IMPACT OF ANTIBIOTIC INDUCED GUT MICROBIOTA ALTERATION ON COGNITIVE ABILITIES AND BEHAVIOURS OF MICE

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ABSTRACT

IMPACT OF ANTIBIOTIC INDUCED GUT MICROBIOTA ALTERATION ON COGNITIVE ABILITIES AND BEHAVIOURS OF MICE

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Recently arising studies with animal models show that there is a bidirectional communication between gut microbiota and central nervous system (CNS) through neural, endocrine and immune pathways which influences brain function and behavior. Apparently, gut microbiota may play a role in the regulation of anxiety, mood, cognition, and pain sensation. Gut microbiota may cause gastrointestinal disorders, such as irritable bowel syndrome (IBS), which can be comorbid with stress-related psychiatric conditions influencing memory and learning processes. In this context, overuse of antibiotics which are known to profoundly change the gut microbiota by reducing the necessary symbiotic bacteria may also influence brain functions leading to the development of anxiety-like-behavior and depression syndromes, and even cognitive deficits. The present study aimed to address this issue using an animal model. Experiments were carried on thirty 21-days old BALB/c mice. Two broad-spectrum antibiotics ampicillin (1g/L) and cefoperazone (1g/L) used in different combinations up to adult age. To screen the fluctuation of the gut microbiota populations denaturing gradient gel electrophoresis (DGGE) technique was applied. By the end of the antibiotic treatment, both the experimental and control mice were subjected to behavioral tests and biochemical assays. The behavioral test included open field (OF), elevated plus maze (EPM), novel object recognition test (NOR), and forced swim test (FST). In biochemical analyses, the level of the brain-

derived neurotrophic factor (BDNF) and Corticosterone, Serotonin receptor 5HT1A and NR2B subunits of N-methyl D-aspartate receptor (NMDA) were determined. The data were analysed comparatively. The results showed that in mice, a repeated antibiotic treatment applied during adolescence changes the gut microbiota composition and in parallel appears to influence the cognitive and affective behavior in young adults.

Keywords: Gut microbiota, DGGE, Cognitive deficits, BALB/c mice, BDNF, NR2B, 5HT1A, Corticosterone, Behavior tests, Anxiety

ÖZ

FAREDE ANTİBİYOTİK KULLANIMINA BAĞLI OLARAK BOZULAN BAĞIRSAK MİKROBİYOTASININ BİLİŞSEL YETİLER VE DAVRANIŞLAR ÜZERİNE ETKİLERİ

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Son zamanlarda özellikle deney hayvanları üzerinde yapılan çalışmalar bağırsak mikrobiyotası ile merkezi sinir sistemi (MSS) arasında çift yönlü bir iletişim olduğunu, bu iletişimin sinirsel, hormonal ve bağışıklık sistemini de kapsamakla beraber aynı zamanda beyin fonksiyonları ve davranış üzerinde de etkilerinin olduğunu göstermektedir. Bağırsak mikrobiyotası ayrıca endişe, ruh hali, bilişsel yetiler ve acıma duygusunun ifade edilmesi üzerinde de rol oynadığı görülmektedir. Bağırsak mikrobiyotası İrritabl Barsak Sendromu gibi mide ve barsak hastalıklarıyla ilişkilendirilmekte, bu durumun stres gibi psikiyatrik sorunları tetiklemesi ile hafıza ve öğrenme süreçlerini de etkileyebilmektedir. Bununla beraber günümüzde aşırı antibiyotik kullanımı mikro biyotada bulunan yararlı simbiyoz bakteri gruplarını uzaklaştırmakla beraber beyin fonksiyonları üzerine etki ederek endişeli davranış sergileme, depresyon sendromları ve hatta bilişsel eksikliklere sebep olabilmektedir. Fakat günümüzde gelişim çağında tekrar eden antibiyotik kullanımının yetişkin dönemde beyin fonksiyonları, bilişsel durumlar ve davranış üzerine olan etkilerini

araştıran çalışmalar halen sınırlı sayıdadır.Yapılan bu çalışmanın amacı yukarıda bahsedilen soruna bir deney hayvanı modeli kullanarak işaret etmektedir. Çalışmada 21 günlük BALB/c fareleri kullanılmıştır. İki farklı geniş spektrumlu antibiyotik amfisilin (1g/L), cefoperazon (1g/L) farklı kombinasyonlarda belli aralıklarla gelişim dönemine uygulanmıştır. Antibiyotik uygulaması boyunca bağırsak mikrobiyotasında değişimler denature gradient jel elektroforezi (DGGE) yöntemi kullanılarak izlenilmiştir. Antibiyotik uygulaması sonunda deney ve kontrol grupları davranış ve biyokimyasal testlere tabi tutulmuştur. Davranış testleri için açık alan, yükseltilmiş artı labirent, yeni obje tanıma ve yüzmeye zorlama testleri uygulanmıştır. Biyokimyasal analizler sonucunda beyinden türetilen nörotropik faktör (BDNF), Kortikosteron, Seratonin reseptörü 5HT1A ve N-metil-D-aspartat (NMDA) alt ünitelerinden NR2B'nin kantitatif seviyeleri belirlenmiştir. Çalışma sonunda elde edilen verilerin karşılaştırılmalı analizleri yapılmıştır. Elde edilen sonuçlar gelişim döneminde farelere uygulanan tekrar eden antibiyotik uygulaması bağırsak florasını değiştirmekle birlikte bu durumun ergin bireyin kognitif ve davranışları üzerinde etkili olabileceğini açıkça göstermektedir.

Anahtar kelimeler: Barsak Florası, DGGE, Bilişsel eksikliler, BALB/C fare, BDNF, NR2B, 5HT1A, Kortikosteron, Endişe

Dedicated to the memory of the ancestors
those who devoted their lives for the well-
being of our nation

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LIST OF ABBREVIATIONS

GM	Gut Microbiota
CNC	Central Nervous System
IBS	Irritable Bowel Syndrome
DGGE	Denaturing Gradient Gel Electrophoresis
EPM	Elevated Plus Maze
OF	Open Field
FST	Forced Swim Test
NOR	Novel Object Recognition
BDNF	Brain-Derived Neurotrophic Factor
GF	Germ-Free
SPF	Specific Pathogen Free
HPA	Hypothalamus–Pituitary–Adrenal
ELISA	Enzyme-Linked Immune Sorbent Assay

CHAPTER 1

INTRODUCTION

Developments in science for centuries have shown that phenomena of different types are both interrelated and have consequences for each other. In modern times, the stress factors affecting people within this interrelatedness and the consequent psychiatric disorders that influence the life quality of human beings have moved to the center of human life itself. World Health Report 2001 shows that depression, among these psychiatric disorders, has become the cause of "the largest amount of non-fatal burden globally" (WHO, 2001; Ustun et al., 2004). Findings of current research show that there is an intrinsic relation of co-morbidity between chronic medical and psychiatric (especially mood) disorders (Moussavi et al., 2007; Lieshout et al., 2009). For example, in addition to being a prime risk factor for myocardial infarction, it has also been observed to increase the risk of death from cardiac disorders. Also, higher rates of obesity, hypertension, dyslipidemia , metabolic syndrome and diabetes have been observed in persons with depression than those without depression (Forsythe et al., 2010).

1.1 Gut-brain communication

Although microbiology and neuroscience have developed historically as separate fields, recent studies show that microbiota; especially within the gut have great influence on physiology in general. Large-scale Metagenomics projects, such as Human Microbiome Project have shown the central role that needs to be attributed to microbiota in issues of health and disease (Dinan and Cryan, 2012). The influence of microbiota on CNS has been revealed in several recent studies (Heijtz et al., 2011; Clarke et al., 2012). According to the reports, neuroactive compounds derived from

the intestinal lumen can permeate the mucosa; cross the blood-brain barrier; and cause cognitive, psychiatric, and behavioral disturbances (Wakefield., 2002). Current research has devoted considerable attention to the role of intestinal microbes on gut-brain communication. (Figure 1.1)

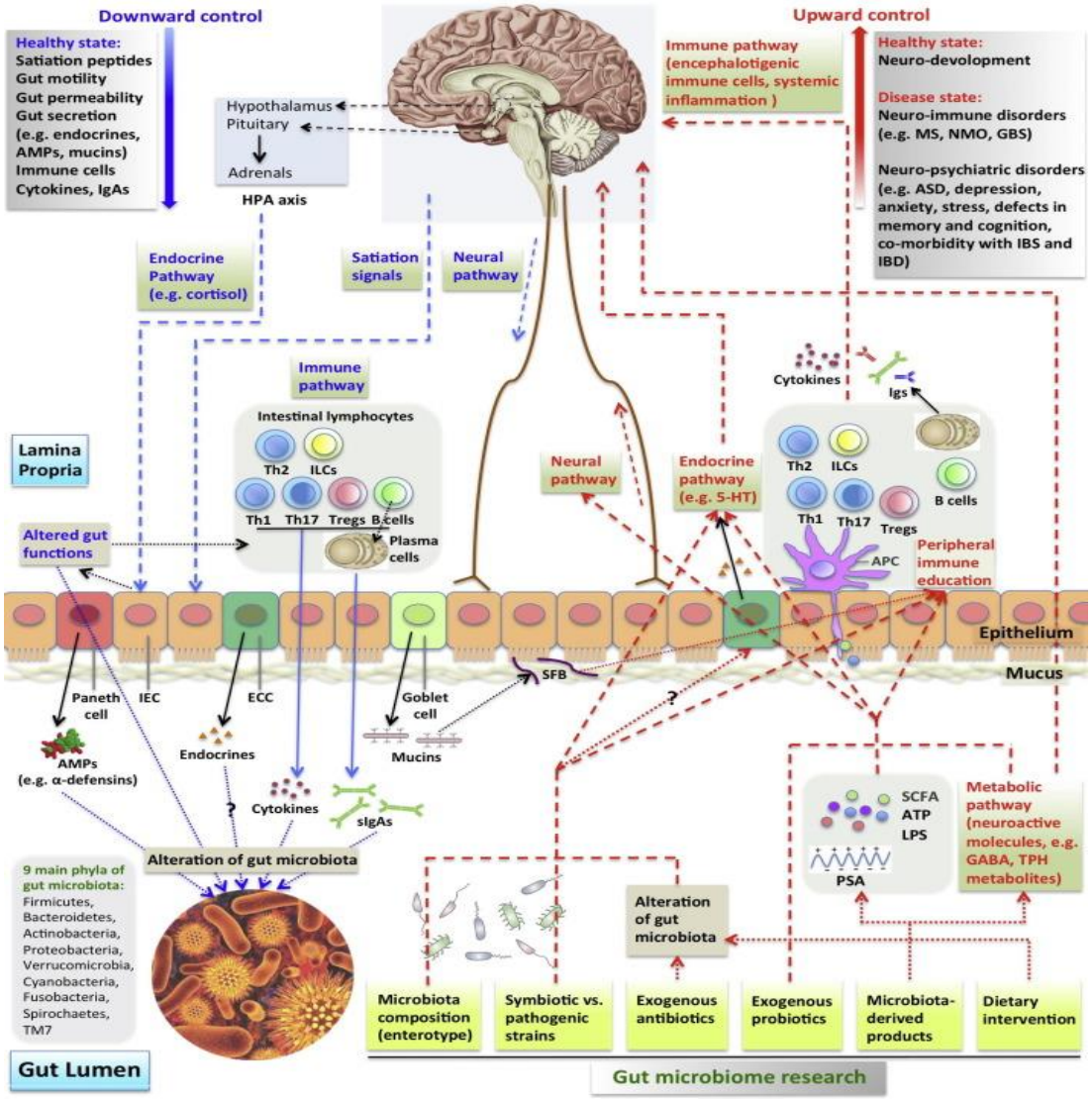


Figure 1.1 Microbiome–gut–brain axis in relation to CNS disorders. (Taken from Wang and Kasper, 2014)

1.2 Bidirectional pathways between brain-gut communications

Bidirectional pathways between brain-gut communications have been known to include autonomic nervous system (ANS), the enteric nervous system (ENS), the neuroendocrine system, and the immune system (Figure 1.2). Within this communicative web between CNS and periphery, recent studies have highlighted the importance of 'bottom-up' influence exerted by microbes on CNS, especially the commensal bacteria (Foster and Mcvey, 2013). By the way, the 'top-down' factors such as anxiety or stress might impair gut functions in such a way that diarrhea, nausea, and discomfort occur (Farmer et al., 2014). Since sporadic changes in dietary habits can cause parallel fluctuations in gut microbiota, they also have an influence on gut-brain axis and which in turn might have an influence on behavior including anxiety and depression (Luna and Foster, 2015).

The link between the gastrointestinal tract and brain functions has been recognized since mid-nineteenth century in the work of researchers such as Claude Bernard, Ivan Pavlov, William Beaumont, William James and Carl Lange. In early in 20th century Walter Cannon, the pioneering researcher in gastrointestinal motility studies had pointed to the effects of brain co-processing in the modulation of gut functions. In current studies, more and more attention is paid to the bidirectional nature of the gut-brain axis which operates through neural, hormonal, immunological routes, and it is postulated that dysfunction in this interactive communication system can have pathophysiological consequences. Also, the gut itself has a dense neural network which is capable of functioning independently, even after the connection to CNS has been cut off. Within the bidirectional network, while signals from the brain have an impact on the motor, sensory, and secretory functions of the gastrointestinal tract, the gut can influence brain functions, particularly stress regulation areas such as the hypothalamus. (Cryan and Dinan, 2012; Reber, 2012)

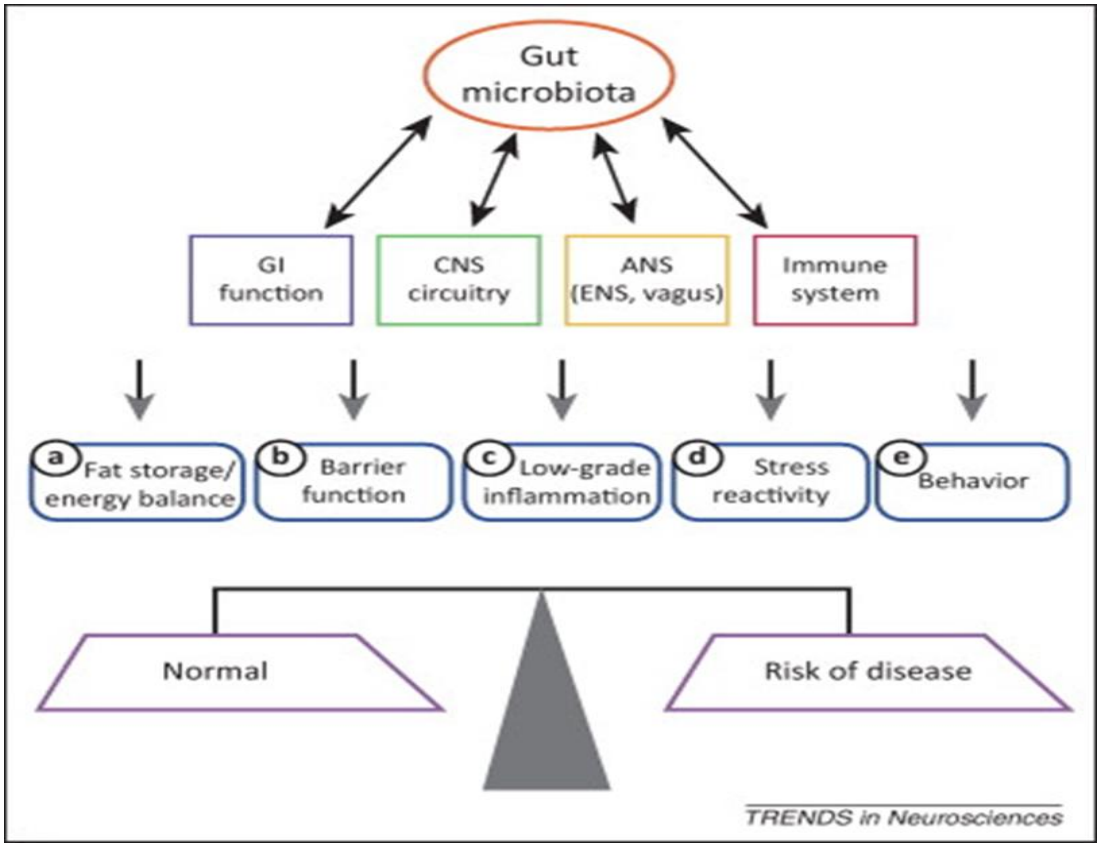


Figure 1.2 Bidirectional communication between gut microbiota and components of the gut-brain axis influence normal homeostasis and may contribute to the risk of disease. Alterations in gastrointestinal (GI). The central nervous system (CNS), autonomic nervous system (ANS), and immune systems by microbiota may lead to alterations in (a) fat storage and energy balance; (b) GI barrier function; (c) general low-grade inflammation (GI and systemic); (d) increased stress reactivity; and (e) increased anxiety and depressive-like behaviors. Each of these mechanisms has implications for the pathophysiology of mood and anxiety disorders. Abbreviation: ENS, the enteric nervous system (Taken from Cryan and Dinan, 2012).

Thanks to detailed research on how the brain regulates the enteric nervous system and gastrointestinal functions, it is known that alterations in brain-gut interactions are associated with gut inflammation, chronic abdominal pain syndromes, and eating disorders. Furthermore, it is postulated that alterations in stress response and behavior can be attributed to changes in gut-brain axis functioning (Rhee, 2009). Co-

morbidity of psychiatric symptoms such as anxiety, and gastrointestinal disorders, including irritable bowel syndrome (IBS) and inflammatory bowel disorder which occur as a result of gut-brain axis malfunctioning prove its particular importance in pathophysiological studies (Reber, 2012). Intervention on the gut-brain axis is becoming a novel means of developing treatments for a wide range of disorders as obesity, mood and anxiety disorders, and gastrointestinal disorders such as IBS (Cryan and Dinan, 2012) (Figure 1.3).

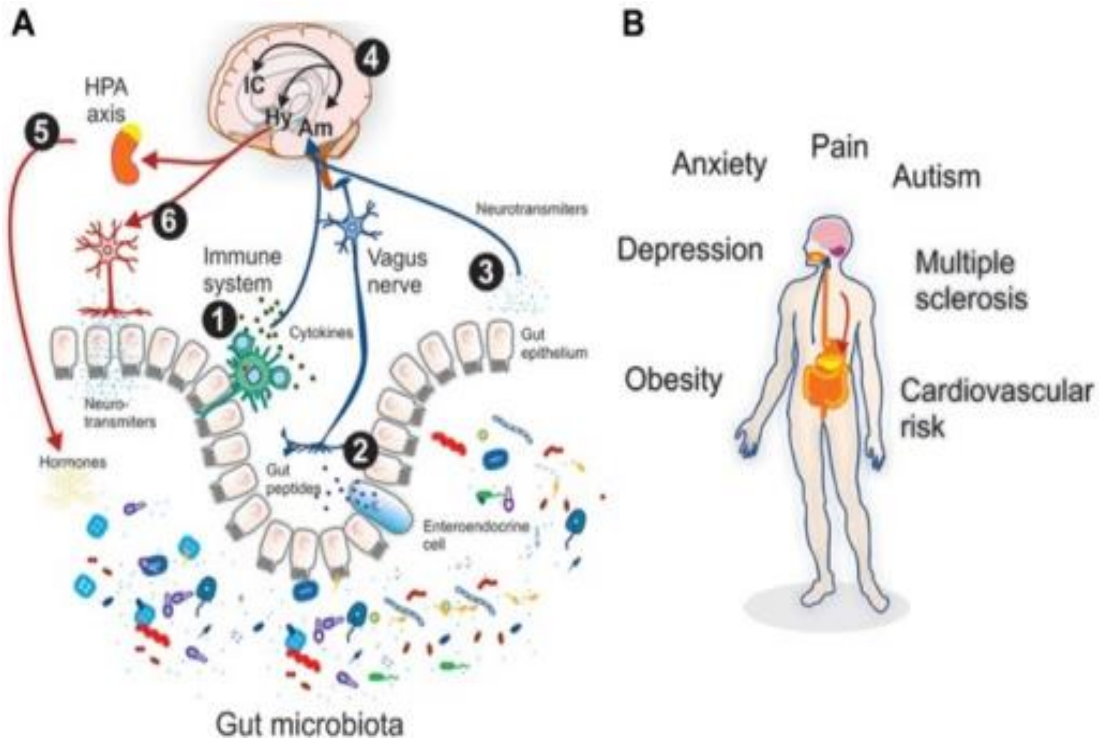


Figure 1.3 Intervention on the gut-brain axis A) Gut-brain axis, 1) lymphocytes, 2) Sensory neuronal terminals, 3) Neurotransmitters, 4) Central, 5) Corticosteroids, 6) Neuronal efferent. B) Health conditions. (Taken from Montiel-Castro et al., 2013)

GI tract and ENS are modulated by the CNS via sympathetic and parasympathetic branches of the ANS, as well as via the HPA axis. It is said that this kind of influence can change the enteric microbiota in an indirect way by modifying its environment and directly via numerous signaling molecules (Figure 1.4) (Rhee et al., 2009). Two types of ANS regulate gut functions through the production of bicarbonates, secretion of acid and mucus, regional motility, the permeability of the intestine, maintenance of epithelial fluid, and the mucosal immune response (Figure 4) (Mayer et al., 2000). Except for cortisol-mediated immune regulation, almost all of these functions are affected by sympathetic and parasympathetic neurons on the circuits of the ENS. Furthermore, the enteric microbiota, nutrient delivery rate (such as prebiotics, including certain dietary fibers and resistant starches), gas composition, and other aspects of the luminal environment are expected to be under regional and overall modifications during GI transit (Mayer et al., 2017).

Impaired intestinal transit has been related to overgrowth in the small intestine that is under parasympathetic modulation (Van Felius et al., 2003). In patients with slow-transit constipation a smaller amount of giant migrating contractions in the colon has been discovered, and in some patients, this is thought to be a likely contributor to the symptoms of IBS as well as constipation. On the other hand, the acceleration of intestinal transit, characterized by a higher number of giant migrating contractions was found in diarrhea cases (diarrhea-predominant IBS (Chey et al., 2001). Besides, factors such as the frequency of food intake, stress, and quality of sleep interfere with the frequency of regular migrations in motor complexes. Furthermore, reduced vagal output to the stomach and increased parasympathetic output to the large and small intestine are also associated with acute stress (Mayer et al., 2000). Although there are yet no studies on the outside setting of bacterial overgrowth, the gut transit alterations usually have a higher impact on the composition and organizational structure of gut microbiota in more than one region of GI tract. It has been detected that the size and quality of the intestinal mucus layer, an important habitat for the biofilm microorganisms, is affected by the ANS-mediated modulation. In this habitat, almost all-enteric microbiota reside (Macfarlane and Dillon, 2007). Also, ANS can modify epithelial mechanisms of the immune system involved in the activation by the gut. This activation can occur in two ways: either directly through

the response modulation of the gut immune cells such as mast cells and macrophages to luminal bacteria with the antimicrobial peptides (Alonso et al., 2008) or indirectly by modifying the access of the luminal bacteria to immune cells of the gut. For instance, many of the preclinical studies have demonstrated that, in the intestinal mucosa, stressful stimuli can aid translocation of luminal organisms, increase the permeability of the intestinal epithelium, and stimulates an immune response (Groot et al. 2000; Keita and Soderholm, 2010).

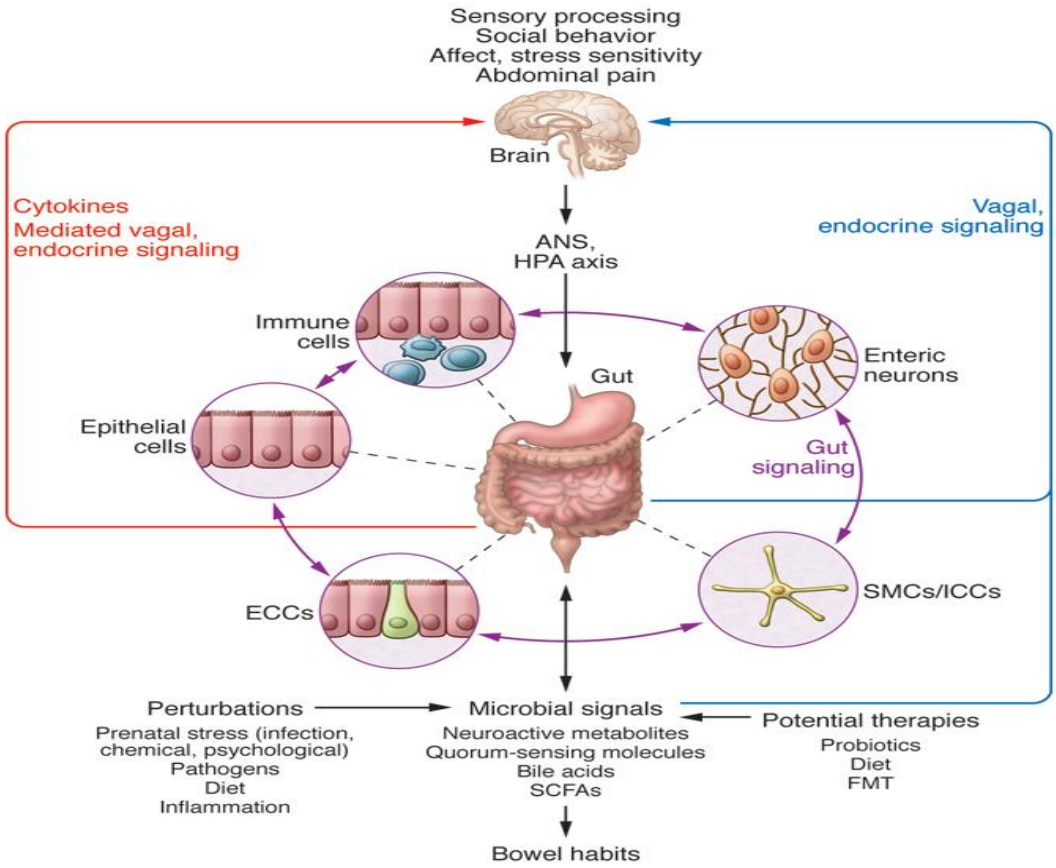


Figure 1.4. Bidirectional interactions within the gut microbiota/brain axis. (Taken from Mayer et al., 2017)

1.2.1 Modulation of gut microbiota by host-derived signaling molecules

CNS-induced changes in the gut composition are modulated by neuroendocrine and neuronal signaling. Signaling molecules, including but not limited to catecholamines, GABA, dynorphin, serotonin, and cytokines; may also be released into the gut lumen by neurons, enterochromaffin, and immune system related cells (Lyte and Freestone, 2010; Lyte et al. 2013; Yang et al., 1992; Santos et al., 1998). Catecholamines are an especially well-investigated case of signaling molecules which enable direct host-to-microbe signaling. Various kinds of stressors can increase plasma and local levels of catecholamines such as norepinephrine and also raise luminal levels in the gut (Alverdy et al., 2000; Hughes and Sperandio, 2008). Some of the pathogens can change their spawning activity in response to external catecholamines in vitro (Lyte et al., 2004). For example, the proliferation of several strains of enteric pathogens can be triggered by norepinephrine. Similarly, the virulent properties of *Campylobacter jejuni* can be increased by norepinephrine (Hughes et al., 2008; Cogan et al., 2007). Still, the influence of catecholamines on nonpathogenic organisms and other microbial signaling molecules on gut metabolic activity and microbiota composition in diseased and healthy individuals is not known (Mayer et al., 2017).

1.2.2 Microbe-to-host signaling by microbial-generated signal molecules

Upon establishing the fact that the gut microbiota can communicate with ENS (Frosythe and Kunze 2013) and the brain, some of the microbe-generated signal molecules have been characterized (Figure 1.4). Quorum-sensing molecules, such as metabolites and neurotransmitter homologs, are used by microbes to communicate with each other. These molecules are also recognized by host cells and may influence immune cells, enteroendocrine cells, and nerve endings in the gut (Rhee et al., 2009). During the immune response, metabolites produced by gut microbiota, including bile acids, SCFAs, and neuroactive substances such as tryptophan precursors, GABA, serotonin, catecholamines, and cytokines are released. They can signal the host via receptors on local cells within the gut (Chey et al., 2001; Bailey et al., 2011). Moreover, a high amount of metabolites found in the circulation

originates from gut microbiota. These metabolites theoretically constitute a basis for a vast gut microbiota–to–brain signaling system (Wikoff et al., 2009).

Fermentable carbohydrates, including acetate, butyrate, and propionate reach into the colon and are transformed into SCFAs (well-known examples of microbe-derived metabolites). Main SCFAs have a number of physiological effects, containing reduction of food intake, modulation of lymphocytes, improvement of glucose tolerance, neutrophil functions, and activation of epithelial cell signaling pathways (Cummings et al., 1987; Nepalska et al., 2012; Cani et al., 2013). For instance, a diet supplemented with *Bifidobacterium breve* caused high fatty acid concentrations in the brain; yet the mechanisms lay beneath these effects are not known yet (Wall et al. 2012).

The microbiota can influence relations between the gut and the nervous system through multiple different mechanisms (Figure 1.5). Without taking the succession of events causing a state of dysbiosis in a particular disorder into consideration, the bidirectional communication between the gut and brain is likely to be affected by alterations in the microbial composition. Such effects may emerge early in life and affect the development of the nervous system. The HPA axis, and the brain's interaction with the intestine; in adults, may act on completely developed circuits (Figure 1.4)(Cryan et al. 2012; Forsythe and Kunze 2013; Bercik et al., 2012). Perhaps, some of these signaling mechanisms operate in the presence of an intact epithelium (e.g., by vagal signaling). However, the effects are likely to be enhanced and changed in the context of increased intestinal permeability induced by mucosal inflammation or stress (Soderholm et al., 2001; Hsiao et al., 2013; Leclercq et al., 2014).

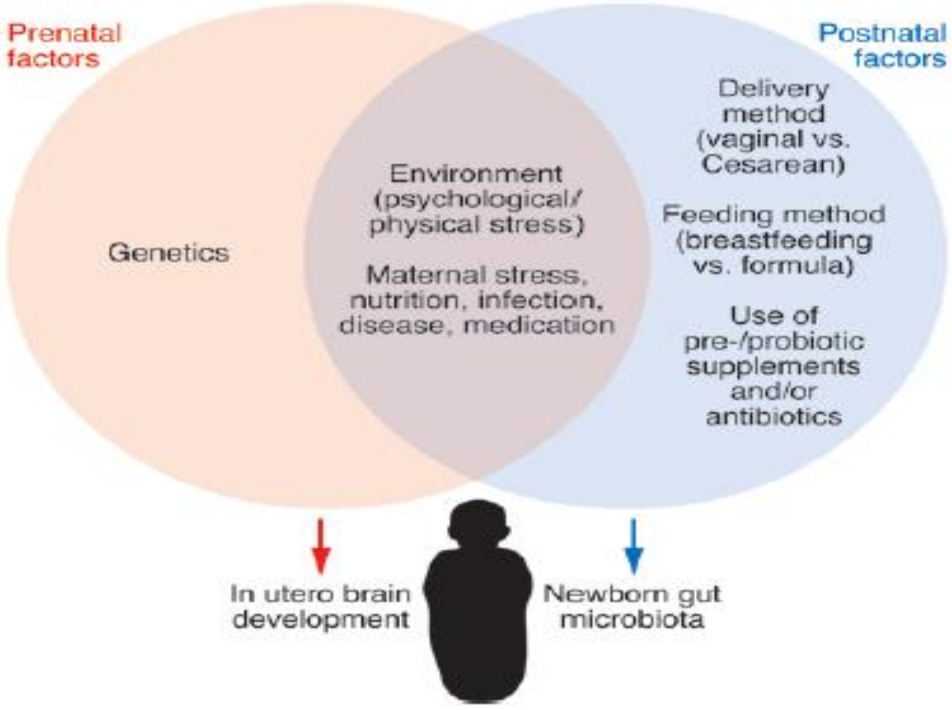


Figure 1.5 Influences on the gut microbiota/brain axis in the perinatal period. (Taken from Mayer et al., 2017)

1.2 The human gut

It is at birth sterile. The human gut plays a role in metabolic and protective functions, ranging from creating important vitamins and amino acids to protecting cells that line the gut from injury (Figure 1.6). Right after birth and within the first year, the bacterial colonization occurs which is a process that shows immense temporal and individual fluctuations of bacterial populations. The bacterial profile established during this period, which in general has a maternal signature, converges with that of the adult, and it has been observed that despite drastic influences by disease, infections, stress, and diet, this intestinal microbiome profile tends to revert to that established in infancy. However, "the core microbiota of the aged individual is distinct from that of the younger adults" (Cryan and Dinan, 2012).

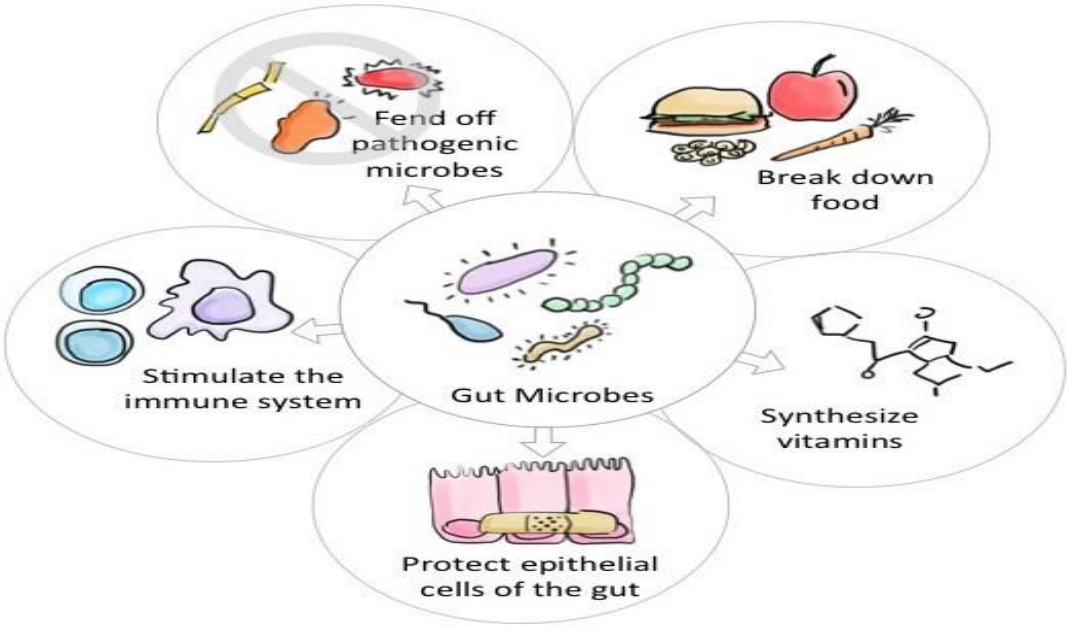


Figure 1.6 Main functions of human gut microbes. (Taken from http://sitn.hms.harvard.edu/flash/2016/second-brain-microbes-gut-may-affect-body-mind/)

The complexity of microbial communities did not allow the application of traditional approaches that rely on isolating microbes in cultures to create a complete map of the intestinal microbiome. This culture-based analysis is only suitable for microbiota that can be cultivated. However, recent developments in molecular microbiology technologies have enabled genetic analysis of complex microbial populations (Forsythe et al., 2010). These culture-independent techniques include sequencing, genetic fingerprinting, fluorescently oligonucleotide probes (FISH), quantitative PCR as well as metagenomics approaches (Archie and Theis, 2011).

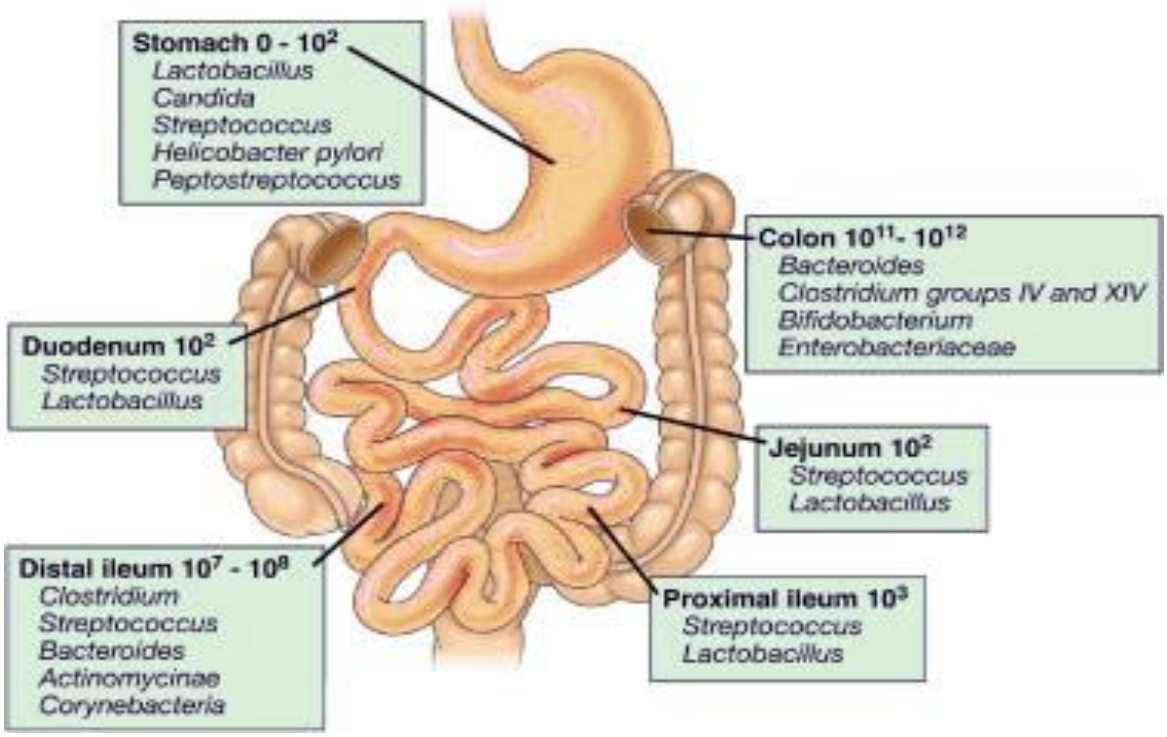


Figure 1.7 Human gut. (Taken from http://www.wright.edu/~oleg.paliy/research.html)

Concerning the species which constitute the gut microbiota, we know that the adult microbiota has more than 1000 species and approximately 7000 strains. Despite the variation of species within the human microbiota Bacteroidetes and Firmicutes are bacterial phyla are found predominantly, whereas Proteobacteria, Actinobacteria, Fusobacteria, Verrucomicrobia phyla appear in relatively low quantities. Furthermore, these partially genetically determined microbiota populations are thought to be divided into three distinct entero-types which are defined by their bacterial composition. Each enterotype is characterized by a relatively high level of single microbial genus: *Bacteroides* spp.; *Prevotella* spp. or *Ruminoccus* spp. (Figure 1.7**).** The balance between the three enterotypes is highly influenced by diet; for example, the *Prevotella* spp. enterotype has been associated with high-carbohydrate diets, whereas the *Bacteroides* spp. has been associated with high fat or high protein diets (Cryan and Dinan, 2012).

1.4 Strategies used to investigate the role of the microbiota-gut-brain axis

Germ-free animals, animals exposed to probiotics or antibiotics, animals with bacterial infections are subjects of studies dealing with the elucidation of the role of gut microbiota on behavior and cognition (Figure 1.8). Because of their role in the psychiatric disorders such as anxiety and depression, most of these studies highlight a role for the microbiota in modulating stress-related behaviors and stress response (Cryan and Dinan, 2012).

Although the microbiota-gut-brain axis is a relatively new concept, studies about communication along this axis have been depicted through different, converging means. To assess how the loss of microbiota during development affects CNS function, germ-free mice have been the most useful experimental animal. It is worth noting that the clinical translation of such analyses is limited, as no equivalent obliteration of the microbiota can be said to exist in humans. However, the impact of a particular entity (for example, a probiotic) on the microbiota-gut-brain axis in isolation can be researched on germ-free mice (Cryan and Dinan, 2012).

Infection studies conducted through activation of the immune system have been used to assess the effects of pathogenic bacteria on behavior and brain (Cryan and Dinan, 2012).

Another method of manipulation of the gut microbiota is by application of probiotics which enhances the microbiota. Administration of various potential probiotic strains in adult animals or humans can be used to assess the effects of these bacterial 'tourists' on the host. A recent study, the investigators fed healthy male Balb/C mice, *L. rhamnosus*, with probiotics, revealed that the mice displayed decreased anxiety-like and depressive-like behavior in the Forced Swim Test (FST), Open Field (OF) and Elevated Plus Maze (EPM) (Bravo et al., 2011). It was observed that these mice spent less time immobile in the FST, made more entries into the open arms than

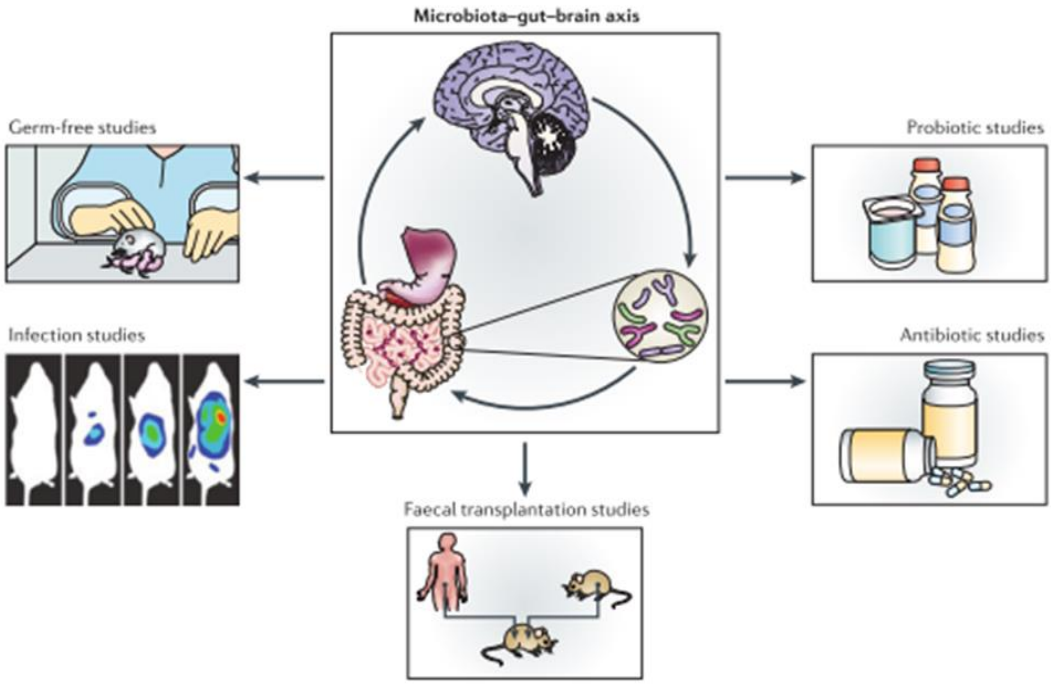


Figure 1.8 Strategies used to investigate the role of the microbiota-gut-brain axis in health and disease (Taken from Cryan and Dinan, 2012).

closed of the EPM, and spent more time in and made more entries into the center of the OF. Another study which separated new-born mice from the maternal environment and treated with probiotic *B. infantism* showed less depressive-like symptoms, which paralleled with mice treated with anti-depressant citalopram (Desbonned et al., 2010).

Finally, administration of antimicrobial (such as antibiotics) drugs can be used to perturb gut microbiota composition in a temporally controlled and clinically realistic manner and is, therefore, a powerful tool to assess the role of the gut microbiota on behavior. Broad-spectrum antibiotics in drinking water have been administered orally to mice to make intestinal dysbiosis. For example, an antibiotic cocktail including neomycin, bacitracin and the antifungal agent primaricin applied to mice resulted in reduced anxiety-like behavior assessed in step down, light/dark and passive avoidance tasks. It is noted that Brain-Derived Neurotrophic factor (BDNF) levels in ATM-treated mice were much higher in the hippocampus and low in the amygdala compared to control mice. When the antibiotic treatment was stopped, the

altered levels of BDNF protein in the amygdala and hippocampus returned to normal, and the mice started displaying normal phenotypic behavior (Bercik et al., 2011).

1.5 Role of gut-microbiota in animal behavior and cognition

The importance of microbiota in the development of brain systems, stress reactivity, and stress-related behavior has been demonstrated in some neuroscience research. The gut-brain axis is influenced by changes in the gut microbiota, and this then influences behaviors including depression and anxiety (Luna and Foster, 2015) (Figure 1.9).

In the past few years, thanks to the animal studies the link between gut microbiota and stress-related behaviors have been revealed (see Table 1.1 for a summary). One of the first studies conducted by Sudo et al. (2004), GF mice showed anxiety-like behavior in the elevated plus maze (EPM), used to test the anxiety-like behavior in mice, compared to specific pathogen free (SPF) mice. Neufeld et al. (2011) demonstrated that the low anxiety-like phenotype could result from long-term changes in plasticity-related genes in amygdala and hippocampus. It also demonstrated that gut-brain interaction influence CNS wiring early in life because of the low anxiety-like behavior phenotype observed in GF mice continued after colonization with SPF intestinal microbiota. Also, using the EPM test Heijtz et al. (2011) reported reduced anxiety-like behavior in GF mice and using the light-dark test, as another way to assess the level of anxiety-like behavior. Nishino et al. 2013 by using a different strain of mice (Balb/C compared to Swiss Webster and NMRI) demonstrated that offspring of colonized GF mice had reduced anxiety-like behavior in the open field OF test when compared to GF mice.

In a different experiment disruption of the microbiota using an infectious bacterium, *Citrobacter rodentium*, caused increased anxiety in the open field apparatus in mice tested 7-8 hours following infection. Additionally, the study found that after 10 and

30 days after infection, mice showed memory dysfunction. Behavioral changes were reversed by administering a probiotic cocktail. Memory dysfunction was also developed in GF mice that were infected with the same infectious agent (Cryan and Dinan, 2012). Such studies suggest that the effect of microbiota can extend to memory and cognition. Similar results were obtained in an experiment comparing GF and SPF mice (Gareau et al., 2011). Furthermore, changes imposed on microbiota through dietary means were shown to influence memory in a study by Li et al., (2009). Mice fed on high content of ground beef for three months showed much higher diversity in gut microbiota compared to mice fed with standard rodent food. Results showed increased anxiety-like behavior, slower speed in seeking food, but improved working and reference memory (Bercik et al., 2012).

1.6 Gut microbiota and the CNS

As it has been stated above, there exists communication between the gut microbiota and the CNS. The channels of this communication include "the neuroendocrine, neuroimmune, the sympathetic and parasympathetic arms of the nervous system and the enteric nervous system" while the gastrointestinal tract provides the framework for these pathways. The bidirectional communication between gut-brain axis includes microbiota influencing CNS function and the CNS influencing the microbiota composition, but which mechanisms are involved in this communication is not fully understood (Cryan and Dinan, 2012).

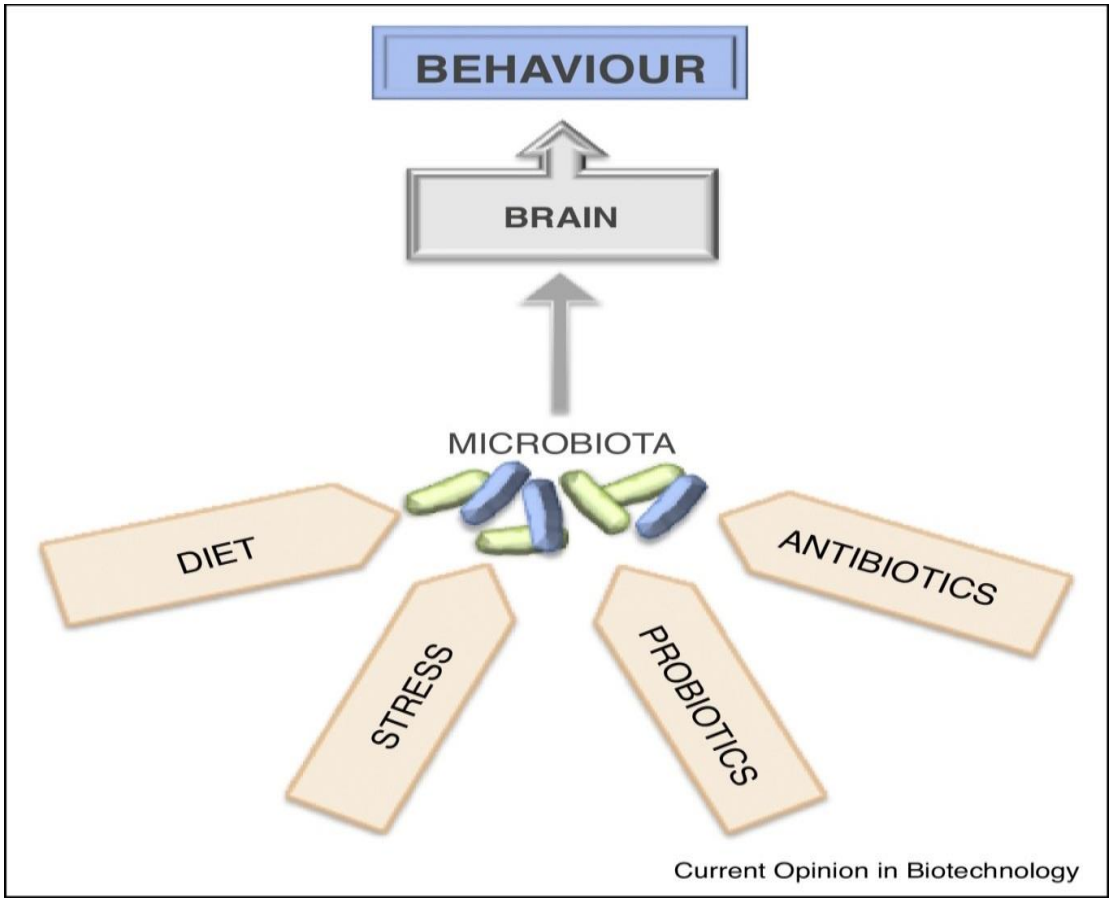


Figure 1.9 Factors influencing the gut-brain axis via microbiota. As reviewed in the article diet, stress, probiotics, and antibiotics have an impact on gut microbiota community. In turn, microbiota influences brain pathways and thereby has an impact on behavior. (Taken from Foster and MvVey, 2013)

1.6.1 Hypothalamus–pituitary–adrenal (HPA) axis

Although it has always been known that hypothalamus-pituitary-adrenal (HPA) axis influences the composition of gut microbiota, up to date research has focused mainly on the impact of microbiota on CNS function and stress perception (Cryan and Dinan, 2012). Another fact known about the HPA is that damage on the HPA system is a major cause of depression. Furthermore, such damage to the system has been proposed as a causal factor in diseases under the "hypothalamic-pituitary-cortisol hypothesis" which is based on the prevalent observation in patients with

Table1.1 Previous studies exploring the link between microbiota and behavior

Reference	Model used	Behavioural tests performed	Conclusions
Sudo *et al*. 2004.	GF, SPF, and gnotobiotic mice	Acute restraint test	Microbial exposure at an early developmental stage regulates the development of the HPA stress response
Heijtz *et al*. 2011.	GF and SPF mice	Open field test; light-dark box test; EPM	Microbial colonization at key developmental time points affects brain development and behavior
Neufeld *et al*. 2011.	GF and SPF mice	EPM	Microbial composition of the gut influences development of behavior
Nishino *et al*. 2013.	GF, gnotobiotic and commensal fecal-microbiota-associated mice	Open field test; marble burying test	Gut microorganisms have an impact on anxiety and modulate brain development and behavior
de Theije *et al*. 2014a,b.	Murine mouse model of ASD	Social behavior score (time spent near unfamiliar gender-matched mouse)	Gut microbiome associated with autism-like behavior found to correlate with decreased serotonin and social behavior scores

Table 1.1 Continued

Park *et al*. 2013.	Surgical intervention mouse models — bilateral olfactory bulbectomy or intracerebroventricular infusion pumps	Tail suspension test; step-down test; open field test; water avoidance stress test	Induced chronic depression alters the microbial profile of the colon via activation of the HPA
Aguilera *et al*. 2013.	Broad spectrum antibiotic-treated mouse model	Water avoidance stress test; intracolonic capsaicin-evoked visceral pain;	Antibiotics combined with stress decreased total bacterial count, altered community composition, and ultimately led to modulation of visceral sensitivity
Xu *et al*. 2014.	Rat model treated with rifaximin	Chronic water avoidance stress test; repeat restraint stress test	Rifaximin led to an abundance of *Lactobacillus* s pp., with treatment preventing intestinal abnormalities and visceral hyperalgesia in response to chronic stress
Desbonnet *et al*. 2010.	Probiotic trial in mouse model of maternal separation (*B. infantis*)	Forced swim test	*B. infantis* reversed some of the behavioral deficits caused by maternal separation
Messaoudi *et al*. 2011a,b.	Probiotic trial in rats and humans (*L. helveticus* and *B. longum*)	Rats — conditioned defensive burying test; Humans — Hopkins Symptom Checklist; Hospital Anxiety and Depression Scale; Perceived Stress Scale; Coping Checklist	Probiotic combination of *L. helveticus* and *B. longum* reduced anxiety-like behavior in rats and had a beneficial effect on signs of anxiety and depression in humans

Table 1.1 Continued

Bravo *et al*. 2011.	Probiotic trial in mice (*L. rhamnosus*)	Stress-induced hyperthermia; EPM; fear conditioning; forced swim test; open field test	Administration of *L. rhamnosus* reduced anxiety and depression-related behaviors
Ait-Belgnaoui *et al*. 2012.	Probiotic-treated rat model (*L. farciminis*)	Partial restraint stress test	Probiotic treatment attenuated the HPA response to acute stress
Ohland *et al*. 2013.	Probiotic trial in mice on standard chow or Western diet (*L. helveticus*)	Barnes maze	The efficacy of *L. helveticus* on the reduction of stress and anxiety is dependent on diet
Hsaio *et al*. 2013.	MIA model of autism treated with *B. fragilis*	Open field exploration; marble burying; social interaction; adult ultrasonic vocalization	*B. fragilis* modulated select gut and behavioral symptoms
Ait-Belgnaoui *et al*. 2012.	Probiotic-treated mouse model (*L. helveticus* and *B. longum*)	Water avoidance stress test	Pretreatment with probiotics can reduce chronic-stress induced abnormal brain plasticity and reduction in neurogenesis

Table 1.1 Continued

Bruce-Keller *et al*. 2014.	Microbiome depletion of mice on a normal chow diet with subsequent microbiota transplant from material obtained from high-fat diet mice	EPM; open field test; marble burying test; fear conditioning memory task	The resulting microbiome from a high-fat diet is capable of disrupting brain physiology and function even in the absence of obesity
Naseribafrouei *et al*. 2014.	Adult patients of Innlandet Hospital, Norway with and without a diagnosis of depression	Montgomery-Asberg Depression Rating Scale to rate severity of depression	Sequences identified as *Oscillibacter* and *Alistipes* spp. showed a significant association with depression

(Modified from Luna and foster 2015)

depression with elevated plasma cortisol levels, elevated corticotrophin-releasing hormone (CRH) levels in the cerebrospinal fluid and increased levels of CRH messenger RNA and protein in limbic brain regions (Burkea et al., 2005; Merali et al., 2004).

Stress related to HPA impairment is considered to be a cause of mood disorders, and it has also been shown to influence the composition of the gut microbiome. Microbial endocrinology has developed as a new field of microbiology that aims at explaining the effects of stress on the microbiome (O'Mahony et al., 2009). To show the effect of gut microbiota on HPA axis, the response of HPA to stress was compared using germ-free (GF), specific pathogen free (SPF) and gnotobiotic mice. When compared to SPF mice, GF showed exaggerated ACTH and corticosterone elevation that was caused by restraint stress (Figure 10). "This hyper-response of the HPA axis was reversed by using a single organism, *Bifidobacterium infantis*" (Forsythe et al., 2010).

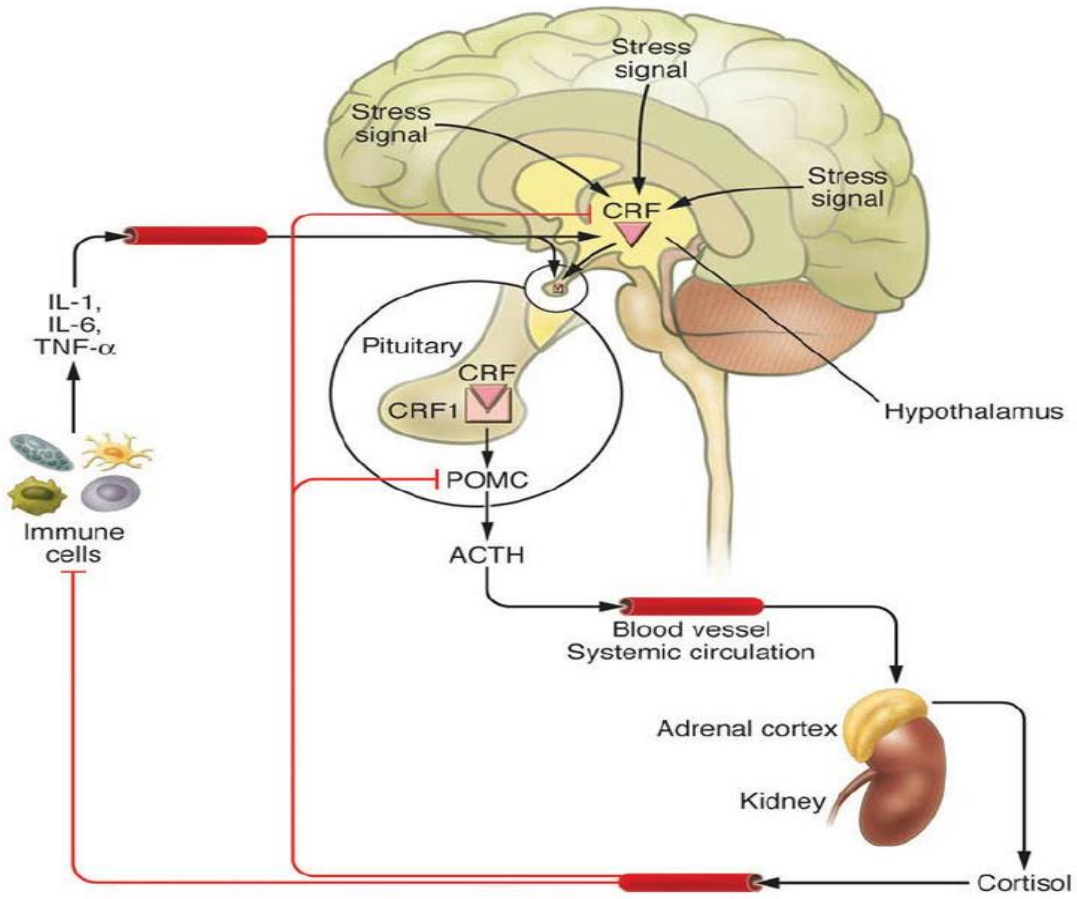


Figure 1.10 Organization of the systemic HPA axis (from Slominski, 2007)

Using germ-free mice has allowed us to achieve valuable information regarding the effect that microbiota has on the development of the HPA. Alternatively, administration of antibiotics or deliberate infection in preclinical studies can be used to bring about the dysbiosis of the enteric flora. Broad-spectrum antibiotics disturb microbiota and intervene with the ongoing process of colonization by reducing the biodiversity. Some studies have used antibiotics to create reproducible changes in the development of the microbiome**.** It is postulated that "the microbiota content of the gut is critical for the development of an appropriate stress response later in life and there is a narrow window in early life where colonization must occur to ensure normal development of the HPA axis" (Cryan and Dinan, 2012).

1.7 Gut microbiota and brain development

After birth, a newborn's gastrointestinal (GI) tract is populated with microbes from the environment that are now known to play a critical role in the development of the brain (Douglas-Escobar et al., 2013). Due to the relevant role-played by perinatal influences during the development of the nervous system and the properly documented adverse effects on early life influences the gut/brain axis, it is important to implicate the gut microbiota in these processes (Figure 1.5) (Clarke et al., 2014; Stilling et al., 2014). There are many studies that have compared adult brain chemistry, behaviors and physiological responses. For instance, activation of the HPA axis, between animals born into and raised in a laboratory cage or GF environment differed in the chemistry, behaviors and physiological responses. However, a small number of studies have documented their findings showing how gut microbiota plays a role in the effect of early adverse life events on adult behavior. Long-lasting consequences of early life perturbations of the gut microbiota on adult phenotypes come from recent studies of the effects of early life antibiotic administration on adult metabolism and visceral pain sensitivity. Prenatal and postnatal stress model in an animal can cause modifications in the composition and total biomass of the enteric microbiota (Cox et al., 2014; O'Mahony et al., 2014).

1.7.1 Prenatal stress model

Wide examples in preclinical literature have shown the effects of perinatal stress on the adult CNS, including the HPA axis (Ladd et al., 2004), and brain systems related to pain modulation (Coutinho et al., 2002), emotion (Meaney et al., 2001), and in intestinal function (Coutinho et al., 2002). The behavioral effects of perinatal stress documented in rodent models. The studies showed high translational validity for various human diseases which include the functional GI disorders (Mayer et al., 2001; Mayer and Collins, 2002) and psychiatric disorders (Heim et al., 2001) in which early young life events appeared to be a decisive factor in vulnerability. Even so, there is plenty of evidence indicating the relation of gut microbiota in the perinatal stressors related to the brain and behavioral changes. The early documents dealing with the issue demonstrated both maternal stress and separation had an

impact on the gut microbiota (Bailey and Coe, 1999). Monkeys subjected to the maternal separation between 6-9 months of age showed microbiota alterations characterized by shedding of lactobacilli three days after separation, followed by the recovery of normal lactobacilli after seven days. Rats in adulthood that went through maternal separation had altered fecal microbial compositions compared to the normally reared control animals (O'Mahony et al., 2009).

1.7.2 Adult stress model

Important discoveries show the role of stress and its mediators in modulating the adult's intestinal microbiota (Bailey et al., 1999; Bailey et al ., 2010; Tannock and Savage, 1974). In adult mice, when psychosocial stress increased, the proportion of clostridia in the cecum along with the proportion of *Bacteroides*. Also, stress-induced increases in IL-6 and chemokine (C-C motif) ligand 2 (MCP1) were observed in the same study, and these changes were related to bacterial species' shifts in number (Bailey et al., 2011). Additional studies are needed to decide if gut microbial alterations observed in preclinical studies and some patient studies with stress-sensitive GI disorders, such as IBS, are the result of the stress induced acceleration of regional intestinal transit, intestinal secretion, or more effects of stress on the intestinal microbiota. These studies support the role of the gut microbiota in modulating emotional, nociceptive, and feeding behaviors in rodents (Mayer et al., 2017)

Also, the microbiota modulates a range of neurotrophins and proteins such as brain-derived neurotrophic factor (BDNF), serotonin, and N-methyl-d-aspartate (NMDA), all previously shown to be involved in brain development and plasticity. Both genetics and environment together guide pre- and postnatal brain development (Heijtz et al., 2011; Douglas-Escobar et al., 2013).

1.7. 3. Brain-derived neurotrophic factor (BDNF)

Germ-free animals were shown to have low levels of brain-derived neurotrophic factor (BDNF). BDNF is a central neurotrophic protein involved in neuronal growth and survival in the hippocampal region. It was found to reduce the expression of N-methyl D-aspartate receptor subunit in the cortex and hippocampus (Suliman et al., 2013). BDNF is a member of the neurotrophin family. It plays a significant role in such processes as the survival and differentiation of neurons, formation of functional synapses, and neuroplasticity during development and adulthood. The quantity of BDNF was shown to be influenced by the gut-brain axis. In some studies, model organisms, whose microbiota profile was altered by means of infection, displayed the low levels of hippocampal BDNF associated with increased anxiety-like behaviors. Researchers concluded that probiotic treatment return BDNF expression to the levels measured in control animals. These findings are parallel to the previous work that associate stress to reduced hippocampal BDNF expression and the restoration of BDNF to normal levels by antidepressant administration (Cryan and Dinan, 2012).

1.7.4 Serotonin receptor 5-HT1A

Serotonin is a primary signaling neurotransmitter in the CNS and a key target for many pharmacologic therapeutics administered in psychiatric conditions. The brain serotonin (5-hydroxytryptamine; 5-HT) system is a powerful modulator of emotional processes and a target of medications used in the treatment of psychiatric disorders (Heisler et al. 2007). Serotonin neurotransmission is involved in the regulation of mood, impulse control, sleep, vigilance, eating, libido, and cognitive functions, such as memory and learning. Also, serotonin is important in the modulation of anxiety and fear, as well as impulsiveness in suicidal and other violent acts (Boldrini et al. 2008; Lemonde et al. 2003). The serotonin-1A (5-HT1A) receptor, in particular, is thought to play an important role in the etiology of anxiety concerning the parts of the brain, such as dentate gyrus, CA1 regions of the hippocampus, amygdala, entorhinal cortex and lateral septum. Additionally, the 5-HT1A agonists have different effects depending on the model of anxiety (Zhuang et al.1999).

1.7.5 N-methyl D-aspartate receptor (NMDA)

NMDA is another neurotransmitter in the CNS. It is also known to play a major role in the pathophysiology of schizophrenia. NMDA glutamate receptor subunits NR1, NR2A, and NR2B have important roles in synaptic development and plasticity, learning and memory, and in the extended amygdala. All these have been thought to be involved in the central expression of anxiety (Neufeld et al. 2011). It is noted that agents that enhance NMDA receptor function reduce negative symptoms and improve cognition (Coyle, 2012). A link between the gut microbiota and NMDA signaling have established through GF mice studies. Compared to the control animals, a decrease in the expression of the NMDA receptor subunit NR2A was found in the cortex and hippocampus of GF animals (Sudo et al., 2004). Besides, in the central amygdala a downregulation of the NMDA receptor subunit 2B has also been reported (Neufeld et al., 2011a,b). NMDA NR2B receptor subunit is also known to play a role in amygdala-dependent fear learning (Rodrigues et al., 2001), and this is considered as one of the reasons to explain the decreased anxiety-like behavior seen in GF mice.

1.8 Gut microbiota and human brain function and behavior

Limited information is available from the human studies despite the emergence of a rich and robust preclinical literature on many different aspects of microbiota-brain interactions. The scarcity of information is perhaps due to the complexity of studying the human microbiota, which is affected by a wide range of variations in diet, gender-dependent differences, environmental influences, and genetic variation. Furthermore, the difficulty of surveying subtle changes in human cognitive and emotional functions along with the underlying functional and structural changes in the human CNS. Gut microbial organization patterns have been related to two clinical phenotypes. A new study in babies with infant colic, often thought to be a risk factor for anxiety disorders and the development of IBS, showed decreased overall diversity, reduced numbers of Bacteroides, and increased density of Proteobacteria compared to the healthy babies (Weerth et al., 2013). Although a causal role of these microbial changes on clinical symptoms has not been

determined, an increasing number of studies in IBS patients have supplied evidence of alterations in gut microbial composition (Simren et al., 2013; Mayer et al., 2014)

1.8.1 Effect of interventions targeting the gut microbiota

A different way of determining the effects of the gut microbiota on brain functions has been to conduct self-reporting measures as a proxy for alterations in the functions after modulating the microbiota with probiotics. A study conducted in healthy men and women reported that anxiety and psychological distress improved after taking a *Bifidobacterium* and *Lactobacillus* containing probiotic, though another study failed to confirm these findings with a different *Lactobacillus* probiotic (Messaoudi et al., 2011a,b; Benton et al., 2007). Such discrepancies in the results may have accounted for the limitations during the design stage of the study including differences between the probiotics, instruments used to collect the mood symptoms, interindividual variation in terms of microbial composition, sample size and baseline mood of the subjects. A different approach included the use of MRI (fMRI) to understand human brain changes while responding to modulation of the gut microbiota. One of these studies reported that chronic ingestion of a probiotic consortium altered functional brain responses in healthy women (Tillisch et al., 2013). In the same study, the response to an emotional face recognition task was measured with fMRI in healthy women before and after four weeks with the ingestion of prebiotics, non-fermented dairy product, or no treatment. A reduced response to the emotional recognition activity across a wide network of brain regions that included emotional and sensory regions reported in the women who had ingested the probiotics. No differences were recognized in self-reporting of symptoms of anxiety or depression between the treatment groups; however, the fMRI alterations demonstrated that a basic modification in responsiveness to negative emotional stimuli the environment. Another brain imaging study investigated the results of gut microbiota modulation via ingestion of a non-absorbable antibiotic in individuals with mild cognitive impairment and hepatic encephalopathy (Ahluwalia et al., 2014). Performance on a cognitive task improved, along with fMRI evidence for increased subcortical brain activity and improved frontoparietal connectivity during the task. Also, in another

study using the same underlying disorder and antibiotic treatment, cognitive function improved after an eight-week treatment route, along with the alterations in serum metabolites presumed to be of bacterial origin (Bajaj et al., 2013). Although the mechanisms operating in these brain changes in response to the experimental perturbations of the gut bacteria is not clear, though it may include some of the gut/brain signaling mechanisms.

1.9. Aim of the study

There is a growing evidence for the influence of the gut microbiota have on brain functions including BDNF expression, development of anxiety-like-behavior and depression syndromes, and even cognitive performance. On the other hand, one of the serious problems in the modern human populations is the over-use of antibiotics which are known to profoundly change the gut microbiota by removing the necessary symbiotic bacteria. However, there are still few studies examining the cumulative effect of repeated antibiotic administration in childhood on the brain status and behavior at the adult age.

The aim of the present study is to address these issues using an animal model.

- ✓ The effect of antibiotic administration in combinations on the gut microbiota
- ✓ The correlation between gut microbiota perturbed by antibiotics and cognitive performances such as learning and memory.
- ✓ The cumulative effect of repeated antibiotic administration in childhood on the anxiety-like behavior and stress response at the adult age.
- ✓ The effect of repeated postnatal antibiotic treatment on CNS, and emotional states (depression-like behaviors).

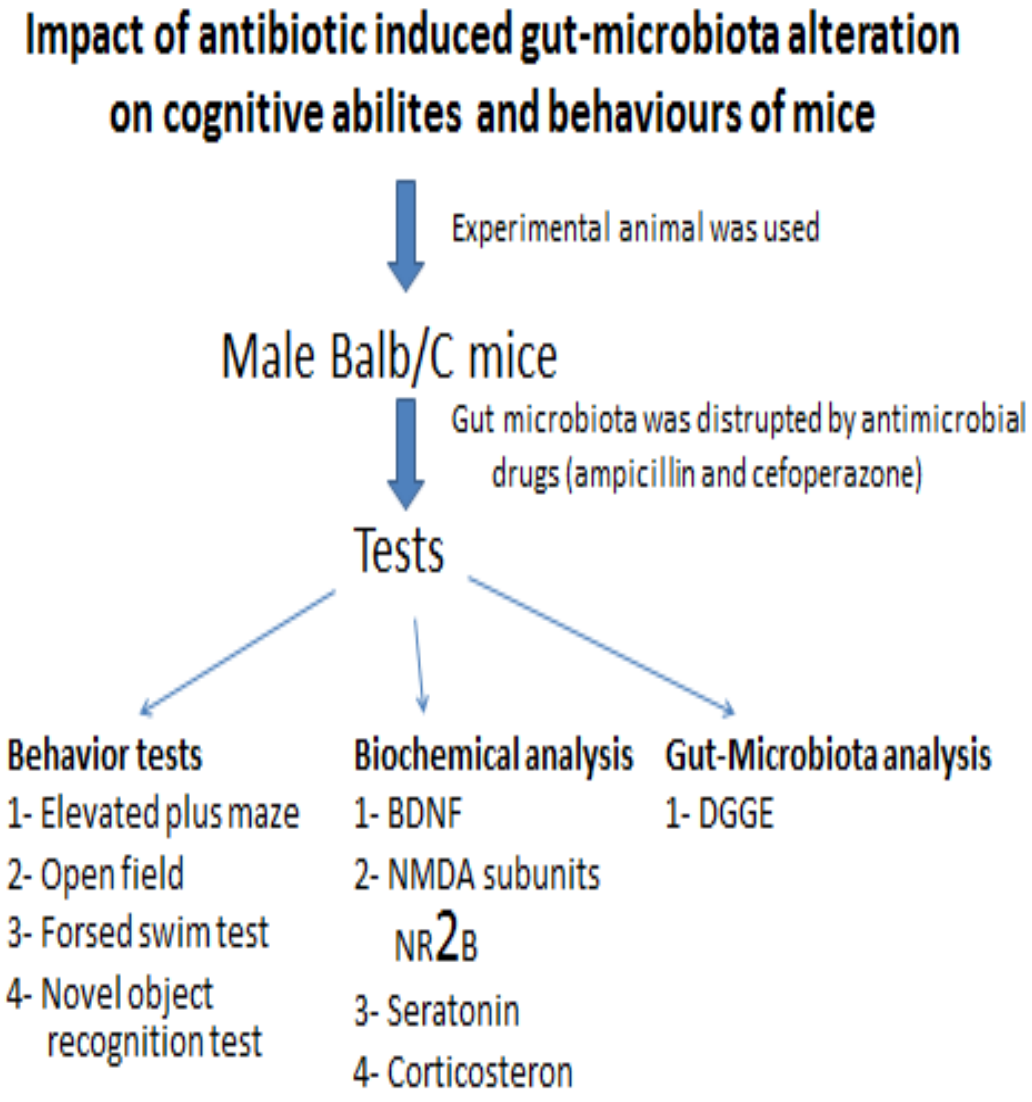


Figure 1.11 The study schedule

CHAPTER 2

MATERIALS AND METHODS

2.1. Subjects

Experiments were carried on 21-day old male BALB/c mice purchased from the Animal Facilities of Ankara University. BALB/c mouse strain is a convenient model for antibiotic studies because significant shifts in bacteria composition may occur in the absence of gut inflammation symptoms (Bercik et al., 2011).To exclude sex as an additional independent variable in the data analysis only the male pups were used in this study. To eliminate the litter effect among the experimental animals, pups coming from 10 different litters were intermingled between groups. The animals were housed in transparent plexiglass cages as five subjects per cage. Throughout the experiments, animals were maintained at a constant temperature (21°C), under a 12 to 12 hours light/dark cycle, with free access to water and food (standard commercial mouse chow, *Korkutelim* TR).

2.2. Antibiotic treatment

Before the experiments, the animals were randomly assigned to an experimental group receiving antibiotics (n=30) and a control group (Cont group) (n=10) receiving no treatment. The antibiotic-treated mice were divided into three groups (n=10 each, five animals per cage) and subjected to the treatments with different antibiotics. The antibiotic treatment consisted of a group receiving cefoperazone (Cef group), ampicillin (Amp group), and the cefoperozone-ampicillion alternation (Cef/Amp group). In the alternation group, 5 mice (one cage) received cefoperazone first, then ampicillin, while the other 5 mice (in the other cage) received antibiotics in the reverse order as ampicillin first, then cefoperazone (Table 2.1). To avoid any

confounding effects resulting from chronic stress induced by oral gavage, antibiotics were administered in the drinking water with free access. The weekly dose of antibiotics was incorporated in 400ml of water. The detailed schedule of antibiotic treatment is presented in Table 2. Water intake was monitored at the end of each week of antibiotic treatment. The antibiotic dosage was determined based on the previous studies where similar antibiotics were administered to mice in drinking water (Desponnet et al., 2015; Farzi et al., 2012; Odeh 2013). The concentration of each antibiotic in the drinking water was 1mg/ml. Water intake per cage, housing 5 mice, was recorded weekly. The average daily water consumption per mouse was 7 ml which correspond to 7mg of antibiotics. First antibiotic treatment was applied on the 21st postnatal day (PD), lasted for a week and was repeated 3 times, one week apart. The timeline of experiments is presented in Figure 2.3.

Figure 2.1 Five mice in one cage.

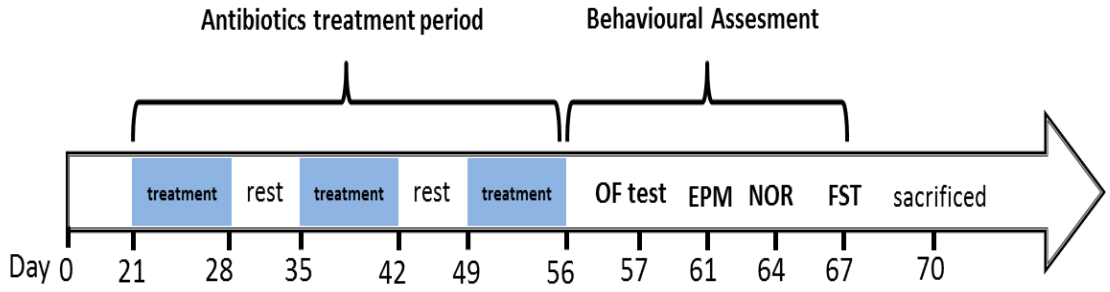


Figure 2.3 Experimental timeline showing antibiotic treatment periods and the order of tests performed on adult mice. (OF) Open field, (EPM) Elevated plus maze, (NOR) Novel object recognition, (FST) Forced swim test.

Table 2.1 Weekly Antibiotic treatment schedule

Weeks	**1. Group (n=10)**	**2. Group (n=10)**	**3. Group (n=10)**	**4.Group (n=10)**	
	Control	**Cefoperazone**	**Ampicillin**	**CefAmpCef**	**AmpCefAmp**
				(n=5)	**(n=5)**
1.Week	Drinking water	Cefoperazone 400ml	Ampicillin 400ml	Cefoperazone 400ml	Ampicillin 400ml
2.Week	Drinking water	Rest	Rest	Rest	Rest
3.Week	Drinking water	Cefoperazone 400ml	Ampicillin 400ml	Ampicillin 400ml	Cefoperazone 400ml
4.Week	Drinking water	Rest	Rest	Rest	Rest
5.Week	Drinking water	Cefoperazone 400ml	Ampicillin 400ml l	Cefoperazone 400ml	Ampicillin 400ml
6.Week	Drinking water	Rest	Rest	Rest	Rest

2.3. Behavioral tests

Behavioral tests were run upon the completion of antibiotic treatment.

2.3.1. Open Field (OF) test

The open field test is used to measure general locomotor activity and at the same time anxiety in rodents. The test was carried out in a square box (60 cm x 60 cm x 50 cm) made of plain wood painted black and illuminated with a bright light from the top. The animal was placed in the middle of one of the side walls facing the wall. Its locomotor activity was recorded by the computerized video tracking system (*EthoVision 3.1 System by Noldus Information Technology*, NL). The open field was divided into 16 equal squares by virtual lines, 12 of which constitute the peripheral zone, and remaining 4, the central zone of the arena. The system recorded time spent and distance covered (ambulation) in each of the zones for 10 min in 5 min intervals (Jakubowska et al. 2008; Elibol et al. 2014; Montiglio et al. 2017).

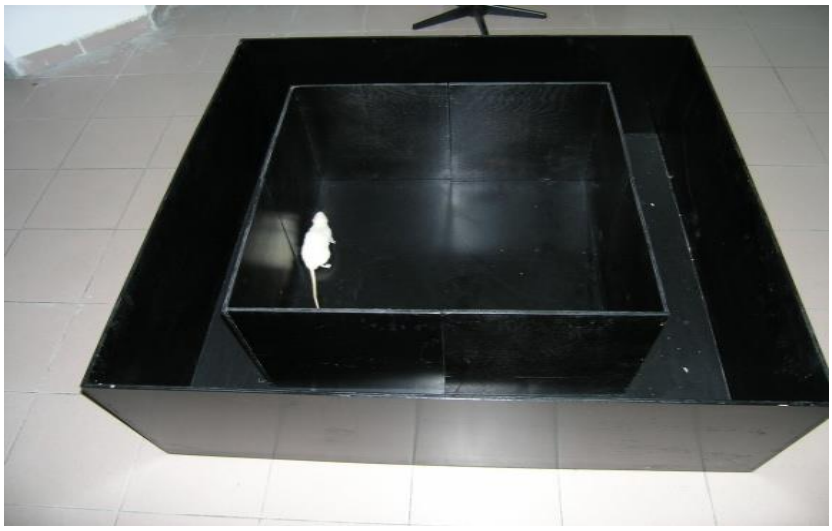


Figure 2.4 Open field test apparatus.

2.3.2. Elevated Plus Maze (EPM) test

The elevated plus maze apparatus is a widely used behavioral assay for rodents, and it has been validated to assess the anxiety levels. (Walf and Frye, 2007).

The elevated plus maze was constructed from polyester (*Commat*, TR). It was positioned 80 cm above the floor and consisted of a central platform (10×10 cm), and four crossed arms (50×10 cm, each): two open and two closed with darkened plexiglass walls extending 30 cm above the maze floor. On a single testing session, each animal was placed in the center of the maze facing an open arm and permitted to explore the maze for 5 min. During this time, the number of entries with all four paws to the closed and open arms, the total time spent in closed and open arms separately, and the total time spent on the central platform were recorded by the computerized video tracking system (*EthoVision 3.1 System by Noldus Information Technology*, NL).

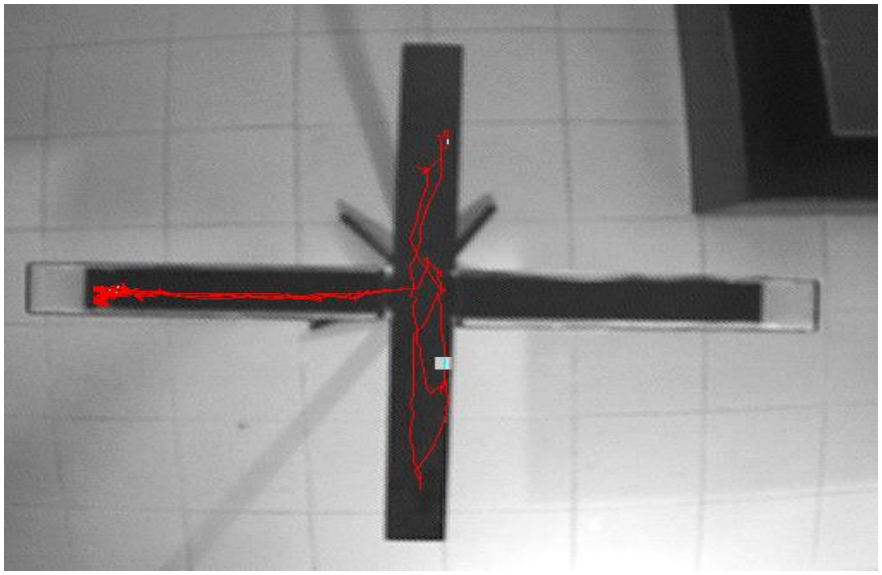


Figure 2.5 Elevated plus maze apparatus

2.3.3. Forced swim test (FST)

FST is considered as a valid test for assessing the susceptibility to a negative mood like depression (Desbonnet et al. 2010). It also provides a valid tool for assessment of the antidepressant efficacy of psychotropic drugs. Each mouse was challenged by placing it in a cylindrical tank (30 cm height ×20 cm diameter), filled up to 19 cm with a tap water at 24 ±1 ◦C. The frequency and duration of periods of immobility were recorded with a video camera. Mice were considered immobile when they became static in the water, except those motions which were vital to hold their head above the water surface. In FST the mice were subjected to a 6 min swimming test, however, for the data analysis only the last four minutes were considered (Can et al. 2012).

In FST there are special considerations for the test mouse species to be used. The swimming ability of mice should not be impaired due to musculoskeletal or other abnormalities affecting swimming performance.

Other important considerations

1. Temperature and water depth:
    a) A depth of 30 cm is commonly recommended so that the animal cannot touch the bottom with its tail or feet.
    b) Water temperature should be between 24-30^{0}C
    c) A warm environment should be supplied to animals for drying after removal from the water. An absorbent towel may be placed in the holding cage to collect water dripping from the animal.

2. Water changes
    a) After each day's tests, the container should be emptied and disinfected. Otherwise urine and fecal material will accumulate in the water.
    b) Fecal material can be removed after each test with a small mesh net.
3. Tests procedures
    a) A wide range of test session durations have been reported (4-20 minutes)

b) The continuous observation of the animals during the swim test is important because if an animal sinks below the surface, it should be removed from the water immediately (http://www.research.psu.edu/arp/experimental-guidelines/rodent-behavioral-tests-1/porsolt-forced-swim-test).

Figure 2.6 Forced swim test apparatus

2.3.4. Novel Object Recognition (NOR) test

To study the memory retention in animal models, the researchers broadly apply the NOR test (Ennaceur, 2010; Antunes and Biala, 2012). Also, this test can be used for the measurement of several other skills and behaviors such as attention, anxiety, and

preference for novelty in rodents (Goulart et al. 2010; Silvers et al. 2007). Furthermore, it is also applied to evaluate the effects of drug treatment and brain injury (Goulart et al. 2010). The apparatus used for NOR test was an open box made of cardboard (45 x 30 x 30 cm). A digital video recording device was used to record the animal behavior. Initially, a habituation trial was carried out with animals freely moving in the box for 10 min. During this time the animal's locomotor activity was recorded. Two hours after the completion of the habituation trial, two identical objects were placed in two corners of the box (approximately 5 cm away from the walls), and the mice were allowed to explore the objects for 10 min. All the mice were confirmed to explore each object for at least 10 s. The testing trial was performed 4 h later. For the testing trial, one of the previously used objects was replaced along with a novel object. The testing trial lasted 10 min. The duration of exploration of each object was scored by trained observers who was kept blind to the experimental treatments. Exploration was accepted as valid when the nose of the mice was directed to the object at a distance of no more than 2,5 cm and/or touching the object with its nose. Sitting on the object was not considered as exploratory behavior. Two sets of objects were used in a balanced manner. Objects were made of hard plastics differing from their shape, color, and patterns. They were mounted on a heavy, flat stone to prevent their displacement by the mice. The ratio b/a+b where "b" is the total exploration time of the novel object and "a" the total exploration time of the familiar object was evaluated as a novel object recognition score (Antunes and Biala, 2012; Uzbay at al. 2013). All of the experimental animal procedures were validated by the animal ethics committees of Ankara University and METU.

2.4. Biochemical tests

Upon the completion of behavioral tests the animals were sacrificed, their blood samples were collected and stored at -80 ^{0}C for the biochemical assays. Brain-derived neurotropic factor (BDNF) (Elfving et al. 2010), corticosterone, serotonin receptor 5-HT1A, and N-methyl D-aspartate receptor (NR2B) concentrations were measured from mouse blood serum using enzyme-linked immune sorbent assay (ELISA) (*ELISA Kits*, *Mybiosource,* US). Apendix A.

Figure 2.7 Novel object recognition apparatus.

2.5. Gut Microbiota analysis

For the microflora tracking at the end of antibiotic treatments, fecal samples were collected from each mouse at an amount of at least four pellets, and those samples were stored at -80 ^{0}C followed by instant freezing in liquid nitrogen.

2.5.1 Extraction of DNA and Amplification of the DNA target sequences

Total bacterial DNA was extracted from 180-220 mg fecal material using *QIAamp DNA Stool Mini Kit* (*Qiagen,* US) according to manufacturer's instructions. DNA concentrations were determined by BioDrop (*BioDrop,* UK). The hypervariable V2-V3 regions of the bacterial 16S ribosomal RNA (rRNA) gene was amplified using polymerase chain reaction (PCR) with universal bacterial primers HDA1 (**GC CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG**ACTCCT ACGGGAGGCAGCAGT-39; the GC clamp is in boldface) and HDA2 (59-GTATT CCGCGGCTGCTGGCAC-39) (Bercik et al. 2011). PCR was performed in 0,2-ml tubes in a high-performance PCR amplification system (*Bio-Rad,* US). For the amplification of the target DNA sequence, the Phusion High-Fidelity PCR Kit (Thermo, US) reaction mixture (20 µl) was used. PCR reactions were performed under the following conditions. One cycle of initial denaturation at 98^{0}C for 30 s, followed by 35 cycles of denaturation at 98 ^{0}C for 10 s, annealing at 55 ^{0}C for 30 s and extension at 72 ^{0}C for 15 s and a final extension at 72 ^{0}C for 10 min (Piwat and Teanpaisan, 2013).

2.5.2 Denaturing Gradient Gel Electrophoresis (DGGE)

The principle of DGGE is based on running the small quantities of DNA in a gel-based electrophoretic system accommodated with a denaturing chemical. During the diffusion over the gel, denaturing chemicals cause melting of DNA at different points enabling the identification of specific sequences. Profiling of PCR derived amplicons of the 16S rRNA genes (V2 and V3 regions) by DGGE is an adequate and competent

approach to distinguish the phylogenetic differences (>1% of total GM) in the GM of animals (Walter et al. 2000; Neufeld et al. 2012).

DGGE was performed with a DCode universal mutation detection system (*Bio-Rad,* US) utilizing 16 cm x 16 cm x 1mm gels. Eight percent polyacrylamide gels were prepared and run with 1X TAE buffer diluted from 50X TAE stock (2 M Tris base, 1 M glacial acetic acid, and 50 mM EDTA). The denaturing gradient was formed by using two 8% acrylamide (acrylamide- bis, 37.5:1) stock solutions (*Bio-Rad,* US). The gels contained a 30 to 50% gradient of urea and formamide increasing in the direction of electrophoresis. A 100% denaturing solution contained 40% (vol/vol) formamide and 7.0 M urea. The electrophoresis was conducted with a constant voltage of 130 V at 60°C for about 4h 30 min. The run was stopped when a xylene cyanol dye marker reached the bottom of the gel. Gels were stained with ethidium bromide solution (5 mg/ml; 20 min), washed with deionized water, and viewed under a UV transilluminator (Walter et al. 2000; Cani et al. 2009; Neufeld et al. 2010).
The details of the application line are given in appendix B.

2.5.3 Sequence and BLAST analyses

Following the visual examination, bands that were markedly different among the compared groups were selected. The DNA in the bands was subjected to further identification followed by sequencing. PCR was repeated under the same conditions on the excised DNA bands using universal primers HDA1 and HDA2. The resulting PCR products were confirmed by agarose gel electrophoresis and sequenced (Macrogene DNA sequencing, *BM laboratory systems,* TR). The 16S rRNA gene sequences were compared via the GenBank database using the Advanced BLAST search program at the NCBI website: http://www.ncbi.nlm.nih.gov/BLAST/ and were aligned with similar sequences (Walter et al. 2000).

2.6 Statistics

Data are presented as means ± standard error (SEM). Statistical analysis was performed using *Student's t-test, Man-Whitney U test* and *One-way ANOVA* followed by Dunnett's test. The degree of significance was denoted as less than or equal to *p $\leq$ 0.05 and **p $\leq$ 0.01. Cefoperazone, ampicillin, and cefoperazone/ampicillin-treated experimental groups were compared with respect to the control groups and with each other.

CHAPTER 3

RESULTS

3.1 Antibiotic, water, and food consumption

There was no a significant effect of applied antibiotic treatment on water and food consumption in mice during adolescence period (Figure 3.1A and 3.1B).

3.2 Body weights

The mean body weights, which at no point were significantly different between the groups, increased steadily and ended at 3± 32g, 3.5± 31g, 4± 33g, and 3± 32g (n = 10) for control mice and mice treated with ampicillin, cefoperazon, and both ampicillin and cefoperazon, respectively. (Figure 3.1C).

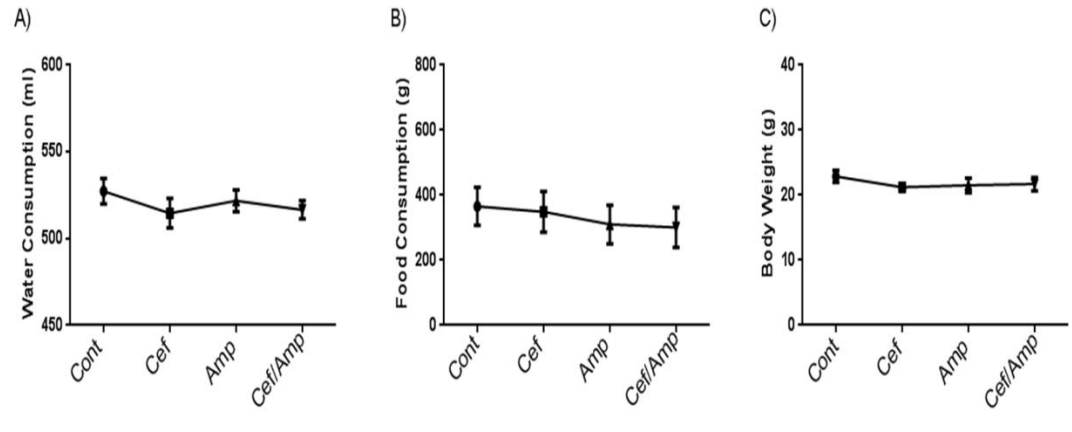
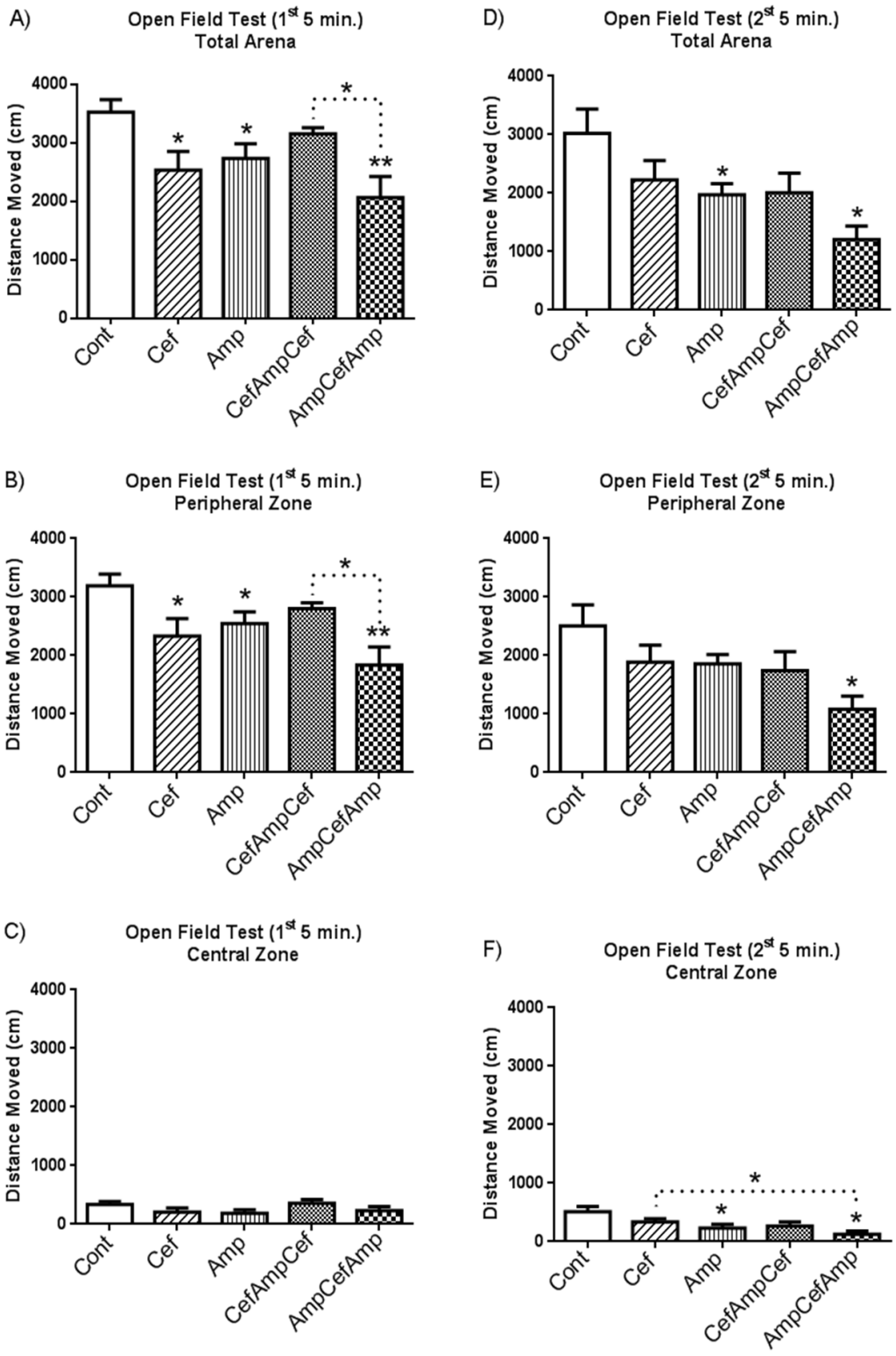


Figure 3.1 Effect of antibiotic treatment on mean total A) water and B) food consumption and C) body weight. Water and food consumption and the weight of each animal were measured weakly throughout the treatment. Data are expressed as means ± S.E.M, n = 10 per group.

3.3 Behavioral Tests

3.3.1 OF Test

Figure 3.2 shows the locomotor activity of the mice in the total arena (A and D), peripheral zone (B and E), and central zone (C and F) of the OF during the two consecutive 5-min intervals. Student's t-test was applied to assess the differences between treatment and control groups for each zone and time interval, independently. A significant difference in the locomotor activity was found between Cef-Amp-Cef and Amp-Cef-Amp subgroups. Except Cef-Amp-Cef subgroup Student's t-test confirmed significantly low locomotor activity in the antibiotic-treated groups compared to the control group for the total arena and peripheral zone during the 1st 5-min interval. Additionally, analysis of the differences between alternation subgroups showed a significantly lower locomotor activity in the total arena and peripheral zone of the OF during the 1st 5 min in the Cef-Amp-Cef group compared to Amp-Cef-Amp. As seen from the Figure 19, during the 2nd 5min interval, mice's locomotor activity exhibited a decreased tendency in the total arena/peripheral zone and increased tendency in the central zone. During the 2nd 5 min interval, in the peripheral zone, locomotor activity was still lower in the treatment groups compared to control. However, the difference between Amp-Cef-Amp and Control groups was significant. Interestingly, during the 2nd 5 min interval, the distance covered in the central zone by Amp and Amp-Cef-Amp treated mice was also significantly shorter. During the 2nd 5 min interval, the distance covered in the central zone by the Amp-Cef-Amp subgroup was significantly shorter compared to Cef only.



Figüre 3.2 The mean distance (±SEM) moved in the total arena (A & D), peripheral zone (B & E) and central zone (C & F) of the Open Field during the 1st (left column) and the 2nd (right column) 5- min intervals in control and antibiotic treated groups. Error bars denote SEM and asteriks the level of significance: * $p \leq 0.05$; ** $p \leq 0.01$.

Figure 3.3 shows the total time spent by animals in the peripheral and central zone during each time interval, independently. Repeated measure ANOVA (treatment x time interval x zone) applied to these data revealed that significantly more time spent in the peripheral than in the central zone.

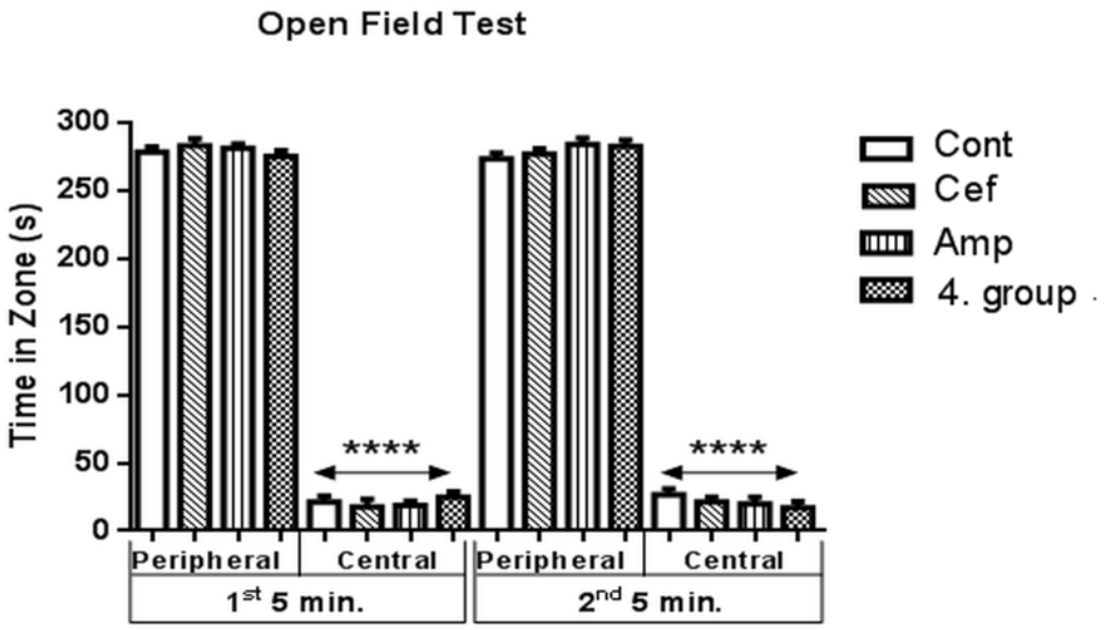
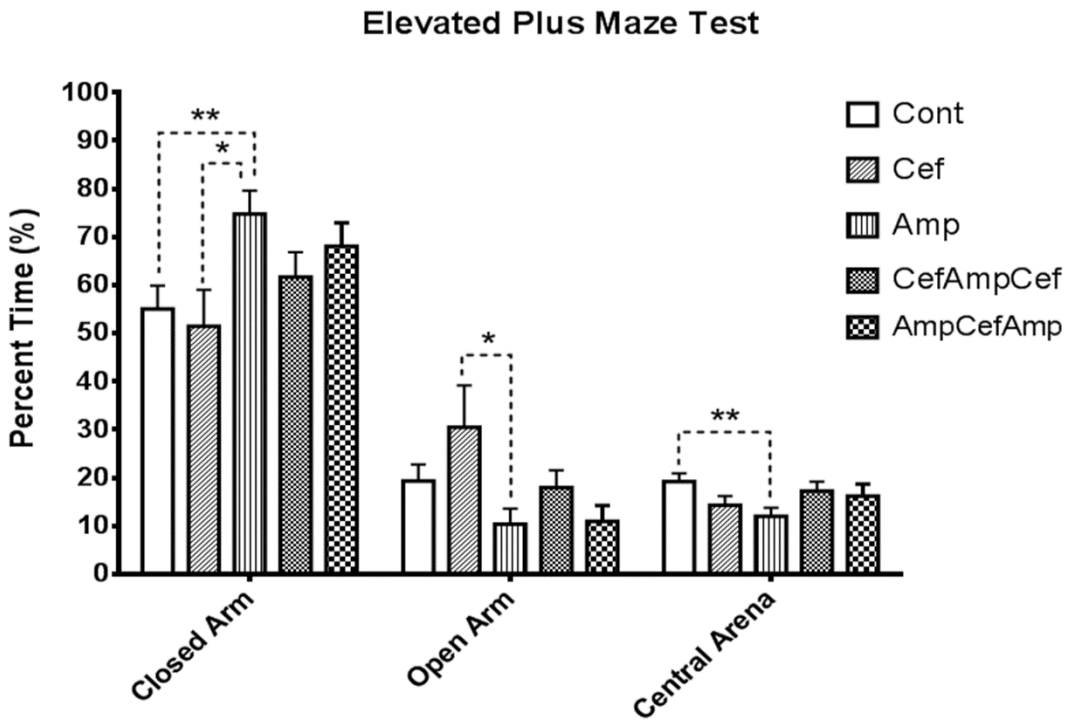


Figure 3.3 Total time spent by animals in the peripheral and central zone during each time interval, independently. Error bars denote SEM and asteriks the level of significance: **** $p \leq 0.00001$.

3.3.2 EPM Test

EPM was the second test run after the antibiotic treatment period. Statistical analysis of the EPM data using Student's t-test revealed that Amp group spent significantly more time in the maze's closed arms compared to control and Cef group and spent significantly less time in the maze's open arms compared to the Cef group (Fig. 3.4). Additionally, Amp group spent significantly less time in the central arena of the maze compared to Control. In the EPM Test, no significant difference was found between Cef-Amp-Cef and Amp-Cef-Amp subgroups.



Figüre 3.4 Comparison of the animal's behavior in the EPM between control and antibiotic groups. Error bars denote SEM and asteriks the level of significance: * $p \leq 0.05$; ** $p \leq 0.01$

3.3.3 NOR Test

Figure 3.5 presents mean ratio of the exploration time spent on a novel object to the total time spent on the exploration of both novel and familiar objects on the test trial. The ratios refer to a novel object recognition score or a learning score. Score 0.5 means that there was no difference in exploration time of novel and familiar object. The score larger than 0.5 means that an animal spent more time on the exploration of a novel object, while the score smaller than 0.5 means that an animal spent more time exploring a familiar object. In all groups, the scores were larger than 0.5 meaning that animals spent more time exploring a novel object. The between-group comparisons by the Student's t-test showed significantly lower learning scores in Cef and Amp groups compared to control group with no difference between the antibiotic groups.

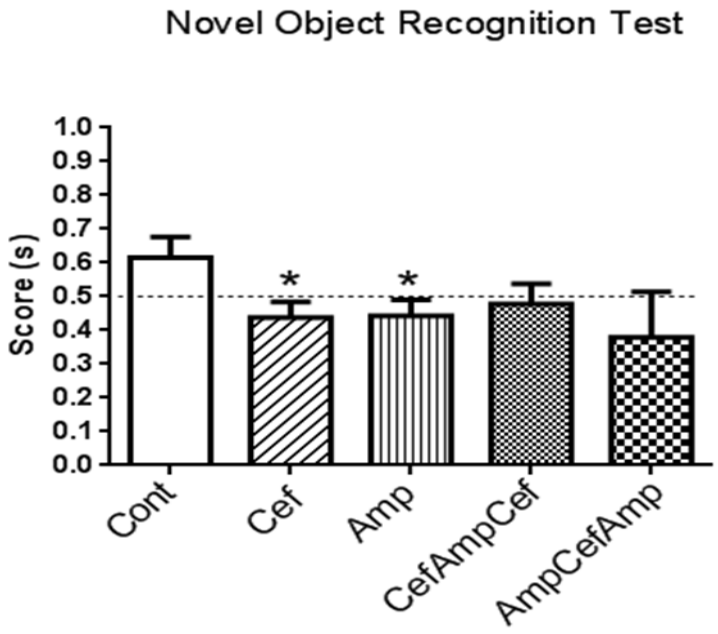
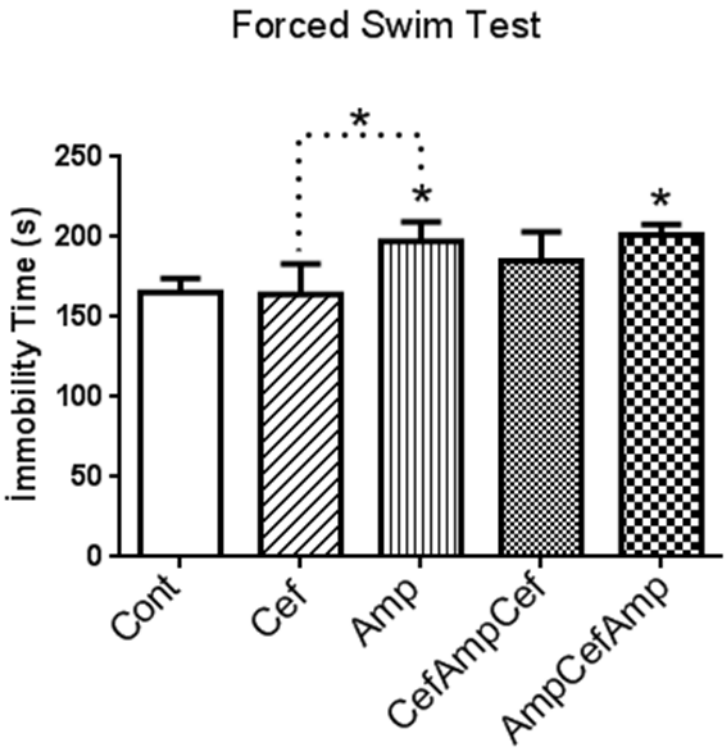


Figure 3.5 Comparison of the animal's behavior in the NOR between control and antibiotic groups. Error bars denote SEM and asteriks the level of significance: * $p \leq 0.05$; ** $p \leq 0.01$.

3.3.4 Forced Swim Test

Figure 3.6 represents mean total immobility time for control and treatment groups. In the ampicillin-treated groups (Amp and Amp-Cef-Amp) significantly longer total time of immobility (passive floating) compared to control group was confirmed by Student's t-test applied to the data. As assessed by the *Mann-Whitney U test* Amp group also floated significantly longer time compared to Cef group.



Figüre 3.6 Comparison of the animal's behavior in the FST between control and antibiotic groups. Error bars denote SEM and asteriks the level of significance: * $p \leq 0.05$; ** $p \leq 0.01$.

3.4 Biochemical Tests

A one-way ANOVA on serum BDNF concentration as an independent variable did not reveal significant differences between control and antibiotic-treated groups (Figure 3.7A). However, serum BDNF concentration in the Cont group was significantly higher compared to Amp with *t test* (p=0.0227) and in the Cef group was significantly higher compared to Amp with *Man-Whitney U test* (p=0.0229). On the other hand, blood serum corticosterone level was the lowest in Cef group (Fig. 3.7B). However, one-way ANOVA applied to the data did not confirm any significant difference between-groups. There were no significant differences in serum NR2B levels between control and antibiotic-treated groups. However, its concentration in the ampicillin-treated group was significantly higher compared to cefoperazone treated group (Fig. 3.7D). As seen in Figure 3.7 B and C, there was also no significant difference between control and antibiotic-treated groups for serum levels of serotonin receptor (5-HT1A).

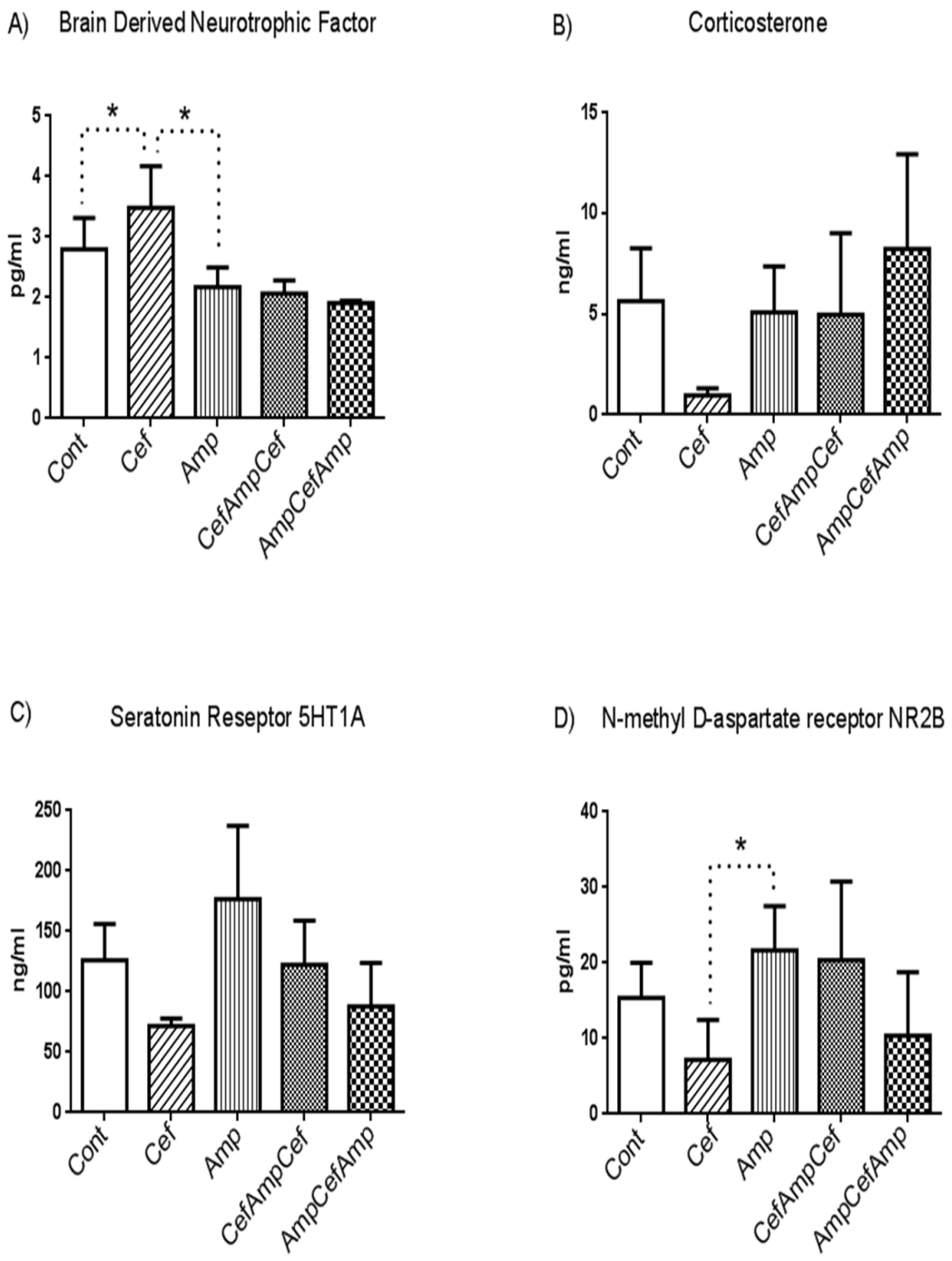


Figure 3.7 Serum BDNF (A), Corticosterone levels (B), Seratonin receptor 5HT1A (C) and N-methyl D-aspartate receptor NR2B in control and antibiotic-treated groups of mice. Error bars denote SEM, and asteriks the level of significance: * $p \leq 0.05$.

3.5 Gut Microbiota Analysis

The hypervariable V2-V3 regions of the bacterial 16S ribosomal RNA (rRNA) gene were amplified using polymerase chain reaction (PCR) with universal bacterial primers HDA1-**GC** and HDA2 are presented in the Figure 3.8. Microbial communities for each experimental group were identified and confirmed using BLAST analysis (Table 3.1).The gut microbiota profile of control and antibiotic-treated mice groups obtained by DGGE of 16s RNA encoding genes is presented in the Fig. 26. The 16s rDNA sequences representing bacterial phylotypes were numbered and labeled on the DGGE gel according to the nomenclature used in Table 3. As can be seen from the Figure 3.9 microbial community profiles found in control mice were altered in the antibiotic-treated groups. The analysis of DGGE gel profile shows that the ampicillin treatment had a more prominent effect on the gut microbiota composition in mice than the cefoperazone. The profile was also affected depending on the starting antibiotics in alternating antibiotic-treatments.

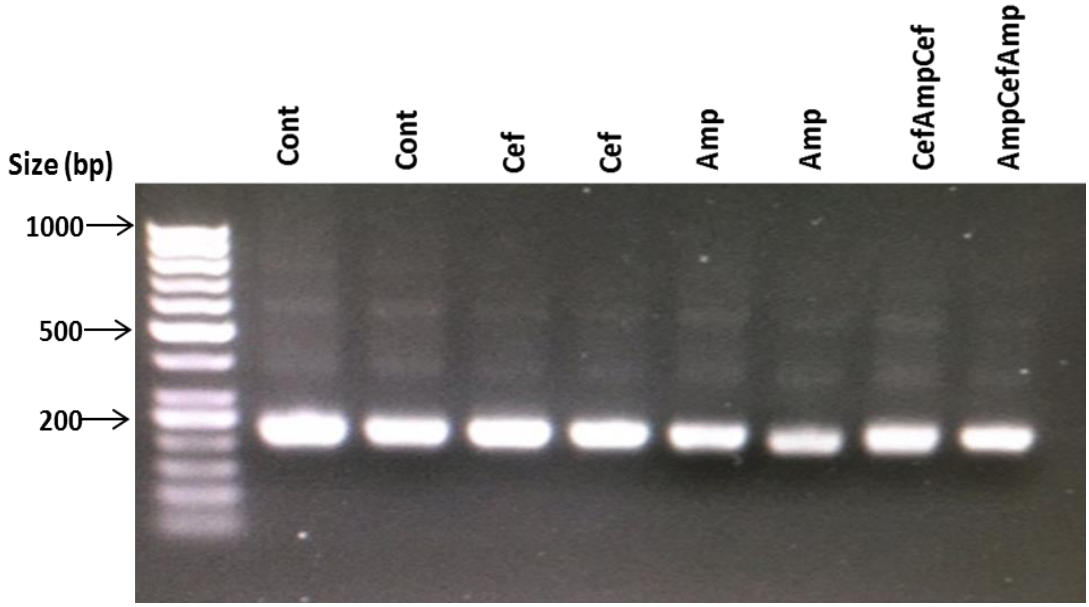


Figure 3.8 Polymerase chain reaction (PCR) with universal bacterial primers HDA1-**GC** and HDA2.

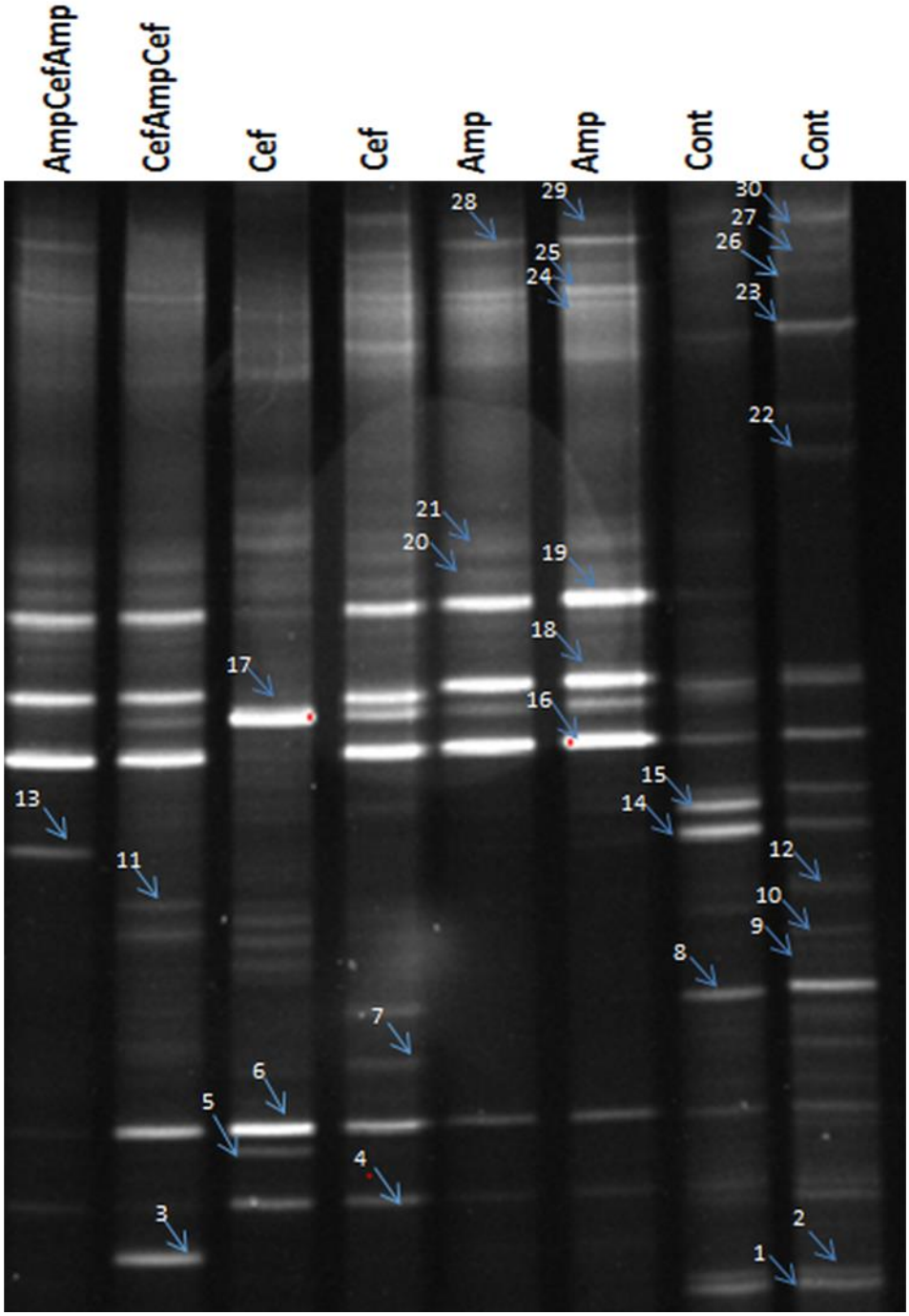


Figure 3.9 Representative DGGE gel of fecal microbiota from control and antibiotic-treated mice. The bands that were markedly different among the groups were selected, excized from the gel, and subjected to identification by sequencing.

Table 3.1 16S rDNA sequence based identification of mouse gut bacteria

Isolates			
	Bacteria	**Name**	**Acc. No**
1	No significant similarity found	K1-8	X
2	Bacteroidetes bacterium	K1-7	KP030834.1
3	Uncultured Helicobacter	M1-1	KX513932.1
4	Uncultured Clostridiales	C2-1	KF508977.1
5	Uncultured Bacilli	C2-2	EF702017.1
6	Uncultured Clostridiales	C1-2	KF508977.1
7	Uncultured Bacteroidales	C1-4	AB702766.1
8	Uncultured Bacteroidales	K2-12	AB702752.1
9	Uncultured Bacteroidetes bacterium, Uncultured bacterium	K1-18	FJ916256.1, AB704736.1
10	No significant similarity found	K1-19	X
11	Uncultured Firmicutes	M1-7	KX057093.1
12	Uncultured Bacteroidetes-	K1-20	FJ650737.1
13	cytochrome oxidase subunit	M2-5	X
14	*Bacteroides vulgatus*	K2-16	KR364743.1
15	Uncultured bacterium	K2-17	JF260492.1
16	*Bacteroidales acidifaciens*	A1-3	KR364740.1
17	No significant similarity found	A1-4	X
18	*Bacteroides acidifaciens*	A1-5	AB599950.1
19	Uncultured bacterium DGGE	A1-8	HE866517.1
20	*Cryphonectria hypovirus*	A2-9	KT868541.1
21	*Bacteroides acidifaciens*	A2-10	KT006899.1
22	Uncultured Bacteroidales	K1-25	AB702765.1
23	Bacteroidetes bacterium	K1-27	KP030834.1
24	Uncultured bacterium	A1-13	JF254331.1
25	*Bacteroides acidifaciens*	A1-14	JJM0207-2
26	No significant similarity found	K1-28	X
27	Uncultured Bacteroidetes bacterium	K1-29	AB611581.1
28	Uncultured Bacteroidales-	A1-15	AB702719.1
29	No significant similarity found	A1-16	X
30	Uncultured Bacteroidetes	K1-30	FJ650737.1

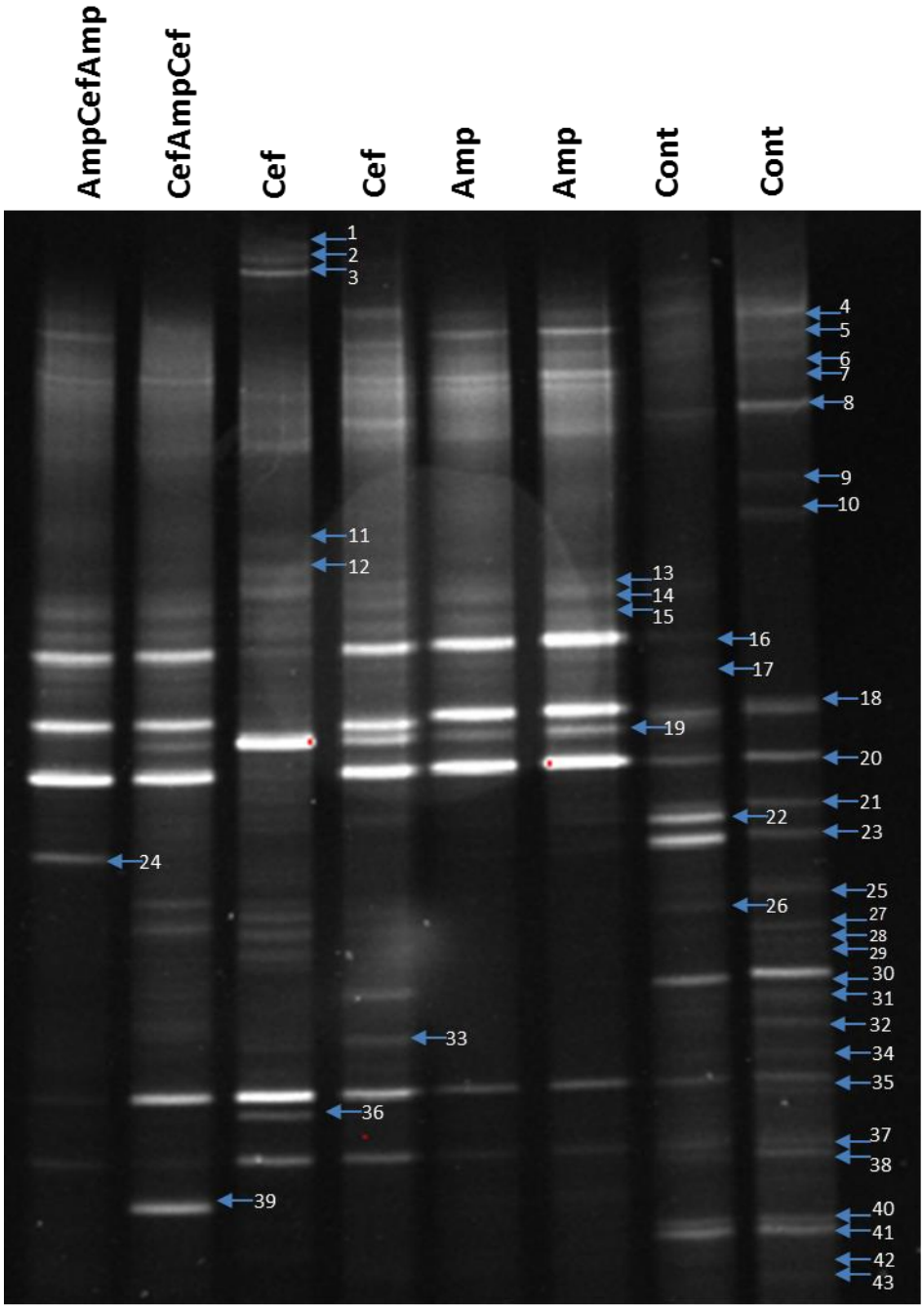


Figure 3.10 16s RNA encoding genes of gut microbiota profile of control and antibiotic-treated mice groups

Table 3.2 Distribution of the 16s RNA encoding genes among the control and antibiotic-treated mice groups

	Control		Ampicillin		Cefoperazone		4.group	
Bands	**1.cage**	**2.cage**	**1.cage**	**2.cage**	**1.cage**	**2.cage**	**CefAmpCef**	**AmpCefAmp**
1						x		
2						x		
3						x		
4	x	x	x	x	x		x	x
5	x	x	x	x	x		x	x
6	x	x	x	x	x		x	x
7	x	x	x	x	x		x	x
8	x	x	x	x	x		x	x
9	x							
10	x							
11						x		
12						x		
13			x	x	x	x	x	x
14		x	x	x	x	x	x	x
15			x	x	x	x	x	x
16		x	x	x	x	x	x	x
17		x	x	x	x	x	x	x
18	x	x	x	x	x		x	x
19			x	x	x	x	x	
20	x	x	x	x	x		x	x
21	x	x						
22		x	x	x	x	x	x	x
23	x	x						
24								x
25	x	x						
26	x	x			x	x	x	
27	x	x			x	x	x	
28	x	x			x	x	x	
29	x	x			x	x	x	
30	x	x			x		x	
31	x	x						
32	x	x						
33					x		x	
34	x	x			x	x	x	
35	x	x	x	x	x	x	x	x
36	x	x				x		
37	x	x	x	x	x	x	x	x
38	x	x						
39	x	x					x	
40	x	x						
41	x	x						
42	x	x						
43	x	x						
total	29	31	16	16	23	20	24	16

CHAPTER 4

DISCUSSION

Few studies have examined the cumulative effect of repeated antibiotic administration in childhood on the brain status and behavior at the adult age. One of the consequences of antibiotic treatment is an impaired microbiota (dysbacteriosis) in the gut leading to impairments in the immune system and overall metabolism. Even so, inadequate amount of information is available in the literature elucidating the interplay between antibiotic usage and brain biochemistry as well as behavior. Since the interaction between the brain and gut is accomplished through the bilateral network, also called gut-brain axis, any changes in intestinal microbiome as a result of antimicrobials will affect the brain. In this thesis project, we have utilized mouse model to investigate the impact of different antibiotics in gut microbiota and their indirect effects on brain and behavioral development. Since the profile and composition of the natural microbiota are specific for individuals, the utilization of germ-free mouse model in microbiota research is not recommended. Although this model is useful in mechanistic studies, it does not reflect the natural and individual circumstances shaping of a huge number of bacterial habitats. Therefore, the use of the antibiotic mouse model gives this research a high pre-clinical impact as it can be directly translated to what is observed in human diseases and disorders. By this purpose, thirty 21-days old BALB/c mice were exposed to three broad-spectrum antibiotics regimes include ampicillin (1g/L), cefoperazone (1g/L) and cefoperazone/ampicillin (1g/L) up to adult age. Antibiotics were delivered for one week, which was followed by one week rest without antibiotics to avoid the side effects. After the antibiotic treatment period, this study is divided into three parts.

In the first part, after the last antibiotic administration, the mice were subjected to behavioral tests. The locomotor activity, anxiety, and depression-like behaviors of mice were identified along with the assessment of learning and memory performances.

In the second part, the levels of the brain-derived neurotrophic factor (BDNF) (associated with memory, learning, and anxiety), Corticosterone (involved in regulation of energy, immune reactions, and stress responses), Serotonin receptor 5HT1A (a factor on emotional behavior and anxiety) and NR2B subunits of N-methyl D-aspartate receptor (NMDA) (playing an important role in synaptic development, plasticity, learning, and memory) were determined.

In the last part, the gut microbiota profile of antibiotic-treated and control groups was obtained through the DGGE method and all the results interpreted together followed by the sequencing of 16S rDNA genes.

4.1 Antibiotics and gut microbiota analysis

Recent literature on rodents grown in a sterile (germ-free) setting showed the power of GM in the development of emotional behavior, stress- and pain-modulation systems, as well as brain neurotransmitter systems (Mayer 2015). Despite the fact that the microbiota is for the most part stable within individuals, after some time, the content can change because of outer factors. Microbiome modification in flora induced by antimicrobials is known as one of the central points that can change the composition of the microbiota, can likewise in a way it influences well-being in the long run (Francino 2016). Antimicrobial agents profoundly affect the microbiota, and their abuse is connected with an expansion of resistant pathogens (Dethlefsen et al., 2008; Sullivan et al., 2001; Clemente 2012). Especially wide range antimicrobial agents decrease bacterial variation due to the rise and fall of the particular indigenous taxa. These fluctuations influence the plenitudes of around 30% of the bacteria in the gut flora, causing fast and huge drops in taxonomic richness, decent variety, and equity resulting in dysbacteriosis (disturbance in composition and functional capacity) (Francino 2016). Nowadays, the utilization of antimicrobials to cure

sickness has turned out to across the borders beginning in early stages of life, proceeding into adulthood. (Cho and Martin, 2012). Early administration of antibiotics decreased the decent variety of the newborns' microbiota and modified their composition, with a weakening of *Bifidobacterium* and marked increases of Proteobacteria (Francino 2016).

In this study, because many bacteria cannot be cultured, a culture-independent identification technique was used. 16S ribosomal RNA targeted polymerase chain reaction–DGGE, and sequencing-based microbial profiling methods were employed to show the potential changes in the gut microbial composition resulting from the three different antibiotic regimes applied to the mice soon after weaning. The obtained data from DGGE showed that the repeated treatment with ampicillin, cefoperazone, and the combination of ampicillin and cefoperazone perturbed the gut microbiota profile differently with all three treatments reducing GM composition. The reduction in microbiota diversity in Amp and Cef groups was about 60% compared to the control microbiota. In the groups receiving Cef-Amp-Cef and Amp-Cef-Amp treatments, loss of microbiota diversity was greater in the group receiving two ampicillin exposures before and after cefoperazone. When the DGGE gel images are examined, it is seen that ampicillin affected the bacterial composition more than the other antibiotics treatment. The composition change also manifested itself in the Cef-Amp-Cef subgroup and the subgroup Amp-Cef-Amp. The DNA bands numbered 16, 17, 18, and 19, appeared with higher intensity in the antibiotic groups than the corresponding bands in the control group on the DGGE gel. The sequence analyses of the bands 16 and 18 showed that these two bands belong the Bacteroides class. The band 19 belonged to a group of bacteria that cannot be cultured. The rDNA in the bands 8, 14, and 15 were expressed more in the control group, bands 8 and 14 belonged to the Bacteroides group whereas the band 15 belongs to an un-cultured bacterium.

In our study, it seems that *Bacterioides* strains such as the ones represented in bands 16, and 18 were expressed more in antibiotic-treated groups compared to the control. In a previous study, a cocktail of antimicrobials was given to BALB/c SPF mice for seven days to reveal the effect of the modified microbiome on rodent attitude (Bercik at al., 2011). Their results demonstrated meaningful disruption of microbiome composition (expansion of *Firmicutes* and *Actinobacteria*, and diminishing in *Proteobacteria* and *Bacteroidetes* populations) measured by DGGE analysis. Similarly, it was recently shown that decreased flora and its diversity in the adult mouse treated with antibiotics started from weaning period (Desponet et al. 2015). According to the measurements conducted at a phylum level, relatively diminished *Firmicutes* and *Bacteroidetes*, while increased *Cyanobacteria* and *Proteobacteria* populations were found as a result of antibiotic treatment. A recent investigation directed by Leclercq et al. 2017 showed that early life antibiotic treatment initiates long-haul changes in microbiome content of both adults and their offsprings. Early life antibiotic-treatments expanded plenitude of Firmicutes (Lachnospiraceae and Erysipelotrichaceae) and Proteobacteria, and diminished wealth of Bacteroidetes (S24-7, Prevotellaceae, and Rikenellaceae) and Lactobacillaceae. In our study, the interpretation of the increase in *Bacterioides* seems to require another verification. In the presented study only DGGE method was employed to show the potential changes in the gut microbial composition. Although DGGE is very well known and powerful technique, it has limitations, and it is not enough alone to interpret our results. To discuss specific changes in bacterial composition at the phylum level, the sequences should be made with by one of the precise techniques such as the multiplexed next-generation sequencing of 16S amplicons. However, with DGGE, effects of different antibiotic regimes on microbiota composition can be analyzed and compared in one gel.

4.2 Behavioral findings

Some of the previous studies suggested that fluctuations in the gut microbiota could lead to modifications in the CNS functions having an impact on the overt behavior in human and experimental animals (Bravo et al., 2012; Heijtz et al., 2011). Our results also demonstrated some behavioral deviations in the antibiotic-treated mice. The overall locomotor activity decreased during the 2nd 5 minutes interval in the OF. This decrease in the locomotor activity was observed especially in the peripheral zone of the OF where the animals spent most of the time during the first 5 minutes interval. As expected, in all groups, due to the habituation to a new environment, the overall locomotor activity decreased during the 2nd 5 minutes interval in the OF. This decrease in the locomotor activity was noted especially in the peripheral zone of the OF where the animals spent most of the time during the first 5 minutes interval. Although due to the habituation to a new environment brings along an overall decrease in the anxiety, during the 2nd 5 minutes interval in the OF, exploration of the central and open area increased in control and Cef groups but not in Amp groups where it was significantly lower than the control and cefoperazone-treated animals. Amp and Cef treated groups also exhibited anxiety-like behavior in EPM test. The Amp group showed increased anxiety, spent significantly more time in the closed arms and central zone but less in the open arms compared to Con and Cef groups. However, Cef group showed decreased anxiety-like behavior in EPM test. Such a discrepancy in anxiety-like behavior has also been seen in related GF studies (Rabot et al., 2016). Most of the information on the relationship between gut microbiota and behavior have been obtained from GF studies. Some of these GF studies showed a complete absence of intestinal bacteria (in germ-free mice) results in decreased anxiety-like behavior (Heijtz et al., 2011; Neufeld et al., 2011**a,b**; Desponnet et al., 2014; Leclercq et al., 2017)

Contrary to our results, the absence of intestinal flora in GF mouse models has been previously reported to have an anxiolytic effect when assessed by an increased plus maze or open-dark preference test (Neufeld et al., 2011; Heijtz et al., 2011; Clarke et al., 2013). However, in a few studies, GF F344 rats but also GF rats were reported to develop anxiety-like behaviors when exposed to OF, social interactions, and marble-

burring test (Clemente et al., 2012; Nishino et al., 2013; Crumeyrolle-Arias et al., 2014). These inconsistent results led to the hypothesis that intestinal microbiota balances behavioral stress responses that reduce anxiety-like behaviors in anxiety-prone strains such as BALB /c mice and F344 rats, and promotes this behavior in anxiety-resistant strains such as Swiss Webster NMRI mice (Crumeyrolle-Arias et al., 2014; Rabot et al., 2016). Per se, microbial transfer experiments have shown that colonization of BALB /c GF mice with microbiota from Swiss mice reduced, whereas Swiss GF mice augmented by microbiota from BALB /c mice elevated anxiety in the recipient animals (Bercik et al., 2011a). Other experiments have shown that inoculation with a single strain of bacteria, such as *Lactobacillus helveticus* r0052 or *Bifidobacterium longum* r0175, may be satisfactory to relieve anxiety-like behaviors (Bercik et al., 2011b; Messaoudi et al., 2011). Our results, however, demonstrated that antibiotic-induced perturbation in the commensal composition of gut microbiota in anxiety-prone BALB/c mice, unlike GF models, may trigger anxiety-like behavior and that these behavioral modulations are specific for antibiotics after ampicillin but not cefoperazone treatment.

Despite the fact that the numbers of studies revealing the relationship between gut microbiota and behavior on GF mice have been increasing, there is still a limited amount of information on the effect of antibiotic-induced microbiota on behavior. A recent study conducted by Leclercq and colleagues (2017) showed that low-dose penicillin taken late in pregnancy and early life of mice offspring changes behavior and the balance of microbes in the gut. They gave pregnant female BALB/c mice low doses of penicillin during their last week of gestation and continued to treat their pups until they weaned a few weeks after birth. The team reported that by the end of six weeks, general locomotor activity did not change between antibiotic exposed and unexposed mice. However, only male mice exhibited decreased anxiety-like behavior. In another study, Bercik and colleagues (2011) perturbed the microbiota by oral administration of the nonabsorbable antimicrobials neomycin and bacitracin along with the antifungal agent pimaricin in adult BALB/c mice. While antibiotic-treated mice showed less anxiety and more exploratory behavior than controls, the overall locomotor activity was not affected.

Past investigations showed a huge connection between the degree of nervousness and the level of emotional depression (Prasad et al., 1997). In our study, an elevated level of behavioral despondency in the FST, and slanting towards depression-like manifestations were recorded in experimental animals treated with Amp. Amp-treated animals demonstrated agitation which was equated to nervousness. The comparable outcome was reported by Hoban and associates (2016) who additionally detailed elevated depressive-like behavior in the FST in young, male Sprague Dawley rats with antibiotic-exhausted microbiome. Then again, probiotic bacteria have been accounted for the recuperation of this condition. Bravo and associates (2011) showed that mice inoculated with *Lactobacillus rhamnosus* (JB-1) exhibited alleviated symptoms of depression in FST. Furthermore, treatment of BALB/c mice with probiotic microorganism- *Bifidobacteria breve* to was accounted for the induction of antidepressant-like behavior in the tail suspension test (Savignac 2014). In parallel with the animal trials, in the current years, evidence from clinical trials indicated that there is a connection between gut microbiota and human depression (Rogers et al. 2016). A differential impact of ampicillin and cefoperazone on nervousness and depressive-like attitude is most likely connected with the spectrum variation of these antibiotics and their diverse effect on the microbiome. Complementary research on particular changes in the microbiota composition after ampicillin and cefoperazone treatments may reveal insight on this inquiry. Also, antibiotic treatment is not simply draining yet it is rebuilding the composition of gut microbiota. Subsequently, it is not conceivable to expect comparable impacts under these two distinct conditions. Ampicillin and additional cefoperazone treatment had some negative impact on the animals' cognitive performance as seen in NOR test, wherein, the exploration time of a novel object was fundamentally shorter in contrast to the control gathering.

Similarly, Fröhlich and associates (2016) recorded a weak performance in the NOR test, memory exercises in grown-up male C57BL/6N mice subjected to a mixture of five antimicrobial agents comprising of ampicillin, bacitracin, meropenem, neomycin, vancomycin. Likewise, cognitive deficiencies were found by another research group in mice treated with a blend of antimicrobials in young animals (from weaning forward) (Desponnet et al., 2015). In the NOR test, animals' reaction to

novelty is assessed based on the exploratory movement. One may expect that animals with slowed general locomotor action would also show diminished exploratory action. Their results were parallel with our findings. In the case of adult BALB/c mice, the disturbance of the microbiota via oral application of neomycin, bacitracin, and antifungal compound-pimaricin prompted an augmentation in the exploratory movement with no change in the general locomotor action (Bercik et al., 2011)

4.3 Biochemical findings

In the present study, elevated behavioral anxiety in ampicillin groups was not accompanied by an increase in the corticosterone levels in the peripheral blood. On the other hand, it is known that an elevated corticosterone level is an indicator of stress (Sudo et al., 2004). Neufeld et al. (2011) reported increased corticosterone levels in the GF mice with respect to the SPF mice. On the other hand, Desbonnet and colleagues (2015) reported that an acute restraint stress (30 min) induced an increase in the serum corticosterone concentration. However, they also did not find a difference in corticosterone blood levels between antibiotic-treated and non-treated mice either at the baseline or following the stressor application. These results suggest that antibiotics may have a greater impact on central rather than peripheral arousal centers.

In contrast to corticosterone, the circulating levels of BDNF were lower in ampicillin-treated animals compared to Cont and Cef groups. The difference between Amp groups and Cef group was also statistically significant ($p \leq 0.05$). The main source of BDNF is the brain tissue, but since it can cross the blood-brain barrier (Pan et al., 1998) positive correlations between peripheral and central BDNF levels were reported in rodents (Karege et al., 2002ab). Therefore, it is postulated that the serum levels of BDNF may be indicative of its concentration in the central nerves system (Suliman et al., 2013). BDNF promotes the differentiation, survival, and proliferation of neurons in the central and peripheral nervous systems (Aydemir et al., 2006) and is related with activity-dependent neuroplasticity (learning and memory) but also with the modulation of affective behavior (Bercik et al., 2011). In the present study, lower serum levels of BDNF were found in Amp groups showing higher anxiety-like

behavior. Our results remain in line with the previous reports of lower serum BDNF levels in the depressed patients compared to the healthy people (Huang et al., 2008; Lee and Kim., 2010).

Although the serum level concentration of NMDA subunit of NR2B and 5-HT1A can not be interpreted for the cognitive performance, anyhow we discussed them here. The NR2B subunit of glutamate NMDA receptor is one of the critical receptors indicating synaptic plasticity and development as well as learning and memory (Neufeld et al. 2011). Also, the antagonists of NMDA receptors are known to block anxiety in rats and mice (Jessa et al. 1995; Kotlinska et al. 1998). Although there were no significant differences between control and antibiotic-treated groups for NR2B subunit of NMDA receptor, ampicillin treated group appeared to be significantly higher than cefoperazone treated group. This might be a reason for the high anxiety-like behavior in the ampicillin-treated group since this group showed an increase in both times spent in the closed arm of EPM and the peripheral zone of OF tests. At the same time, lower NR2B levels in cefoperazone treated group may be a contributor to their courageous behavior in the open arm when measured in EPM, and the time they spent in the central zone of OF test.

In the etiology of anxiety the serotonin-1A (5-HT1A) receptor has crucial functions in various brain parts, particularly in the dentate gyrus, CA1 regions of the hippocampus, amygdala, entorhinal cortex and lateral septum. Furthermore, with respect to the model of anxiety, the 5-HT1A agonists exert non-identical/diverse actions (Zhuang et al.1999). In our study, we measured total 5-HT1A levels from the blood serum, because of the limitations and difficulties mentioned before. Although there were no significant differences in the 5-HT1A levels both in up and down direction when antibiotic treated groups were compared to control, it seems to have a noticeable difference between ampicillin and cefoperazone treated groups. In spite of this difference, ampicillin and AmpCefAmp treated groups showed depression-like behavior when measured in FST compared to control and cefoperazone treated groups.

CHAPTER 5

CONCLUSION

The major findings of the present work evidently showed that in mice;

- ✓ The administration of two different antibiotics namely, ampicillin and cefoperazone lead to prominent alterations in whole GM of adolescent mice.
- ✓ Remarkably, ampicillin was found to be more destructive to the microbial community than cefoperazone.
- ✓ In parallel, attitudes of mice were also affected differently for each antibiotic as measured in cognitive tests. Diminishing in overall locomotor activity was recorded in every antibiotic-treated group except Cef-Amp-Cef group.
- ✓ The administration of both antibiotics ampicillin and cefoperazone amplified the depressive attitudes in young adults with ampicillin specifically enhancing anxiety- and depressive-like responses.
- ✓ A repeated antibiotic treatment applied during adolescence, parallel to the changes in GM affects cognitive skills in young adults, attention and memory impairments observed after both ampicillin and cefoperazone treatments
- ✓ The low concentrations of serum BDNF were correlated with the affected attitudes, while corticosterone concentrations were not influenced as a result of antibiotic treatments.
- ✓ The increased concentration of serum NR2B in the ampicillin-treated group was considered as a contributor reason to anxiety-like behavior compared to cefoperazone.

Further studies may require thorough investigation of the hormone and neurotransmitter levels in the brain tissue which could be correlated with the cognitive motifs.

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APPENDIX A

PROCEDUR OF ELISA KITS

MyBioSource Mouse 5 Hydroxytryptamine (5-HT)

Catalog Number: MBS723181

THEORY OF THE ASSAY

5-HT ELISA kit applies the competitive enzyme immunoassay technique utilizing a monoclonal anti-5-HT antibody and an 5-HT-HRP conjugate. The assay sample and buffer are incubated together with 5-HT-HRP conjugate in pre-coated plate for one hour. After the incubation period, the wells are decanted and washed five times. The wells are then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. Finally, a stop solution is added to stop the reaction, which will then turn the solution yellow. The intensity of color is measured spectrophotometrically at 450nm in a microplate reader. The intensity of the color is inversely proportional to the 5-HT concentration since 5-HT from samples and 5-HT-HRP conjugate compete for the anti-5-HT antibody binding site. Since the number of sites is limited, as more sites are occupied by 5-HT from the sample, fewer sites are left to bind 5-HT-HRP conjugate. A standard curve is plotted relating the intensity of the color (O.D.) to the concentration of standards. The 5-HT concentration in each sample is interpolated from this standard curve

Sensitivity: The sensitivity in this assay is 1ng/mL

Detection range: The detection range of this kit 6,25-200 ng/Ml

SPECIMEN COLLECTION AND STORAGE

Used a serum separator tube and allowed samples to clot for 2 hours at room temperature or overnight at 2-8°C. Centrifuged at approximately 1000 × g (or 3000 rpm) for 15 minutes. Removed serum and assay immediately or aliquot and stored samples at -20°C or -80°C.

ASSAY PROCEDURE

Please read Reagents Preparation before starting assay procedure. It is recommended that all Standards and Samples be added in duplicate to the microtiter plate. It is strongly recommended to do a preliminary experiment before measuring all samples.

1) Secure the desired numbers of coated wells in the holder then add 100 µL of Standards or Samples to the appropriate well in the antibody pre-coated Microtiter Plate. Add 100 µL of PBS (pH 7.0-7.2) in the blank control well.

2) Dispense 10 µL of Balance Solution into 100 µL specimens, mix well. (NOTE: This step is required when the sample is cell culture supernatants, body fluid and tissue homogenate; if the sample is serum or plasma, then this step should be skipped.)

3) Add 50 µL of Conjugate to each well (NOT blank control well). Mix well. Mixing well in this step is important. Cover and incubate the plate for 1 hour at 37°C.

4) Wash the microtiter plate using one of the specified methods indicated below:

a) Manual Washing: Remove incubation mixture by aspirating contents of the plate into a sink or proper waste container. Fill in each well completely with 1× wash solution, and then aspirate contents of the plate into a sink or proper waste container. Repeat this procedure five times for a total of FIVE washes. After washing, invert plate, and blot dry by hitting the plate onto absorbent paper or paper towels until no moisture appears. Note: Hold the sides of the plate frame firmly when washing the plate to assure that all strips remain securely in frame. Complete removal of liquid at each step is essential to good performance.

b) Automated Washing: Wash plate FIVE times with diluted wash solution (350-400 µL/well/wash) using an auto washer. After washing, dry the plate as above. It is

recommended that the washer be set for a soaking time of 10 seconds and shaking time of 5 seconds between each wash.

5) Add 50 µL Substrate A and 50 µL Substrate B to each well including blank control well, subsequently. Cover and incubate for 10-15 minutes at 37°C. (Avoid sunlight If the color is not dark, please prolong the incubation time. But the longest time is 30min).

6) Add 50 µL of Stop Solution to each well including blank control well. Mix well.

7) Determine the Optical Density (O.D.) at 450 nm using a microplate reader immediately.

CALCULATIONS

The calculated concentrations at the 450nm statistically analyzed by using GraphPad statistic program.

MyBioSource Mouse glutamate NMDA reseptor subunit NR2B ELİSA Kit

Catalog Number: MBS921793

THEORY OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for GRIN2B has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any GRIN2B present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for GRIN2B is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of GRIN2B bound in the initial step. The color development is stopped and the intensity of the color is measured.

Detection range: 31.25 pg/ml -2000 pg/ml.

Sensitivity: 7.8 pg/ml

SAMPLE COLLECTION AND STORAGE

Use a serum separator tube (SST) and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1000 ×g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.

ASSAY PROCEDURE

1. Prepare all reagents, working standards, and samples as directed in the previous sections.

2. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and seal the ziploc, store unused wells at 4°C.

3. Add 100µl of standard and sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at 37°C. A plate layout is provided to record standards and samples assayed.

4. Remove the liquid of each well, don't wash.

5. Add 100µl of Biotin Biotin Biotin Biotin Biotin -antibody antibody antibody antibody antibody antibody (1x) 1x) 1x) to each well. Cover with a new adhesive strip. Incubate for 1 hour at 37°C. (Biotin Biotin Biotin Biotin Biotin -antibody antibody antibody antibody antibody antibody (1x) 1x) may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.)

6. Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 2 minutes, complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash Buffer by aspirating ordecanting. Invert the plate and blot it against clean paper towels.

7. Add 100µl of HRP-avidin (1x) 1x) 1x) to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37°C.

8. Repeat the aspiration/wash process for five times as in step 6.

9. Add 90µl of TMB Substrate to each well. Incubate for 15-30 minutes at 37°C. Protect from light.

10. Add 50µl of Stop Solution to each well, gently tap the plate to ensure thorough mixing.

11. Determine the optical density of each well within 5 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. Subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATIONS

The calculated concentrations at the 450nm statistically analyzed by using GraphPad statistic program.

MyBioSource Mouse Corticosterone (CORT) ELISA Kit

Catalog Number: MBS703441

PRINCIPLE OF THE ASSAY

This assay employs the competitive inhibition enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with goat-anti-rabbit antibody. Standards or samples are added to the appropriate microtiter plate wells with an antibody specific for CORT and Horseradish Peroxidase (HRP) conjugated CORT. The competitive inhibition reaction is launched between with HRP labeled CORT and unlabeled CORT with the antibody. A substrate solution is added to the wells and the color develops in opposite to the amount of CORT in the sample. The color development is stopped and the intensity of the color is measured.

Detection range: 0.1 ng/ml 0 -20 ng/ml.

Sensitivity: Is typically less than 0.05 ng/ml.

SAMPLE COLLECTION AND STORAGE

Use a serum separator tube (SST) and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1000 ×g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.

ASSAY PROCEDURE

1. Prepare all reagents and samples as directed in the previous sections.

2. Determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and seal the ziploc, store unused wells at 4°C.

3. Set a Blank well without any solution.

4. Add 50µl of Standard or Sample per well. Standard need test in duplicate.

5. Add 50µl of HRP-conjugate to each well (not to Blank well), then 50µl Antibody to each well. Mix well and then incubate for 1 hour at 37°C.

6. Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 10 seconds, complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating ordecanting. Invert the plate and blot it against clean paper towels.

7. Add 50µl of Substrate A and 50µl of Substrate B to each well, mix well. Incubate for 15 minutes at 37°C. Keeping the plate away from drafts and other temperature fluctuations in the dark.

8. Add 50µl of Stop Solution to each well, gently tap the plate to ensure thorough mixing.

9. Determine the optical density of each well within 10 minutes, using a microplate reader set to 450 nm.

CALCULATIONS

The calculated concentrations at the 450nm statistically analyzed by using GraphPad statistic program.

MyBiosource Mouse Brain Derived Neurotrophic Factor (BDNF) Elisa kit

Catalog number: MBS724249

THEORY OF THE ASSAY

BDNF ELISA kit applies the quantitative sandwich enzyme immunoassaytechnique. The microtiter plate has been pre-coated with a monoclonal antibody specific for BDNF. Standards or samples are then added to the microtiter plate wells and BDNF if present, will bind to the antibody pre-coated wells. In order to quantitatively determine the amount of BDNF present in the sample, a standardized preparation of horseradish peroxidase (HRP)-conjugated polyclonal antibody, specific for BDNF are added to each well to "sandwich" the BDNF immobilized on the plate. The microtiter plate undergoes incubation, and then the wells are thoroughly washed to remove all unbound components. Next, substrate solutions are added to each well. The enzyme (HRP) and substrate are allowed to react over a short incubation period. Only those wells that contain BDNF and enzyme-conjugated antibody will exhibit a change in color. The enzyme-substrate reaction is terminated by addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. A standard curve is plotted relating the intensity of the color (O.D.) to the concentration of standards. The BDNF concentration in each sample is interpolated from this standard curve

Detection range: This assay has high sensitivity and excellent specificity for detection of BDNF. No significant cross-reactivity or interference between BDNF and analogues was observed. NOTE: Limited by current skills and knowledge, it is impossible for us to complete thecross-reactivity detection between BDNF and all the analogues, therefore, cross reaction may still exist in some cases.

Sensitivity: The sensitivity in this assay is 1.0 pg/mL

ASSAY PROCEDURE

1. Secure the desired numbers of coated wells in the holder then add 50 µL of Standards or Samples to the appropriate well in the antibody pre-coated Microtiter Plate. Add 50 µL of PBS (pH 7.0-7.2) in the blank control well.

2. Dispense 5 µL of Balance Solution into 50 µL specimens, mix well. (NOTE: This step is required when the sample is cell culture supernatants, body fluid and tissue homogenate; if the sample is serum or plasma, then this step should be skipped.)

3. Add 100 µL of Conjugate to each well (NOT blank control well). Mix well. Mixing well in this step is important. Cover and incubate the plate for 1 hour at 37°C.

4. Wash the microtiter plate using one of the specified methods indicated below:

a) Manual Washing: Remove incubation mixture by aspirating contents of the plate into a sink or proper waste container. Fill in each well completely with 1× wash solution, and then aspirate contents of the plate into a sink or proper waste container. Repeat this procedure five times for a total of FIVE washes. After washing, invert plate, and blot dry by hitting the plate onto absorbent paper or paper towels until no moisture appears. Note: Hold the sides of the plate frame firmly when washing the plate to assure that all strips remain securely in frame. Complete removal of liquid at each step is essential to good performance.

b) Automated Washing: Wash plate FIVE times with diluted wash solution (350-400 µL/well/wash) using an auto washer. After washing, dry the plate as above. It is recommended that the washer be set for a soaking time of 10 seconds and shaking time of 5 seconds between each wash.

5. Add 50 µL Substrate A and 50 µL Substrate B to each well including blank control well, subsequently. Cover and incubate for 10-15 minutes at 37°C. (Avoid sunlight If the color is not dark, please prolong the incubation time. But the longest time is 30min).

6. Add 50 µL of Stop Solution to each well including blank control well. Mix well.

7. Determine the Optical Density (O.D.) at 450 nm using a microplate reader immediately.

CALCULATIONS

The calculated concentrations at the 450nm statistically analyzed by using GraphPad statistic program.

APPENDIX B

DETAILS OF DGGE APLICATION

DGGE system of BioRad Company was used for DGGE operation. The electrophoresis system was appropriately prepared and sealed using "spacers" allowing the formation of 1 mm polyacrylamide gels. Then 0% and 100% DGGE stock solutions were prepared. The 40% and 60% solutions were prepared from these stock solutions and mixed using a gradient forming device so that the solutions would be formed into a gradient gel. The increased concentration of gelatin ensures that it has a urea and formamide gradient. The denaturant concentration in the denaturing gradient gel is set to increase from 30% to 50%. The comb was placed in the gel and allowed to stand for at least 2 hours for polymerization. During the polymerization, the electrophoresis tank was filled with 1X TAE buffer and the temperature was expected to reach 60 °C. The DNA concentration in the DGGE PCR products was previously determined using the Low mass DNA ladder marker and the sample was prepared in an amount corresponding to 500 ng. 10 µL of gel loading paint was added to the pits and allowed to walk for 4.5 hours at 130 V. After electrophoresis, polyacrylamide gel was washed with ethidium bromide solution (1 µg / mL) for 15 minutes, then washed twice in distilled water for 10 minutes and observed with UV light in transilluminator. The bands in different positions in the jellies were cut into gels with sterile scalpel under UV light and taken into sterile ependorf tubes. Solvent buffer was added at the gel weight ratio. After incubation at 37 °C for 1 night, the section DNA isolation method was applied after Fenol: Chloroform: Isoamylalcohol (25: 24: 1) step and amplified with DGGE primers without GC tail. The PCR obtained products were sent to sequence analysis.

APPENDIX C

SOLUTIONS OF DGGE APLICATION

100% DGGE stock solution

Urea	42 g
Deionized Formamide	40 mL
Acrylamide/Bisacrylamide (40%)	20 mL
TAE (50X)	2 mL

mQ su ile 100 mL'ye tamamlanıp +4 °C'de saklanmıştır

0% DGGE stock solution

Acrylamide/Bisacrylamide (40%)	20 mL
TAE (50X)	2 mL

In mQ water 100 mL final volume, stored at +4 °.

Solvent Solution

Amonium acetate	0.5 M
Magnesium acetate	10 mM
EDTA (pH 8.0)	1 mM
SDS	% 0.1

CURRICULUM VITAE

Personal information

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Education

PhD	Middle East Technical University (METU), Graduate school of Natural and Applied sciences, School of Biology, Ankara (CGPA 3.43/4), **2012- 2017**
MSc	Yüzüncü Yıl University (YYU), Graduate school of Natural and Applied sciences, School of Biology (MS), Van (CGPA 89/100), **2008- 2010**
MD	Istanbul University (IU) Graduate school of Natural and Applied sciences, School of Biology (CGPA 70/100), **2003- 2008**

Computer knowledge

Microsoft Office Programs, Various Statistical and other scientific analysis programs such as GraphPad, SPSS.

Language skills

YDS-2015 English- **66.5**

- English- fluent

Other skills

Training and tutoring for students (graduate and undergraduate) in the research laboratory.

Developed succinct Report Writing Skills through project assignments.

Learnt to speak efficiently in formal meetings through my course representative duties.

Gained insight into how teams can develop strategies to deal with problems through participating as a team member in project assignments.

Independent conduction of scientific experiments
Possess experience in microbiology, molecular biology

Publications

Academic Thesis and Dissertation

- **MS thesis:** Isolation, characterisation and identification of *amycolatopsis* and *streptomyces spp*. İsolated from soil.

- **PhD dissertation:** Impact of antibiotic induced gut microbiota alteration on cognitive abilities and behaviousr of mice

Articles

Taha Ceylani, Ewa Jakubowska-Doğru, Rafig Gurbanov, Hikmet Taner Teker, Ayse Gul Gozen, "The effects of repeated antibiotic administration to juvenile BALB/c mice on the microbiota status and animal behavior in young adults", Neuroscience letter, (Submitted at 09.12.2017)

Conference Papers and Proceedings (Presentations and posters)

- Taha Ceylani, Ewa Jakubowska-Doğru, Rafig Gurbanov, Hikmet Taner Teker, Ayse Gul Gozen, "Bowel-brain axis affected by cefoperazone and ampicillin treatment in mice", 6th International Congress of Molecular Medicine Congress, May 22-25, 2017, Istanbul, Turkey. p.71 (oral presentation).

- Taha Ceylani, Ewa Jakubowska-Doğru, Rafig Gurbanov, Hikmet Taner Teker, Ayse Gul Gozen, "Brain biochemistry and gut microbiota (GM) influenced by antibiotic treatment during adolescence period of mice", 6th International Congress of Molecular Medicine Congress, May 22-25, 2017, Istanbul, Turkey. p.152 (poster presentation).