed with anti-ZO and anti-β-actin. Cntrl: controls/4 rat samples, CAF: cafeteria diet group/4 rat samples b) Quantitative analysis of band intensity, which were normalized to β-actin expression. Experiments were repeated 3 times, n=3.

3.6.2 Mucus Investigation

3.6.2.1 RT-PCR Analysis of Muc1 and Muc2

Mucus secretions and their degradation process is crucial for both establishment and host-microbial communication. Our results have shown that expressions of Muc1 were significantly decreased at 20 day and 30 day and normalized at 90 day old (Figure 28) CAF group offspring vs the controls. For *Muc2*, expressions were statistical significantly decreased at 20 day and increased at 30 day and normalized at 90 day old CAF group offspring vs. the controls (Figure 29). However, Western blot analysis of mucin2 have not show statistically significant changes (Figures 30, 31, 32).

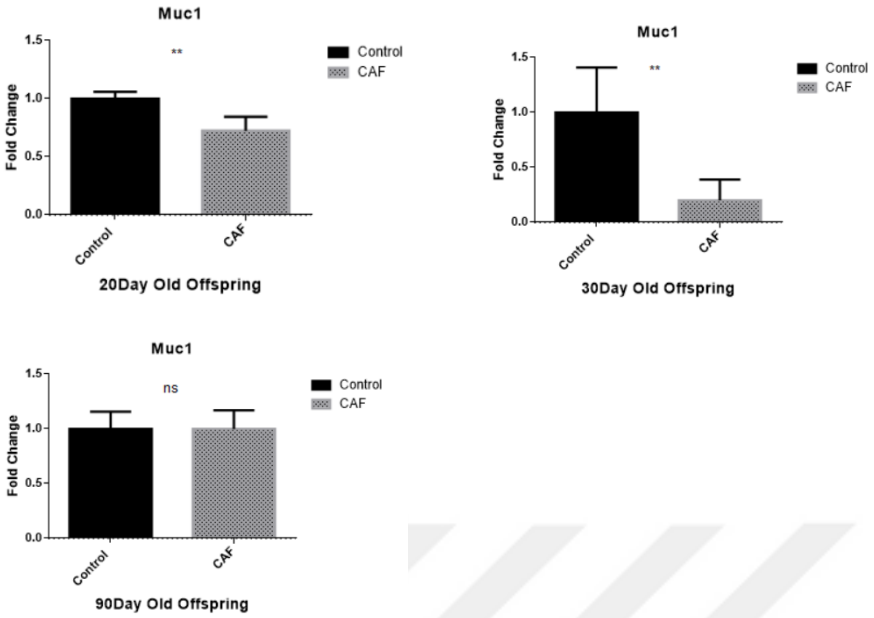

Figure 28: Relative expression of Muc1 by RT-PCR. *Muc1* expression levels were significantly decreased for the CAF offspring at 20 and 30 day for the CAF offspring. At 90 day old offspring *Muc1* expression levels were normalized.

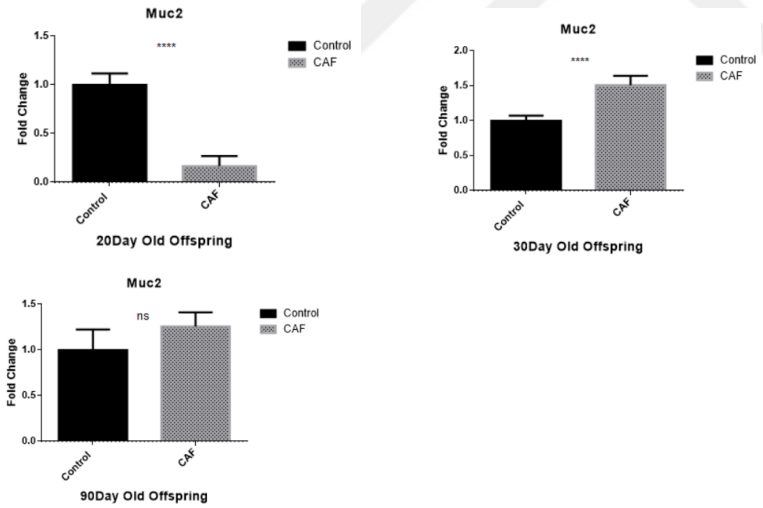

Figure 29: Relative expression of *Muc2* by Rt-PCR. *Muc2* expression levels were significantly decreased for the CAF group at 20 day. At 30 day-old, *Muc2* expression levels were significantly increased for the CAF group offspring and at 90 day old normalization were determined.

3.6.2.2 Western Blot Analyses of Muc2

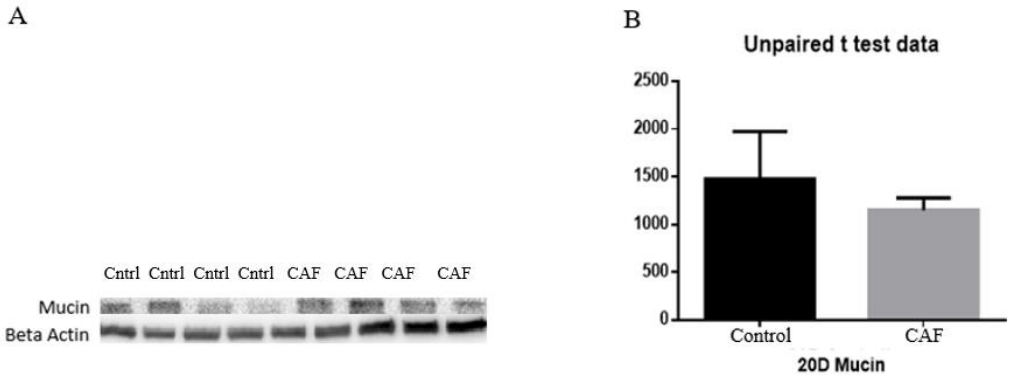

Figure 30: Western blot analysis for Muc2 for 20 day old offspring.A) The blot was probed with anti-Muc2 and anti beta-actin. Cntrl: control/4 rat sample, CAF: the CAF group/4 rat samples. b) Quantitative analysis of band intensity of Muc2, which were normalized to β-actin expression. Experiments were repeated 3 times, n=3.

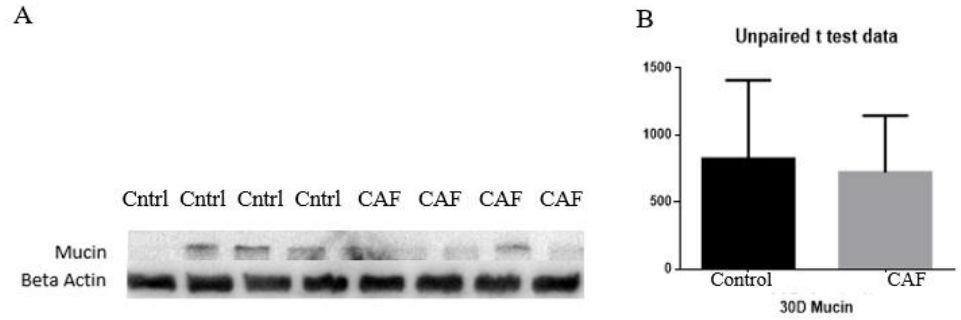

Figure 31: Western blot analysis for Muc2 for 30 day old offspring. A) The blot was probed with anti-Muc2 and anti beta-actin. Cntrl: control/4 rat sample, CAF: the CAF group/4 rat samples. b) Quantitative analysis of band intensity of Muc2, which were normalized to β-actin expression. Experiments were repeated 3 times, n=3.

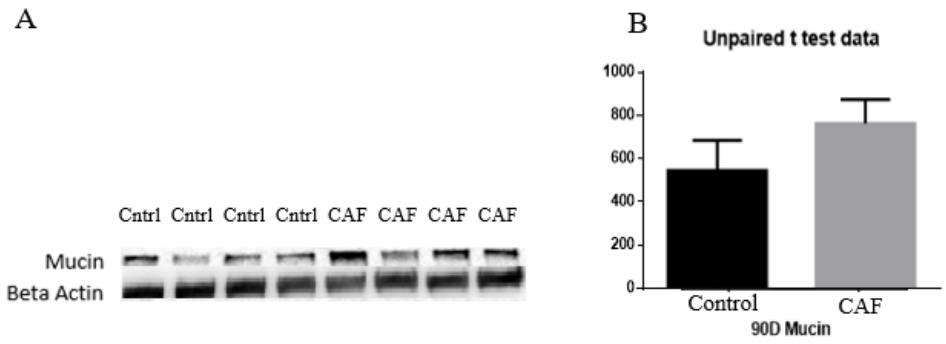

Figure 32: Western blot analysis for Muc2 for 90 day old offspring.A) The blot was probed with anti-Muc2 and anti beta-actin. Cntrl: control/4 rat sample, CAF: the CAF group/4 rat samples. b) Quantitative analysis of band intensity of Muc2, which were normalized to β-actin expression. Experiments were repeated 3 times, n=3.

CHAPTER 4

DISCUSSION

Maternal diabetes as a chronic metabolic disease, generally observed at the gestational period all over the world. The National Birth Defects Prevention Study (NBDPS) has revealed that pre-gestational and/or gestational diabetes mellitus might be the most dangerous risk factor for birth defect conditions and might generate life-long physiological effects offsprings[178].

Each individual has special gut flora that plays important functions in protecting against pathogens, nutrient metabolism, the integrity of the gut barrier, and immunomodulation. Each gut microbiota is shaped in early life as their composition depends on infant transitions and external factors[179].

In parallel with gut microbe expansion, many essential functions for human health evolve; vitamin biosynthesis, dietary energy extraction, a function of the gut barrier, and maturation/education of the immune system. Neonates requires careful exposure of gut bacteria to develop properly immune system. Mothers health status might have huge roles at this point because disease-associated microbe communities might be translocated to offspring and generate altered microbe composition in infant [180].

Establishment of microbial species in the intestine at early stages plays a critical role in host development, metabolic, immune, endocrine systems, and other systems[181]. However, the microbiota of infants is very different from adult stages and shows interindividual variability[182]. After weaning, microbiota establishment shows gradual changes according to age; similarities appear around 1 year of age and more resemble adult-like microbiota for humans. Throughout these changes, microbial community gains compositional stability, and this compositional stability progressively generates colonization resistance in which newly introduced bacteria

barely gain rarely gain access to those regions in the intestinal lumen and fail to become a permanent member of this ecosystem[22].

Changes in early infant gut microbiome has shown correlation with the development of childhood disease conditions like; obesity, autoimmune conditions, including asthma, allergies, and diabetes. With recent studies, it is realized that maternal diabetes might be the most dangerous risk factor for establishment of the microbiome during early stages[182].

Studies conducted for understanding mother to offspring microbial transitions have faced the correlation problems, and also maternal, and child microbiota could not show statistically significantly stable results; as significant differences obserced between infant and typical adult gut microbial compositions [183]. In another study, it was found that bacterial compositions from maternal skin and vagina colonize only transiently, and the infant continues to acquire microbes from distinct sources[184]. Even such difficulties, in one study, it is identified that from gestationally diabetic human candidates, a few *Bacteroides* and *Blautia* oligotypes were significantly shared by the GDM mothers and their offspring. But infant *Bifidobacterium* oligotypes of the newborns were not present in the feces of the GDM women and shows post-natal acquisition[185]. These results may imply that host-directed factors decide which microbiome to establish gains much more attention during developmental period[144,142].

Gut microbiota balance is essential for health status, but the mechanisms for acquiring such homeostasis are incompletely understood. Recent studies has shown that host has crucial roles in shaping microbiota for host's microbial balance. At this point, epithelial cells have huge impacts on imposing ecological control mechanisms, which involve the release of AMPs by small-intestinal Paneth cells. These peptides not only protect from infection and inflammation but also modulate the metabolic activity and functionality of the microbiome. Such epithelial control mechanisms for therapeutic means could provide a novel approaches for dysbiosis[186]

Antimicrobial peptides and proteins are gene-encoded crucial effector and modulatory molecules of innate immunity. They are found at all developmental periods; in the cells and tissues of the uterus, fetus, and the neonate. The presence of all developmental periods implicates their crucial role in immunity during pregnancy and in early development[142].

Studies are showing that aberrant expression of antimicrobial peptides in epithelial tissues is associated with barrier dysfunction in the GI tract, bacterial ecological changes, and also changes in bacterial metabolic and functional actitivities[27].

In our study, we focused on the effect of maternal diabetes and obesity as metabolic disorder conditions on offspring intestinal AMPs expressions, which shape microbial diversity established. Also, it was investigated of that offspring glucose regulation, intestinal barrier function, and changes in certain probiotic species.

As an initial step, maternal diabetes and obese condition before the gestational period were determined that the CAF group mothers were obese and diabetic after long-term CAF diet. Next, it was investigated that blood glucose and plasma insulin levels of 20, 30, and 90 day old offspring. Results showed that blood glucose levels of 20 days old CAF group were significantly decreased but at 30 day old, individuals, blood glucose levels were significantly higher when compared to the control group. Towards to adulthood, these changes were normalized. Plasma insulin levels for both 20 and 30 day old CAF groups were significantly high, but at 90 day it became normalized. All these results showed that even both groups consume the same calori from the SC; the CAF group of offsprings at 20 and 30 day tend to secrete much more insulin at the same amount of glucose. The results indicate that at 20 and 30 day old, offspring of the CAF group, rats might be prone to develop metabolic diseases (the CAF group rats were not obese, data not shown). However, insulin secretion was tended to normalized at 90 day, adulthood.

Because of the role of microbiota on offspring metabolism and development, for further investigation, 7 probiotic bacteria were determined of that

Bifidobacterium spp., Bacteroides fragilis group, Lactobacillus spp., Akkermansia mucinophilia, Faecalibacterium prausnitzii, Enterobacteriaceae.

In our study, it was found that Akkermansia mucinophilia were increased both diabetic mother and 20 day but normalized at 30 and 90 day old offspring. *Akkermansia mucinophilia* normally has beneficial effects[187] and at certain studies it was found that the abundance of this bacteria decreases at obese and type II diabetic animal models[188]. The use of the bacterium as a probiotic is beneficial to the host. In one study it was shown that protein isolated from the outer membrane can interact with TLR2 and improves the gut barrier[189]. Although in our study we observed diabetic mothers and 20 day old CAF group offspring has higher level olf Akkermansia mucinophilia, the similar conflicting result also observed by others also[190–192]

Bacteroides fragilis was increased both in 30 and 90 day old offspring of the CAF group. A similar result observed in one adult animal CAF study also[193]. Conflicting results also are seen in literature; at one human study, it was shown that *Bacteroides fragilis* abundance decreased for obese subjects[194]. Also, in one study, it is shown that reductions in the genus Bifidobacterium correlated with elevated body mass index in later childhood[195]. In our study we have found that *Bifidobacterium spp*. was decreased at 30 day old offspring of the CAF group.

Enterobacteriaceae numbers were increased for 30 day old offspring of the CAF group and tended to decrease for diabetic mothers. In the literature, one similar CAF diet study, it was shown a decrease in *Enterobacteriaceae* number for adult animals[196].

Faecalibacterium prausnitzii was increased for 20 day old offspring of the CAF diet group. Similar results were also observed in human obese child studies[197,198].

Studies have shown that higher *Lactobacillus* colonization of gut microbiota within 3 months of birth predicted risk for child obesity[89] In our study, we observed that Lactobacillus spp was increased for 20 day old offspring of the CAF group but decreased at 90 day old offspring of the CAF group vs. the controls.

As a microbiome shaper and immune system modulator, it was studied that changes and fluctuations on antimicrobial peptide expressions. To our knowledge this is the first study in literature after birth investigates the effect of maternal metabolic condition on offspring developmental periods. To see all antimicrobial family fluctuations, whole genome microarray analyses were performed. Our microarray studies showed that defensin family member (defensin RatNP-3 precursor, defensin beta 27, defensin alpha-like 1,defensin NP-4 precursor,defensin beta 43,defensin beta 51,defensin beta 52, defensin alpha 24, defensin alpha 8, defensin alpha 9) and reg family members (regenerating islet-derived 3 beta,regenerating islet-derived 3 gamma, regenerating islet-derived 1 alpha) and also C-type lectin domain family members (C-type lectin domain family 2 member D-like 1,C-type lectin domain family 2 member D2,C-type lectin domain family 2 member G, C-type lectin domain family 1 member A) were all down regulated for 20day old offsprings. For 30 day old offspring have showed also lower expression of defensin family member defensin beta 22,defensin RatNP-3 precursor,defensin alpha-like 1,defensin NP-4 precursor,defensin beta 43,defensin beta 52, defensin alpha 8, defensin alpha 9,defensin alpha 10, defensin alpha 7. The whole-genome microarray analysis for 20 and 30 day old offspring might indicate highly deficient conditions for both microbiota and immune regulations. Yet, all those changes were normalized at 90 day old offspring in the CAF group vs. RT-PCR results were validated for 7 antimicrobial peptides with respect to their change of significance in RT-PCR analysis.

Reg3 family belong to C-type lectins of the AMPs, that function as protecting body surfaces against microorganisms and also affect bacterial colonizations[199]. Reg3 mainly expressed throughout the small intestine, modulate the host defense process via bactericidal activity. It is also shown that specific knock out study of Reg3-gama have shown that REG3 gama can bind to Gram-negative and Gram-positive bacteria and influence mucus distribution in the ileum[200]. And its depletion results in increased epithelial contact with the microbiota resulting in low-grade inflammation[201]. In our study, both Reg3b and Reg3g were significantly decreased

at 20 day and normalization observed at 30 day old offsprings of the CAF group but towards to adulthood significantly higher expressions were detected at 90 day old of the CAF group vs the controls. A similar study conducted for investigation of maternally obese fetuses has shown that Reg3g expression levels increased at 19 day old fetuses[202]. Also, in our study another C-type lectin domain family member clec2g was significantly decreased at 20 and 30 day, but significantly higher expressions at 90 day old were determined.

Enteric defensins are accepted as essential regulators of microbial ecology[203]. In our study, microarray corrected defb52, defb43, defb1, defa8 were analyzed with RT-PCR. Defb52 was significantly decreased at 20 day and significantly fluctuated and increased at 30 day old offspring of the CAF group but normalized at 90 day old offspring. Defb43 was significantly higher at both 20 and 30 day old offsprings but normalized at 90 day old offspring. Defb1 was significantly decreased at 20 day and significantly increased at 30 day old offspring of the CAF group and continued significantly higher expressions at 90 day old offspring. Defa8 was significantly decreased at 20 day and 30 day, but at 90 day old offspring of the CAF group expression levels were significantly higher than control groups. At literature, similar study in which only 19 day old fetuses were analyzed for effect of maternally obesity, had found that increased gene expression level of Defa1 as defensin member of proteins[202].

Intestinal barrier function and mucosal secretions were investigated that as the mucus secretions and their degradation process is crucial for both establishment and host-microbial communication[108]. Our results have shown that the expression of Muc1 was significantly decreased at 20 day old and 30 day old and normalized at 90 day old offspring of the CAF group vs. the controls. For Muc2 expression; significantly decreased at 20 day similar to another study in which only 19 day old fetuses investigated[202]. At 30 day Muc2 have shown significantly increase and lastly normalized at 90 day old offspring. Interestingly, mucin2 protein levels were not changed statistically significant compare to the controls.

Lastly, intestinal barrier were studied via analysis of tight junction proteins

of occludin and zone-occludin from intestinal ileum region. At literature aberrations in gut barrier integrity observed in many study[204–208]. In our study we found that a significant decline of occludin levels at 20 day and 30 day and then normalization at 90 day old offspring of the CAF group vs the controls. Nevertheless, zone-occludin levels were only decreased at 30 day old offspring of the CAF group.

Since AMPs controls and inhibit bacterial contact with the gut epithelial[209], a reduction in their expression has been associated with increased bacterial adherence to the barrier [210] and bacterial translocation[211–213]. In our study we observed both decreased levels of Reg3b, Reg3g, clec2g, defb52, defb1, defa8 at 20 day may predict early bacterial translocation with the decreased gene expression level of both muc1 and muc 2 and also decreased occludin levels. Only defb43 highly expressed at 20 day old offsprings might have compensational roles.

Because Reg3g is critical to Muc2 distribution and spatial segregation of the gut epithelium and bacteria[201,214,215], if there is downregulation on these genes due to obesogenic diet, mucus layer and gut barrier defenses against microbes could be reduced and might result in bacterial contact with the epithelium, increased inflammation, tissue damage, and microbial translocation[201].

At 30 day defb52, defb1 and also muc2 expression levels became higher might be for further compensational roles. But at 30 day; both occludin and ZO-1 protein levels were also low for barrier protection.

At 90 day, Reg3b, Reg3g, defb1, defa8 clec2g became highly expressed and might cause systemic normalization as observed in both mucin gene expressions and TJ molecules. Reg3g might have a huge role for this point [201,202]. Due to the lack of formed early response mechanisms, at 90 day as increasing the expression of AMP genes organism may to regulate and protect themselves from the pro-inflammatory environment observed in high fat diets[202].

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APPENDIX

A. AMP RT-PCR Raw Datas

For microarray validation, a set of 7 genes were selected with respect to significant changes observed at microarray analysis. Selected genes analyzed with RT-qPCR. Raw data of obtained fold changes with respect to groups were shown at table A1, A2 and A3 for the 20 day (A1),30 day (A2), 90 (A3) old offsprings.

Table A1: Raw data of relative expression of selected genes: effect of maternal diabetic condition on 20 old offsprings antimicrobial peptides for CAF offspring comapre to the control group.

GENE SYMBOL	CAF 20 D4	CAF 20 D4	CAF 20 D6	CAF 20 D6	CAF 20 D7	CAF 20 D7	CAF 20 D8	CAF 20 D8	CAF 20 D10	CAF 20 D10
GENE SYMBOL										
ywhaz	16.48	18.18	19.23	18.54	20.11	19.07	20.29	19.73	19	18.57
ppip	17.2	17.76	18.54	18.54	19.65	19.03	19.08	19.79	18.66	18.52
reg3g	23.07	22.85	20.28	20.29	19.64	19.31	24.4	25	24.98	25.59
reg3b	20.73	23.31	22.34	23.43	19.43	18.2	21.97	22.89	22.04	22.04
Clec2g	22.55	22.55	21.33	21.28	24.42	23.35	23.58	24.24	23.02	22.68
Muc1	26.07	27.37	25.53	25.58	28.35	27.17	28.31	29.51	27.32	26.69
Muc2	17.68	17.79	18.4	18.45	20.63	18.11	20.32	20.32	18.94	20.27
Defb1	25.23	24.78	27.12	26.43	25.95	25.95	27.52	27.52	27.87	27.87
Defb43	28.99	31.76	33.5	32.98	28.52	28.18	28.5	28.29	27.84	27.77
Defb52	30.24	31.6	26.93	26.88	30.33	31.01	29.83	30	33.3	35.18
Defa8	17.01	17.49	17.03	16.99	20.06	18.94	20.45	19.73	19.27	19.15
	Control 20 D8	Control 20 D8	Control 20 D6	Control 20 D6	Control 20 D5	Control 20 D5	Control 20 D2	Control 20 D2	Control 20 D4	Control 20 D4
ywhaz	22.64	22.51	20.76	20.59	20.76	20.64	19.38	21.13	21.12	20.07
ppip	18.23	19.42	18.36	18.02	18.67	18.78	19.99	18.26	20.84	21.41
reg3g	14.52	13.55	17.4	16.06	14.63	13.55	18.45	18.45	17.71	17.69
reg3b	14.94	14.66	15.6	15.14	13.17	13.4	18.99	17.32	20.02	20.02
Clec2g	22.48	22.68	23.15	22.95	22.24	22.66	24.41	24.41	24.08	24.08
Muc1	28.11	28.62	27.26	27.62	27.63	27.63	28.11	28.11	28.43	28.23
Muc2	16.9	16.87	17.63	16.29	16.95	16.85	17.93	17.93	20.22	19.87
Defb1	33.23	33.23	29.61	29.61	32.86	32.62	32.22	28.53	32.26	32.26
Defb43	29.57	29.57	28.94	33.03	32.47	32.18	32.1	29.45	32.49	32.49
Defb52	32.34	33.13	29.23	29.02	32.91	31.92	27.13	26.02	27.25	27.9
Defa8	17.16	15.12	15.53	15.54	14.16	14.16	16.52	15.7	18	17.87

Table A2: Raw data of relative expression of selected genes: effect of maternal diabetic condition on 30 old offsprings antimicrobial peptides for CAF offspring comapre to the control group

GENE SYMBOL	CAF 30D5	CAF 30D5	CAF 30D8	CAF 30D8	CAF 30D10	CAF 30D10	CAF 30D9	CAF 30D9	CAF 30D7	CAF 30D7
GENE SYMBOL										
ywhaz	19.58	20.225	22.53	21.79	20.04	19	18.42	18.38	19.52	19.7
ppip	21.51	18.955	22.53	22.53	18.83	19.15	17.96	17.96	18.47	18.39
reg3g	17.28	18.38	18.86	17.2	18.33	17.21	16.5	15.76	17.91	17.6
reg3b	16.2	16.715	18.51	18.51	16.91	15.71	15.88	15.53	16.96	16.41
Clec2g	22.25	22.845	24.26	23.76	22.67	22.67	21.92	21.78	22.05	21.96
Muc1	27.07	27.28	31.79	31.79	28.62	28.39	27.01	27.78	28.66	28.66
Muc2	17.41	17.74	18.81	18.59	17.11	17.13	17.11	16.99	17.06	16.94
Defb1	31.31	29.32	30.05	30.18	31.74	29.05	30.06	28.35	28.7	29.57
Defb43	29.14	29.14	27.59	27.59	29.11	29.78	31.19	32.2	32.95	32.45
Defb52	25.35	25.95	27.86	27.98	31.71	31.78	24.09	30.78	30.78	31.56
Defa8	16.19	17.22	20.51	20.51	17.11	16.36	15.7	15.62	16.41	16.39
	Control 30D5	Control 30D5	Control 30D6	Control 30D6	Control 30D8..3	Control 30D8..3	Control 30D4	Control 30D4	Control 30D2	Control 30D2
ywhaz	21.89	22.65	20.11	19.76	20.31	20.75	19.22	19.16	20.21	20.36
ppip	19.41	19.35	19.78	20.08	19.82	18.02	18.99	18.74	19.41	19.4
reg3g	18.34	18.55	16.44	15.77	16.14	15.45	15.95	18.16	19.12	19.3
reg3b	37.62	39.04	17.66	17.66	16.38	18.12	17.68	17.67	15.92	16.07
Clec2g	22.36	21.75	22.59	22.59	22.12	23.18	21.98	21.98	22.32	22.27
Muc1	27.58	27.86	31.28	29.72	29.26	27.62	17.35	17.41	26.94	27.16
Muc2	18.89	19.78	17.7	18.69	18.5	17.1	28.08	27.6	17.76	17.87
Defb1	31.85	31.26	29.06	28.7	29.58	30.82	30.82	30.82	31.22	31.71
Defb43	34.3	34.96	30.39	30.39	30.29	30.49	30.13	29.7	28.52	28.45
Defb52	33.01	32.34	31.42	31.56	32.36	32.36	31.44	31.44	30.21	29.62
Defa8	16.57	16.18	16.79	15.93	15.4	15.59	14.94	15.02	16.04	16.08

Table A3: Raw data of relative expression of selected genes: effect of maternal diabetic condition on 90 old offsprings antimicrobial peptides. CAF offspring comapre to the control group

GENE SYMBOL	CAF 90D 1	CAF 90D 1	CAF 90D 4	CAF 90D 4	CAF 90D 5	CAF 90D 5	CAF 90D 3	CAF 90D 3	Control 90D 2	Control 90D 2
GENE SYMBOL										
ywhaz	21.09	22.12	18.91	20.48	26.95	26.82	22.63	22.74	23.52	23.02
ppip	20.12	20.13	19.25	18.99	20.03	20.03	19.94	19.66	21.53	21.48
reg3g	14.65	14.72	16.17	16.49	15.09	15.15	15.09	14.92	18.63	19.09
reg3b	13.97	14.01	12.45	12.45	22.03	21.82	15.12	15.12	18.73	18.86
Clec2g	22.56	22.54	21.71	22.18	27.83	31.07	24.18	23.84	22.89	22.91
Muc1	26.86	27.58	27.21	27.8	31.43	31.43	30.44	30.55	31.29	31.48
Muc2	15.98	15.81	15.75	15.7	23.4	23.37	18.7	19.57	18.8	18.85
Defb1	31.07	31.02	30.05	30.08	31.15	31.17	30.84	30.84	32.04	32.019
Defb43	32.23	31.26	30.84	30.84	31.14	32.49	27.07	25.35	31.43	32.17
Defb52	32.31	32.69	29.24	29.29	31.61	31.61	30.12	30.16	28.05	27.32
Defa8	15.21	15.46	13.89	13.83	21.77	21.87	18.07	18.07	16.83	16.95
	Control 90D 7	Control 90D7	Control 90D 4	Control 90D 4	Control 90D 2	Control 90D 2	Control 90D 3	Control 90D 3	Control 90D 5	Control 90D 5
ywhaz	20.96	21.07	19.38	19.62	21.12	20.07	22.74	22.74	24.11	23.2
ppip	19.09	20.07	18.02	18.91	19.89	19.58	20.92	21.31	21.97	21.67
reg3g	14.98	15.39	12.89	13.38	15.68	15.93	17.92	17.98	19.48	19.71
reg3b	14.62	14.48	12.97	12.95	17.14	17.42	17.18	17.09	19.77	19.95
Clec2g	23.12	22.87	21.87	21.94	22.69	25.89	24.53	24.61	24.81	24.65
Muc1	29.82	28.93	27.01	27.07	28.01	28.23	29.83	30.78	29.48	30.36
Muc2	17.43	17.15	15.27	15.01	16.49	17.11	18.47	18.45	21.85	21.75
Defb1	30.62	30.45	31.16	31.37	30.88	30.88	32.12	32.18	30.41	30.38
Defb43	32.06	32.06	30.7	32.62	27.85	27.85	30.31	30.31	26.1	28.09
Defb52	32.77	26.62	31.65	33.12	28.08	28.04	29.14	29.24	28.75	28.75
Defa8	15.13	15.02	14.12	14.13	16.46	16.46	17.92	17.77	20.36	20.34

B. Western Blot Images

For intestinal barrier investigation, occludin and zone-occludin1 as TJs; and for mucus level Muc2 was investigated at protein level. Whole membrane with beta actins were shown at figüre A1-A9.

Occludin

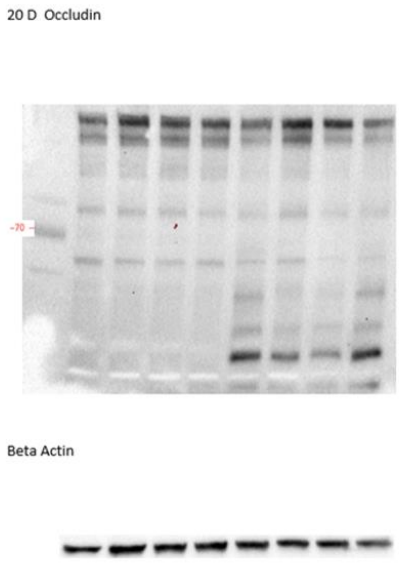

Figure A1: Western blotting analysis of occludin and β-actin expression for 20 day old offsprings. From left to right; first four column control and last four column represents CAF group offsprings.

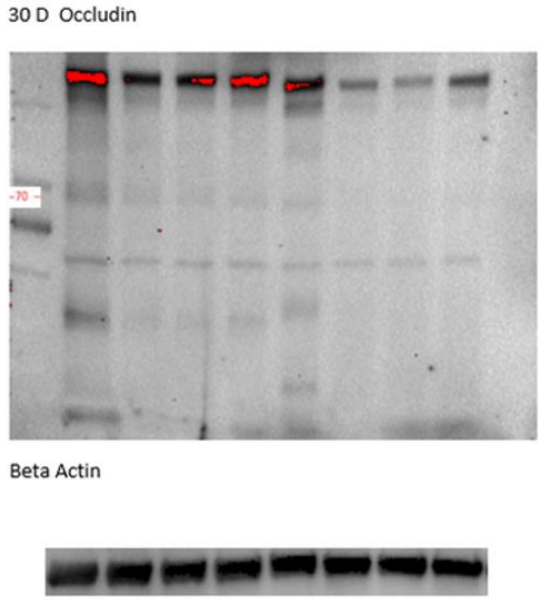

Figure A2: Western blotting analysis of occludin and β-actin expression for 30 day old offsprings. From left to right; first four column control and last four column represents CAF group offsprings.

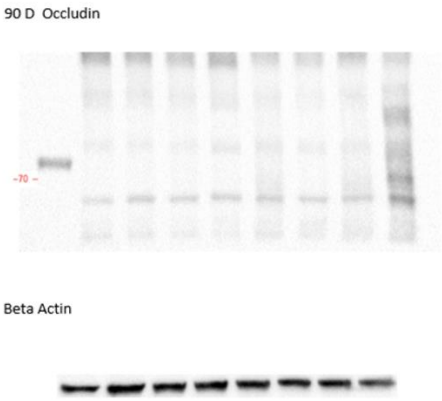

Figure A3: Western blotting analysis of occludin and β-actin expression for 90 day old offsprings. From left to right; first four column control and last four column represents CAF group offsprings

Zone-Occludin

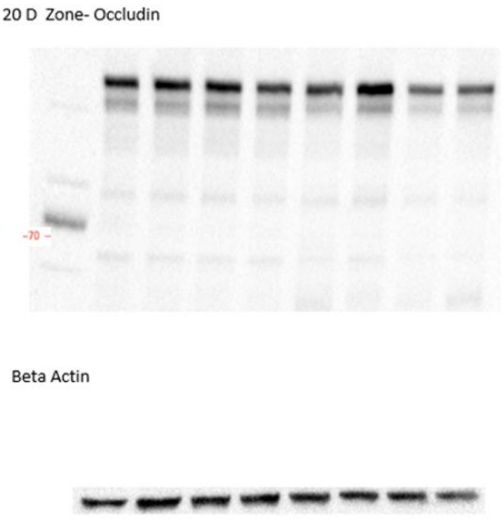

Figure A4: Western blotting analysis of ZO-1 and β-actin expression for 20 day old offsprings. From left to right; first four column control and last four column represents CAF group offsprings

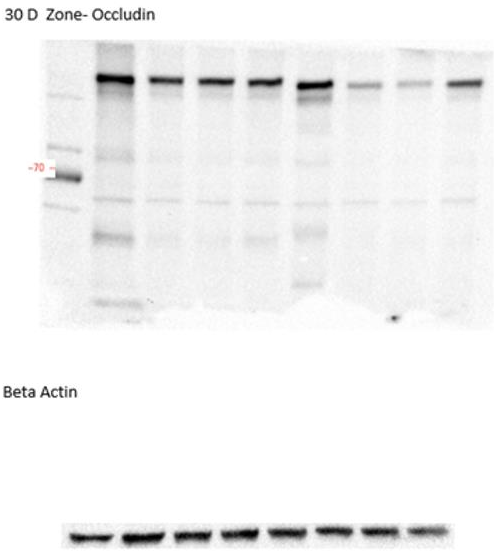

Figure A5: Western blotting analysis of ZO-1 and β-actin expression for 30 day old offsprings. From left to right; first four column control and last four column represents CAF group offsprings

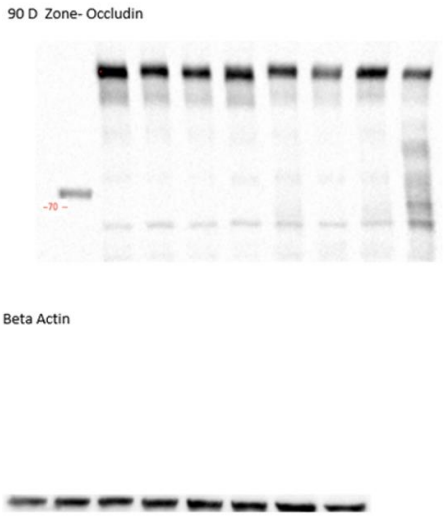

Figure A6: Western blotting analysis of ZO-1 and β-actin expression for 90 day old offsprings. From left to right; first four column control and last four column represents CAF group offsprings

Muc2

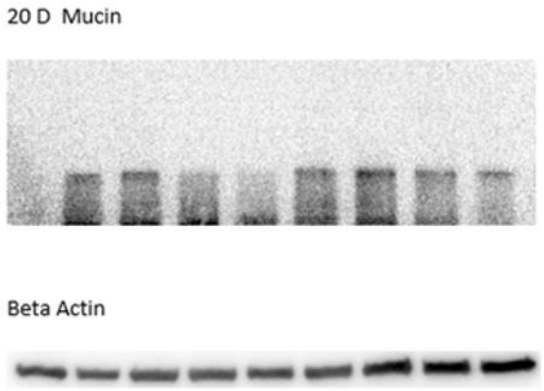

Figure A7: Western blotting analysis of Muc2 expression for 20 day old offsprings. From left to right; first four column control and last four column represents CAF group offsprings

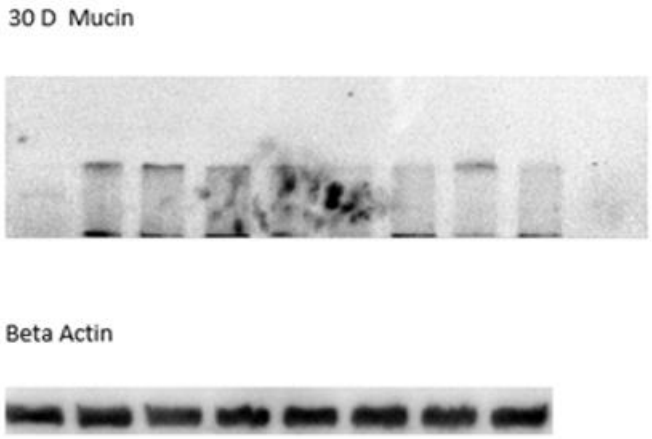

Figure A8: Western blotting analysis of Muc2 expression for 30 day old offsprings. From left to right; first four column control and last four column represents CAF group offsprings

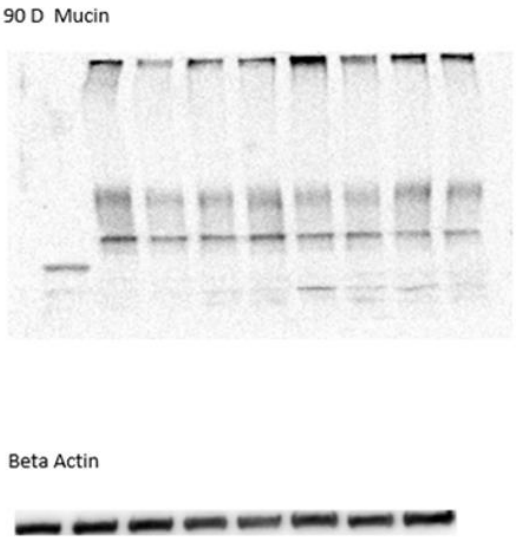

Figure A9: Western blotting analysis of Muc2 expression for 90 day old offsprings. From left to right; first four column control and last four column represents CAF group offsprings

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MS	METU Biotechnology	2014
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Year	Place	Enrollment
2020	Çankaya University	Part Time Instructor
2019	Çankaya University	Part Time Instructor
2018	Genz Biotechnology	Researcher
2018	Cellovative Biotechnology	Founder
2018	Doku Biotechnology	Researcher
2017	Umut Biotechnology	Researcher

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PUBLICATIONS

Ulum, B., **Teker, H. T.**, Sarikaya, A., Balta, G., Kuskonmaz, B., Uckan-Cetinkaya, D., Aerts-Kaya, F. (2018). *"Bone marrow mesenchymal stem cell donors with a high body mass index display elevated endoplasmic reticulum stress and are functionally impaired." Journal of Cellular Physiology*, 2018 Nov;233(11):8429-8436. doi: 10.1002/jcp.26804. PubMed PMID: 29797574.

Ceylani, T., Jakubowska-Dogru, E., Gurbanov, R., **Teker, H. T**., Gozen, A. G. (2018). *"The effects of repeated antibiotic administration to juvenile BALB/c mice on the microbiota status and animal behavior at the adult age." Heliyon*, 2018 Jun 4;4(6):e00644. doi: 10.1016/j.heliyon.2018.e00644. PubMed PMID: 29872772.

Duzkale N, Eyerci N, Oksuzoglu B, **Teker T**, Kandemir O. "*Novel BRCA2 pathogenic genotype and breast cancer phenotype discordance in monozygotic triplets."* Eur J Med Genet. 2019 Sep 26:103771. doi: 10.1016/j.ejmg.2019.103771. PubMed PMID: 31563594.