Characterization of Gut Microbiota in Newly Diagnosed Patients with Depression

Does the Depressed Gut Exist?

Knudsen, Julie Kristine

DOI (link to publication from Publisher):
10.54337/aau455013245

Publication date:
2021

Document Version
Publisher's PDF, also known as Version of record

CHARACTERIZATION OF GUT MICROBIOTA IN NEWLY DIAGNOSED PATIENTS WITH DEPRESSION

DOES THE DEPRESSED GUT EXIST?

BY
JULIE KRISTINE KNUDSEN

DISSERTATION SUBMITTED 2021

AALBORG UNIVERSITY
DENMARK

CHARACTERIZATION OF GUT MICROBIOTA IN NEWLY DIAGNOSED PATIENTS WITH DEPRESSION

DOES THE DEPRESSED GUT EXIST?

by

Julie Kristine Knudsen

AALBORG UNIVERSITY
DENMARK

Dissertation submitted June 15th, 2021

Dissertation submitted: 15.06.2021

PhD supervisor: Ass. Prof. Suzette Sørensen,
Centre for Clinical Research,
North Denmark Regional Hospital, Hjørring, Denmark
Department of Clinical Medicine, Aalborg University,
Aalborg, Denmark

Assistant PhD supervisors: Prof. René Ernst Nielsen,
Aalborg University and Psychiatry,
Aalborg University Hospital, Aalborg Denmark

Ass. Prof. Simon Hjerrild,
Aarhus University and Psychosis Research Unit,
Aarhus University Hospital, Aarhus, Denmark

PhD committee: Associate Professor Kristine Allin (chair)
Aalborg University

Dr. Gerard Clarke
University College Cork

Professor Michael Eriksen Benros
Copenhagen University Hospital

PhD Series: Faculty of Medicine, Aalborg University

Department: Department of Clinical Medicine

ISSN (online): 2246-1302
ISBN (online): 978-87-7210-956-5

Published by:
Aalborg University Press
Kroghstræde 3
DK – 9220 Aalborg Ø
Phone: +45 99407140
aauf@forlag.aau.dk
forlag.aau.dk

Printed in Denmark by Rosendahls, 2021

PREFACE

All Disease Begins in the Gut – as famously said by the Father of Medicine, Hippocrates. Recently, this idea has grown roots and spread its ideology across the medical research field like a weed, and most non-communicable and/or chronic diseases are now being investigated for involvement of the intestinal bacteria, also popularly referred to as the gut microbiota. While this notion seems wild, and it surely did to me when I was first introduced to the subject, I realized after a few months at the Centre for Clinical Research that this may be the new 'antioxidants' of the 2000s – the cause of every disease with unknown or complicated etiology, and therefore, naturally, the solution to it.

To me, neurology and psychiatry have always been the most fascinating medical subjects, due to their complicated nature. How can simple neurochemical fluctuations result in melancholia? How can emotional trauma undealt with lead to life-long suffering despite the constant, everchanging nature of the surrounding world? This fascination never left me after I finished my Master's degree with a focus on schizophrenia, and it was therefore a no-brainer that I would jump at the opportunity to explore further what the mind had to offer. Although depression has historically been recognized for millennia, the origin of it is still unknown and thought to consist of complex interactions between several organic systems. Imagine my surprise when I found that even the intestinal bacteria may play a vital role in this disorder as well! I had to delve deeper into this subject and discover something that might add to the current knowledge in this scientific field.

My research objective throughout the last four years have been to examine the gut microbiota and its involvement in depression. I will say now, in hindsight, that I succeeded in what I set out to do, although my perspective has changed radically during the study. In the beginning, I was enthusiastically convinced that the intestinal microbes certainly were involved in the development and severity of depression. During the study design, the recruitment of participants, the transplantation of fecal matter into rats, the data synthesis and the article writing, nuances kept appearing to give me a broader perspective. Occam's Razor is not always the solution, and the gut microbiota is such an intricate system that it was foolish and naïve to think that it may provide me with a simple answer to a complex question: Is there a depressed gut? Read the thesis to see how I tried to answer that question.

The clinical work was conducted at the Department of Psychiatry, Aalborg University Hospital, Aalborg, Denmark, and the animal research was conducted at Translational Neuropsychiatry Unit, Aarhus University, Aarhus, Denmark. All laboratory work was performed at the Centre for Clinical Research, North Denmark Regional Hospital, Hjørring, Denmark.

ENGLISH SUMMARY

Background: In recent years, it has been suggested that the gut microbiota may be involved in major depressive disorder (MDD) by both animal and clinical studies. However, the studies conducted so far have only provided limited results depending heavily on which methods were used to determine the gut microbiota composition. Therefore, it is necessary with additional studies consisting both clinical experiments, as well as new animal models, to conclude if there is indeed an association between the gut microbiota and MDD

Aims: The overall aim of the study was to analyze the possible association between gut microbiota and MDD. Furthermore, we wished to explore if the gut microbiota of patients with MDD could elicit a depressive-like behavior in an animal model.

Methods: Initially, we performed a systematic literature search to evaluate the studies conducted on patients with MDD thus far. In the animal study, we transplanted fecal material from patients with MDD (FMT-MDD) or non-depressed individuals (FMT-Healthy) into rats thrice a week for three weeks. Additionally, we included two control groups. After the three weeks of FMT, we analyzed the depressive behavior of the animals. In the clinical study, we recruited young patients diagnosed with MDD with no prior antidepressant treatment, as well as non-depressed individuals (nonMDD). Participants donated fecal samples at baseline, and at four and twelve weeks follow-up. Patients commenced antidepressant treatment after delivery of the baseline sample. The gut microbiota composition was evaluated by 16S rRNA gene sequencing in both the animal and the clinical study.

Results: The literature study revealed that most previous studies observed significantly different gut microbiota in their patients with MDD compared to non-depressed controls. However, there was a lack of consensus on which bacteria were significantly altered between the two groups. In the animal study, there was a difference in depressive behavior between the FMT-MDD and the FMT-Healthy rats, but not between the FMT-MDD rats, and control rats. There were several individual taxa, originating from the human donors, which were significantly different between the FMT-MDD and the FMT-Healthy rats. In the clinical study, we also found bacteria which were significantly different between the MDD and nonMDD groups. Moreover, some bacterial taxa changed in the MDD group during the twelve weeks follow-up.

Conclusions: In all three studies, it was possible to separate the MDD and its control group based on individual bacteria. Nevertheless, it was not possible in the experimental studies to separate the two groups based on overall microbial diversity. Our findings therefore suggest that changes induced to the gut microbiota is visible in distinct taxa rather than in the overall gut microbiota diversity.

DANSK RESUME

Baggrund: I de seneste år er en sammenhæng mellem tarmens mikrobiota og depression (MDD) blevet foreslået igennem både dyrestudier og kliniske studier. Dog har studierne publiceret indtil videre kun givet begrænsede resultater, hvilket er dybt afhængigt af hvilke metoder de har brugt til at karakterisere sammensætningen af tarmbakterier. Derfor er det nødvendigt med yderligere studier i både et klinisk setting, og med nye dyremodeller, for at kunne konkludere om der vitterligt er en sammenhæng mellem tarmens mikrobiota og MDD.

Formål: Det overordnede formål med studiet var at analysere den potentielle sammenhæng mellem tarmens mikrobiota og MDD. Yderligere ønskede vi at undersøge om tarmbakterier fra patienter med MDD kunne inducere en depressiv-lignende adfærd i en dyremodel.

Methods: Først lavede vi en systematisk litteratursøgning for at evaluere de studier der var udført på patienter med MDD indtil videre. I dyreforsøgene transplanterede vi fæces fra patienter med MDD (FMT-MDD) eller ikke-depressive individer (FMT-Healthy) ind i rotter tre gange over tre uger. Derudover inkluderede vi også to kontrolgrupper af rotter. Efter tre ugers FMT analyserede vi dyrenes adfærd. I det kliniske studie rekrutterede vi unge patienter med MDD uden tidligere antidepressiv behandling, samt ikke-depressive individer (nonMDD). Deltagerne donerede afføringsprøver ved baseline, samt ved fire og tolv ugers opfølgning. Patienterne opstartede deres antidepressive behandling efter indleveringen af baseline prøven. Tarmens mikrobiota sammensætning blev undersøgt via 16S rRNA gen sekventering i både dyrestudiet og det kliniske studie.

Resultater: I litteraturstudiet fandt vi at næsten alle tidligere studier havde observeret bakterier der var signifikant forskellige mellem MDD og non-depressive individer. Dog var der ingen konsensus om hvilke specifikke bakterier der var de signifikant forskellige mellem de to grupper. I dyrestudiet fandt vi at der var en forskel i den depressive adfærd mellem FMT-MDD og FMT-Healthy rotterne, men ikke mellem FMT-MDD gruppen og de to kontrolgrupper. Der var flere bakterier der var signifikant forskellige mellem FMT-MDD og FMT-Healthy rotterne, som kunne genfindes fra de humane donorer. I det kliniske studie fandt vi også bakterier der var signifikant forskellige mellem MDD og nonMDD gruppen. Yderligere fandt vi at nogle af bakterierne ændrede sig under de tolv ugers opfølgning.

Konklusioner: I alle tre studier var det muligt at separere MDD og kontrol gruppen baseret på individuelle taxa. Dog var det ikke muligt i de eksperimentelle studier at separere de to grupper baseret på den overordnede mikrobiel diversitet. Vores fund indikerer derfor at ændringerne i den overordnede bakterielle sammensætning primært kan ses i individuelle bakterier, end på den generelle diversitet.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude everyone who has contributed to this project, be it academically, technically, or emotionally. It has been four wonderfully exciting, but also incredibly challenging and demanding, years.

First and foremost, I would like to thank my main supervisor Suzette Sørensen for giving me the opportunity to conduct my studies under her supervision and learn a great deal on how to conduct scientific experiments, as well as her collaborative support. I am also greatly appreciative towards my two co-supervisors René Ernst Nielsen and Simon Hjerrild, for whom the clinical part of the study would not have been possible, and whom have taught me how to think like a clinician. Furthermore, I would like to express my gratitude towards my mentor from Aarhus University, Gregers Wegener, for letting me conduct my animal experiment s at his facility with both his support, supervision and great feedback. Finally, I would like to express my gratefulness towards Peter Leutscher, who gave a young, newly graduated scientist a chance to fulfil her dream of conducting a PhD study.

A special thanks to all former and current staff at both the Centre for Clinical Research, the Translational Neuropsychiatry Unit, Aarhus University, Aarhus and the Department of Psychiatry, Aalborg University Hospital, Aalborg for their assistance on the clinical and/or the animal studies. This study was not possible without the great help you provided me with, be it academic sparring or technical support. A huge thanks to Caspar Bundgaard-Nielsen, Annemarie Villadsen, Louise Rold, Louise Arenholt, Thomas Yssing Michaelsen, Sandra Tillman, and Amanda Eskelund for academic discussions and feedback. A grateful thank you to our technical staff Maria Jensen, Bente Jensen, Signe Østergaard, Katrine Bech Lauritzen, Marit Nielsen, Per Mikkelsen, Stine Dhiin and Tessa Rasmussen, as without you, this study would have been impossible to complete. And furthermore, thank you to Gustav Bizik, Anelia Larsen, Simone Madsen, and Winni Abildgaard for assisting with recruitment of patients to the study, which was the vital part of the entire thesis. I will forever remember my time in each department with joy, but especially Centre for Clinical Research will always have a dear place in my heart as being where I started my career. Finally, I would like to express gratitude towards my friends and family, especially my mom and dad, whom have listened endlessly to my complaints, my highs and lows, and my achievements. I would not have been able to complete these last four years had you not carried me some of the way.

This study was supported by North Denmark Regional Hospital, Aalborg University and Aarhus University, Aalborg University Hospital, Horsens Regional Hospital, Savværksejer Jelle Juhl og Hustru Ovita Juhls Mindelegat, Svend Andersen Fonden, Grosserer L. F. Foghts Fond, Marie Pedersen og Jenseine Heibers Legat, Else og Mogens Wedell-Wedellborgs Fond, Torben og Alice Frimodts Fond and Frimodt-Heineke Fonden.

CONTENTS

Chapter 1. Background 1
1.1 Gut microbiota 1
1.1.1 Composition 1
1.1.2 Development 2
1.1.3 Influence of intrinsic and external factors 3
1.1.4 Functions of the gut microbiota 5
1.1.5 Dysbiosis 7
1.2 Depression 9
1.2.1 Prevalence, symptoms and burden 9
1.2.2 Pathophysiology 10
1.2.3 Common treatment options 12
1.3 Association between gut microbiota and depression 14
1.3.1 Preclinical assessment and investigations 14
1.3.2 The leaky gut theory 15
1.3.3 Systemic inflammation 17
1.3.4 Gut-brain axis 17
1.3.5 Antidepressants and the gut microbiota 19
Chapter 2. Aims and study parts 21
Chapter 3. Methods 23
3.1 Study I – Design overview and outcomes 23
3.2 Study I – Critical evaluation of methods 23
3.3 Study II – Design overview and outcomes 25
3.4 Study II – Critical evaluation of methods 26
3.4.1 Animal models 26
3.4.2 Fecal microbiota transplantation 27
3.4.3 Behavior 28
3.4.4 Markers of altered intestinal permeability 29
3.5 Study III – Design overview and outcomes 30
3.6 Study III – Critical evaluation of methods 31
3.6.1 Recruitment of participants 31

3.6.2 – Evaluation of depressive and gastrointestinal symptoms 32
3.6.3 Antidepressant treatment..... 33
3.7 Methods common for the animal and clinical studies..... 33
3.7.1 Gut microbiota characterization 33
3.7.2 Statistical and bioinformatical analyses 35
3.7.3 Ethical considerations 36
Chapter 4. Results 37
Study I..... 37
Study II..... 39
Study III 42
Chapter 5. Discussion 45
5.1. The gut microbiota in patients with MDD is significantly different in taxa and diversity..... 45
5.1.1. Limitations and strengths 47
5.2 FMT from healthy donors increased struggling in FRL recipient rats 48
5.2.1 Limitations and strenghts 50
5.3 Clinical relevance of gut microbiota in patients with MDD 51
5.3.1 Limitations and strenghts 53
Chapter 6. Conclusions.....55
Chapter 7. Perspectives 57
Chapter 8. Reference list 59
Chapter 9. Appendices..... Fejl! Bogmærke er ikke defineret.

LIST OF ABBREVATIONS

ASV	Amplicon sequence variants
BDNF	Brain-derived neurotrophic factor
CUMS	Chronic unpredictable mild stress
DNA	Deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of Diseases
FRL	Flinders Resistant Line
FSL	Flinders Sensitive Line
GABA	γ-aminobutyric acid
GF	Germ-free
ICD	International Classification of Diseases
IL	Interleukin
LPS	Lipopolysaccharide
MDD	Major depressive disorder
MDI	Major Depressive Inventory
OTU	Operational taxonomic unit
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
rRNA	Ribosomal ribonucleotide acid
SCFA	Short chain-fatty acid
TJP	Tight junction protein
TNF-α	Tumor necrosis factor alpha

CHAPTER 1. BACKGROUND

1.1 GUT MICROBIOTA

Microbiota is a term covering the entirety of all microorganism living within a habitat, such as bacteria, viruses, and eukaryotes (1). Usually, the term 'gut microbiota' is used interchangeably applying to all living microorganisms in the gastrointestinal tract (2), but commonly, the term is used to refer to the collective bacterial population in the gut (3).

1.1.1 COMPOSITION

Overall, it is estimated that there is roughly one bacterial cell per human cell in an adult male body. Correspondingly, with a total of 200 grams of bacteria in the human body, this makes up only 0.3% of the total human body weight (4). Nonetheless, the number of bacteria of the human gut microbiota depends on the specific segments of the gastrointestinal tract. The upper gastrointestinal tract, consisting of the oral cavity, the stomach and the small intestine, has an estimated 10^3-10^4 bacteria per gram of intestinal content (5). In the lower gastrointestinal tract, as transit time slows down, the number of bacteria rises exponentially to 10^{10}-10^{11} bacteria per gram of intestinal content (5).

Most studies investigate the human gut microbiota by collecting fecal samples (6). Early studies of the individual humans found that there was a high diversity of bacteria in the human gut microbiota analyzed from fecal samples (7). Several large consortia has attempted to generalize the healthy human gut microbiota using next generation sequencing techniques. The Human Microbiome Consortium (8) and the MetaHit Consortium (9) were the some of the first large-scale American and European studies, sampling and analyzing the gut microbiota of 242 and 124 adult humans, respectively. Since then, several large-scale population studies have performed next generation sequencing of the gut microbiota. Recently, a study sampled 11,850 adults and found that the majority of bacteria belonging to the human gut belong to the phyla Firmicutes, Actinobacteria, Bacteroidetes, Tenericutes and Proteobacteria, with a small fraction belonging to other phyla (10) (see *Figure 1* for the relative abundance of bacterial taxa). With higher resolution through next generation sequencing, it became obvious that several of the human gut bacteria still remain unclassified and uncultured (10).

The exact composition of the human gut microbiota remains difficult to define, as it is highly personalized and individually adapted to its host genotype (11). Therefore, there is a high degree of inter-individual variation (8). Some studies suggested that the gut microbiota can be divided into 'enterotypes' defined by their relative ratio of specific genera, namely *Bacteroides* (type I), *Prevotella* (type II) or *Ruminococcus* (type III) (12). These enterotypes consist of distinctive clusters of bacteria dependent on which nutrients they rely on as their primary energy source. Initially, next generation sequencing analyses struggled to define a 'core set' of species-level

bacteria present in all humans (13, 14). More recently though, population-level analyses of pooled datasets from national and global studies have found bacteria putatively present in all study subjects (15, 16). Twin studies have confirmed an interplay between host genetics and gut microbiota composition (17, 18), which has likewise been examined in mother-infant cohorts during the development of the human gut microbiota. As a core microbiota has been identified amongst humans, and that it has been shown to be affected by genetic dispositions, the gut microbiota may also be associated with several diseases.

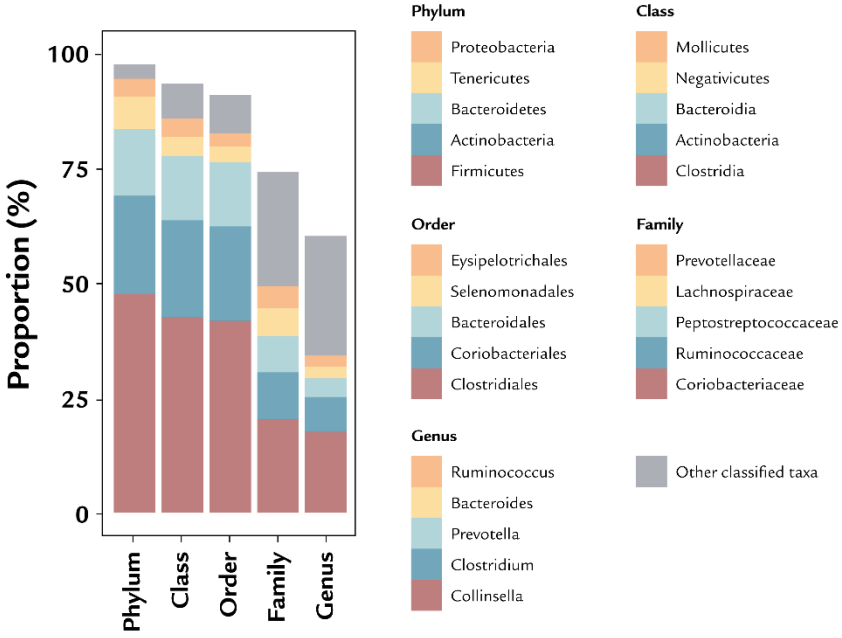

***Figure 1* – Relative abundance of bacterial taxa in the human gut at taxonomic levels phylum, class, order, family and genus.** Adapted from (10)

1.1.2 DEVELOPMENT

It is well-known that the human gut microbiota is obtained primarily by vertical transfer from the mother (19), but it is continuously disputed if the colonization begins *in utero* or at birth (20). Some argue that *in utero* transmission of maternal gut microbiota is confirmed by detection of bacteria in the placenta (21), fetal membranes (22), umbilical cord blood (23) or amniotic fluid, while others confer these results to extra-maternal contaminants (24). However, birth is a main impact of the development and structure of the gut microbiota. Mode of delivery, gestational age at delivery and nutritional type were found to impact the development of the gut microbiota up until

24 weeks of age in a study (25). In vaginally-delivered children, the infant is colonized by bacteria enriched in the maternal vagina, such as the genera *Prevotella*, *Lactobacillus* and *Sneathia* (26). Conversely, children born by cesarean section are dominated by bacteria predominantly inhabiting the skin of the mother, such as the genera *Streptococcus* and *Propionibacterium* (25, 27). In vaginally-delivered children fed with breastmilk, the neonatal gut microbiota is characterized by low diversity. Colonization by facultative anaerobes such as *Streptococcus*, and *Lactobacillus* drives the transformation of the gut lumen towards anaerobic conditions (19).

As the infant ages, the gut microbiota diversifies to include several obligate anaerobes such as the genera *Bifidobacterium* and *Clostridium*, which colonize the gut within the first weeks of life (28, 29). The majority of these taxa derive their energy from human milk oligosaccharides (30). When solid foods are introduced into the diet, a further shift is driven in the gut microbiota diversification, giving rise to increases in genera such as *Bacteroides* (31, 32). At one year of age, the gut microbiota of the infant resembled that of their mother, although with increased complexity and diversity than during the first weeks of life (33). The gut microbiota stabilizes into a complete, fully developed entity at approx. three years of age (34) and is henceforth temporally stable during adulthood (35), unless disturbed by external factors. Even though there is still a dispute on whether the colonization in the infant begins *in utero* or at birth, it is nevertheless clear that the structure remains stable in the adult. Therefore, structural differences between groups of people may be clinically relevant for studies of diseases with complex etiologies.

1.1.3 INFLUENCE OF INTRINSIC AND EXTERNAL FACTORS

Several intrinsic and external factors can influence the growth, and expansion of the gut microbial composition, as illustrated in *Figure 2*. The local environment, such as pH, oxygen concentration, transition time, and water content affects the gut microbiota composition. The large density and diversity of the colonic microorganisms is partially explained by constant and active mixing through colonic muscle contractions, which limits complete bacterial expulsion upon defecation (36, 37). As roughly half of the bacterial biomass is lost daily through defecation (38), colonic bacterial replication and growth is necessary to replenish the lost mass.

Fluctuations in local pH, which happens segmentally throughout the gastrointestinal tract, can affect the growth rate of specific bacterial taxa, and this is regulated by secretions of bicarbonate by the intestinal epithelium (36). For example, the lower the local pH, the higher the competitive advantage for taxa belonging to the *Firmicutes* phylum, as these bacteria tolerate slightly acidic environments better (39). The local pH is also influenced by luminal water content, which in turn is regulated by transit time and colonic motility (40), affecting the gut microbiota composition (41). Bacterial richness is inversely correlated with increased fecal water content, and species belonging to the *Bacteroidetes* phylum have the competitive advantage at higher pH, which often follows higher water content (15, 41). Transverse oxygen gradients from the colonic lumen to the apical epithelial surface also impacts the gut microbiota composition, and aerotolerant bacteria are primarily associated with the mucosal surface, while obligate anaerobes are enriched in the bowel lumen (42).

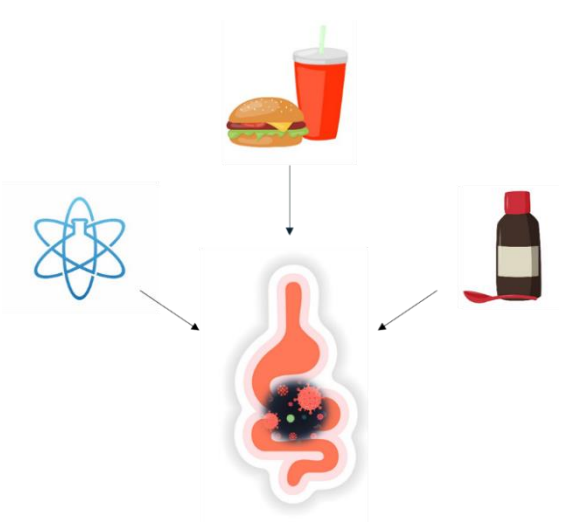

***Figure 2* - Environmental effects on gut microbiota composition.** Left: biochemical. Middle: dietary. Right: xenobiotic.

While local biochemical variations can affect the competitive advantage of different bacterial taxa, external environmental factors also impact the overall composition and function of the gut microbiota. Diet is widely known to transform the intestinal microbes depending on the primary source of nutrients (43). Diets rich in refined sugars and animal-derived fats and proteins, often termed the western diet, has been linked with loss of bacterial diversity (44). This is furthermore recognized in studies assessing the gut microbiota of different geographical locations by comparisons between rural populations and developed countries such as the United States (34, 45, 46). Dietary restrictions and preferences are the primary driving factors of significant differences in bacterial composition rather than geographical location (47). This is observable in the western diet for example, as there is a loss of *Bacteroidetes*, compared to a diet richer in fibers and vegetables (48), and dietary restrictions also correlate with enterotypes (49).

Just as nutrient intake afflicts the microbial composition, types and duration of medical treatments also influences the bacterial population (15). Virtually all

xenobiotics has an impact on individual bacterial taxa, as was illustrated in an *in vitro* study of 40 gut isolates incubated with 1,079 different compounds. The majority of the drugs are given as oral administration, and have human cell molecular targets, yet still impose an anti-bacterial effect on gut microbes (50). The medical treatment with the highest impact on gut microbiota is without question antibiotics given to combat infectious diseases. Both narrow- and broad-spectrum antibiotics can have sustained community-wide effects on the gut microbiota composition such as permanent or prolonged loss of distinct taxa (51-53). Some effects were conserved amongst all patients, such as loss of bacterial diversity, while others were highly individualized, such as the recovery of individual taxa. Other such effects are increased susceptibility to pathogenic growth due to loss of colonization resistance by the gut microbiota (54), as well as diarrhea imposed by increased gut motility due to serotonin depletion (55). Additionally, ingestion of xenobiotics stimulate temporal expression of genes involved in drug resistance and drug metabolism in the gut microbiota (56). The effect of xenobiotics on the gut microbiota may affect the structural properties, which could be either beneficial in a disease perspective, or detrimental to health-beneficial bacteria.

1.1.4 FUNCTIONS OF THE GUT MICROBIOTA

While the gut microbial structure constitutes a high diversity of bacteria, there is a high degree of functional redundancy amongst members of the gut microbiota, with conservation of several microbial genes across genetically diverse species (57). Recently though, the functional redundancy of the intestinal microbes has been explained by some researchers as a resilience method in response to external perturbations (58). Despite the functional redundancy, the gut microbiota has a wide variety of mutualistic functions, such as priming and regulating the immune system, protection from foreign pathogenic invaders, and production of host-beneficial vitamins and metabolites (see *Figure 3*).

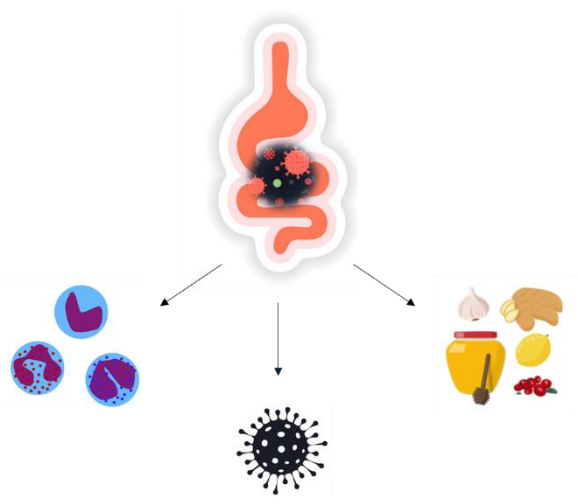

***Figure 3* – Functions of the gut microbiota.** Development of the immune system (left), protection against foreign pathogens (middle) and production of host-dependent nutrients and vitamins (right).

Just as the gut microbiota changes composition and diversity during the first years of human life, the intestinal commensals themselves coordinate the development of a fully functioning immune system. This is not only a complete necessity to fully combat pathogenic infections later in life, but is equally as important in the maturation of tolerance to self-, commensal- or environmentally-associated antigens (59). If the immune system is not primed to tolerate the gut microbiota, this would result in an overt, chronic inflammatory state along the entirety of the gastrointestinal tract due to the constant microbe-host interactions. Lymphoid tissue along the gastrointestinal tract develop during the first years of life as a result of commensal interaction with epithelial cell receptors (60). The innate immune response is refined by priming by several molecular-associated molecular patterns. For example, bactericidal c-type lectins are expressed by Paneth cells in response to gram-positive bacterial peptidoglycans (61), while specialized secreted immunoglobulin A compartmentalize intestinal bacteria to the gut lumen by agglutination (62, 63). The adaptive immune response is also dependent on bacterial-host interactions for maturation. Prolonged exposure to bacterial components, such as the gram-negative cell wall molecule lipopolysaccharide (LPS), is registered in the neonatal gastrointestinal tract by Toll-like receptors. This leads to impairment of inflammatory mediators (64) and a subsequent hypo-responsiveness to receptor activation in adulthood (65). Likewise, the gram-negative capsule component polysaccharide A assists in the maturation and regulation of a proper T_H1/T_H2 immunological balance (66). Even after complete immune function maturation, several species of the gut microbiota confer continuous regulatory effects, such as the anti-inflammatory properties of *Faecalibacterium prausnitzii* (67).

Colonization resistance of foreign pathobionts in the gastrointestinal system by the gut microbiota are facilitated by several mechanisms (68). The first ‘defense’ mechanism by the gut microbiota is limiting nutritional components, such as the ability to utilize specific sugars, thereby gaining the competitive advantage against genetically similar species lacking this ability (69). This has furthermore been examined in animal models, wherein restriction of iron availability by *E. coli* limited pathogenic infection by *Salmonella typhimurium* (70). Another mechanism is limitation of host-derived signaling molecules necessary to induce virulence genes (71). This ability to hinder opportunistic infections can therefore be transiently lost during antibiotics exposure (54). Alterations in microbial functionality have not yet fully been explored for other xenobiotics (72). Host-produced antimicrobial components can be metabolized by the gut microbiota to further enhance their properties or target them against specialized bacterial strains. Species belonging to *Clostridium* can for example convert primary bile salts into deoxycholic acid, which is highly bactericidal against taxa such as *Staphylococcus aureus* (73, 74). Taxa of the gut microbiota can also produce antimicrobial peptides such as bacteriocins with species-specific bactericidal effects (75). One such bacteriocin is the *Lactobacullis*-derived lantibiotic nisin which targets lipid particles in the bacterial cell wall (76).

In the gastrointestinal tract, bacterial inhabitants are evolutionarily adapted to survive under harsh living conditions (77). Just as the intestinal bacteria are affected by several parameters in the local milieu, they also regulate the local biochemical balance. The majority of bacteria of the gut microbiota produce energy through fermentation, and their acidic end-products are secreted into the gut lumen, lowering the local pH (36). These short chain-fatty acids (SCFAs), namely acetate, propionate and byturate, are metabolized from undigestible carbohydrates by several species in the gut (78) and are known for different functions. Firstly, SCFAs serve as the primary energy source of colonic enterocytes (79). Secondly, they are also involved in microbial cross-feeding (80), promoting the growth non-SCFA producers such as some species of *Bifidobacterium* (81), further expanding the diversity of the gut microbiota. Moreover, SCFA signaling in enterocytes promotes mucin production (82), improves intestinal barrier function by stimulating intercellular tight junction protein (TJP) expression (83), and regulate several inflammatory responses (84). Bacterial signaling additionally occurs between intestinal taxa and foreign bacteria, where SCFAs downregulate virulence factors (85). Other than SCFAs, the gut microbiota additionally mutually benefit the host by biosynthesis of essential amino acids (86) and vitamins (87, 88), which cannot be produced *de novo* by the host. Structural differences in gut microbiota composition leading to alterations in the functional capacity may be involved in disease development, severity and response to treatment.

1.1.5 DYSBIOSIS

Normally, gut microbiota in homeostasis with its human host is termed as eubiotic, or in eubiosis. Recently, the term ‘dysbiosis’ has been used to characterize disruptions in the gut microbiota composition. But consensus on the use of the term, and its distinct definition, has not yet been established in the research community, which is illustrated in the paper by Hooks et al., as seen in *Figure 4*. Here, they found that dysbiosis is loosely interpreted as corresponding to changes, overall or specific in the gut microbiota structure, or more diffusely as an imbalance (89). Historically, dysbiosis was first used in the context of microbiology by the microecologist Helmut Haenel, whom described ‘dysbiotic state of the gut’ diverting from that of a normal, eubiotic state (90). The practice of using probiotics, bacterial strains with beneficial properties, stem from the belief that the effectiveness of such a treatment is the amelioration of the underlying dysbiosis (66). Additionally, other prominent researchers have argued how a shift in relative abundance of core bacterial taxa could result in a skewed development of T_H17 and T_{reg} cells, which regulate immune responses, resulting in manifestations of disease (91, 92). Dysbiosis is also broadly defined as expansion of pathogens at the expense of beneficial taxa, as well as reduced bacterial diversity (93). This is however still being debated, as several researchers argue that it is impossible to discern between the chicken and the egg – whether dysbiosis should be considered the cause of the effect (94). Nevertheless, dysbiosis is largely used as a term for significantly different gut microbiota in one population cohort compared to another, and this thesis will employ the term in such a context.

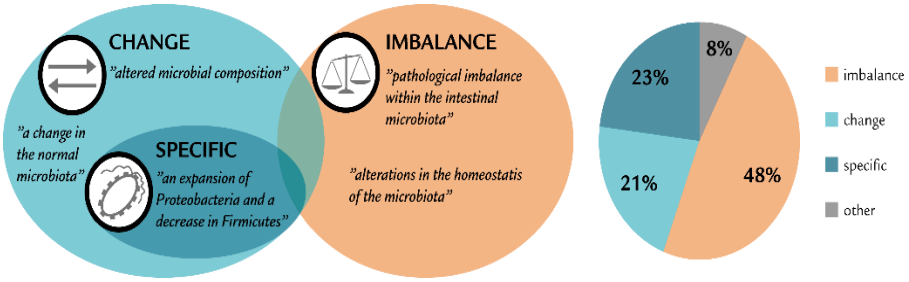

***Figure 4* – Definitions of dysbiosis.** Adapted from (88)

Transient dysbiosis can be defined based on time and external influential factors. It is widely recognized to arise in patients receiving antibiotic treatment, defined as depletion of bacterial strains, extensive loss of diversity, and in some cases only partial recovery (51-53). Furthermore, antibiotics use may have long-term consequences, as epidemiological studies have found increased risk of developing immunological, psychiatric, or metabolic disorders later in life if the gut microbiota was disrupted during childhood (95-98). Another external influence on gut microbiota dysbiosis is diet. In animal studies, supplementing animals with a high-fat diet corresponding to the beforementioned western diet resulted in structural variations in 57 % of the gut microbiota, which was associated with the development of metabolic syndrome (99). The western diet is not only correlated to lower intestinal bacteria diversity in humans, but is also associated with different diseases, such as inflammatory bowel disorders (100, 101). Dietary preferences and restrictions may therefore exacerbate dysbiotic states (102), which has also been associated with the development or aggravation of chronic diseases (102, 103).

In general, the gut microbiota composition appears to play a major role for our well-being as investigated by several clinical studies. Intriguingly, the gut microbiota is altered in several psychiatric disorders, such as depression (104-121), schizophrenia (122), autism (123), attention deficit-hyperactivity disorder (124, 125) and bipolar disorder (126, 127). A significantly different gut microbiota composition has likewise been observed in autoimmune diseases such as inflammatory bowel disorders (128-130) and rheumatoid arthritis (131), endocrine disorders such as obesity and metabolic syndrome (17, 132-134) and type 1 and type 2 diabetes, (135-138), cardiovascular diseases (139) and neurological disorders such as Parkinson's disease (140, 141) and multiple sclerosis (142).

1.2 DEPRESSION

1.2.1 PREVALENCE, SYMPTOMS AND BURDEN

Depression – overview of generalized symptoms
Affective – feelings of worthlessness, sadness, diminished interest in everyday activities, excessive guilt, suicidal thoughts
Cognitive – difficulty concentrating, loss of executive functioning such as planning and reasoning, working memory and attention deficits
Vegetative - psychomotor agitation or retardation, pain, insomnia or hypersomnia, elevated or diminished appetite, gastrointestinal disturbances

***Box 1* – An overview of symptoms in depression**

Depression is a mood disorder diagnosed according to criteria defined by the Diagnostic and Statistical Manual of Mental Disorders (143) or the International Classification of Diseases (144). There are slight variations in their definitions of core symptoms, but they include depressed mood, loss of energy and/or anhedonia during a minimum period of two weeks in combination with several of the symptoms described in *Box 1*. Generally, major depressive disorder (MDD) is characterized by a combination of affective, cognitive and vegetative symptoms (145, 146). There is a high degree of inter-variability in symptom profiles amongst patients with MDD, which was exemplified in a study of the "Sequenced Treatment Alternatives to Relieve Depression" participants (147). Here, it was found that the combination of the nine different symptoms defining MDD according to the Diagnostic and Statistical Manual of Mental Disorders could create 1,030 unique symptom profiles out of the 3,703 included patients (147). There are several risk factors for developing depression, such as female sex, as women are almost twice as likely as men to be diagnosed with MDD (148). Other risk factors include poor socio-economic status (149), a family history of MDD (150), perceived social isolation (151), smoking (152) and low physical activity (153), as well as obesity (154) and poor dietary choices (155). MDD can be a singular episode, recurrent, or chronic (156, 157) and relapse has been found to be the rule rather than the exception (158).

The global prevalence of depression varies between countries, with lifetime rates from 1.5 per 100 adults in Taiwan to 19.0 per 100 adults in Beirut (159). In the Danish population, the most recent publication estimated that 3.2 % of the adult population was diagnosed with MDD with an incidence rate of 270 per 100,000 capita between 2011-2015 (160), and surveys estimated a similar prevalence (161). In Europe, direct and indirect costs of MDD also vary widely, possibly due to differences in study design (162), while the Danish annual direct cost was calculated to €1.2 billion (160). Not only is MDD one of the leading economic burdens in medical expenses, but it is also a primary cause of disability and low quality of life (163, 164). The Global Burden of Disease Study from 2010 found that MDD was the cause of 2.5% of global disability-adjusted life years, which is the sum of years lost to premature mortality, and years lived with disability (165). Cross-national assessments have also reported high comorbidity rates between chronic physical disorders and MDD, which was

furthermore associated with significantly lower health scores (166). Nevertheless, while the global economic and life quality burden of MDD is high, intervention is cost-effective in averting disability-adjusted life years (167).

1.2.2 PATHOPHYSIOLOGY

MDD is believed to be caused by environmental stressors in combination with genetic susceptibility (168). Although no evidence-based, definitive combination of genetic predispositions have been proposed, several risk variants have been identified for MDD (169), some of which are sex-specific (170). These reside in loci involved with neuronal development, gene expression regulation in the brain, synaptic function and transmembrane adhesion (169). Environmental factors include emotional traumas, although separation from genetic influence is difficult (171). Gene-environmental interactions have been consistently reported, with one of the more investigated associations in the literature being polymorphisms in the 5-HTT gene in combination with stressful life events and subsequent diagnosis with MDD (172). A high degree of patient variations in disease presentation nonetheless confound generalizability, which furthermore emphasize the difficulty in building a reliable model of pathophysiology for MDD. This is emphasized by a study examining single nucleotide polymorphisms, where no gene X environment interactions were found to reach statistical significance (173). Functionally though, many neurobiological hypotheses are evidence-supported, albeit they cannot sufficiently and independently of each other explain the individual disease presentation.

Neurotransmitter alterations

It has been suggested that the heterogeneity of MDD may arise from neural circuit dysfunctions in response to external stimuli (174), which can derive from altered neurotransmitter signaling and availability. A relation between specific neurotransmitter dysfunctions and specific symptoms has also been proposed: Obsession, compulsion and anxiety due to serotonin deficiency; energy loss, low alertness, inattention, cognitive dysfunction and difficulty concentrating due to lack of norepinephrine; and reduced motivation, pleasure and reward seeking due to depleted dopamine (175).

Historically, the monoamine hypothesis of MDD arose in the 60s when the excitatory neurotransmitters catecholamines, and the indolamine serotonin were proposed as deficient in MDD brain signaling (176, 177). This is supported by increased serotonin transporters in the prefrontal cortex in MDD (178), and reduced binding potential of serotonin receptors (179). This suggests limited serotonin availability and thereby reduced neural activation. One of the catecholamines, norepinephrine, binds to the α_{2A}-adrenoceptors. These were found to have altered density and binding potential (180), while the norepinephrine transporter was decreased (181) in autopsy studies of MDD. This suggests that depletion of norepinephrine leads to depressive symptoms. One of the core symptoms of MDD, anhedonia, is believed to be linked to dysfunction in the reward system, which is primarily facilitated by dopamine signaling (182).

Patients with MDD have reduced striatal response to reward (183), and lower striatal dopamine transporter binding (184), possibly by downregulation due to depletion of dopamine (185). Combined, lack of neurotransmitter signaling by serotonin, norepinephrine and dopamine are well-known components of MDD pathology.

Newer hypotheses introduce other neurotransmitters as manifestations of MDD features. Neurogenesis in the hippocampus is partly mediated by γ-aminobutyric acid (GABA)-mediated proliferation and maturation (186, 187). As reduced neurogenesis is implicated in the etiology of MDD, this suggests loss of GABA-mediated signaling (188). Reductions in GABAergic neurons in prefrontal (189) and occipital cortices suggests a change in ratio of excitatory (serotonin/norepinephrine/dopamine) and inhibitory (GABA) neurotransmitter levels (190). The excitatory neurotransmitter, glutamate, has likewise been proposed to be involved in MDD signaling (191), as glutamatergic synapses are involved in emotional cognitive processing (192). Abnormal glutamate transmission has been associated with maladaptive changes in MRI scans of neuroplasticity (193), and there is aberrant glial reuptake and metabolism of glutamate in several brain regions involved in MDD (194-196). Overall, several neurotransmitter systems are implicated to be deficit in MDD.

Neuroplasticity reductions

Neuroimaging studies have provided great insight in anatomical and functional deficits in neural systems in MDD. Loss of overall cerebral volume in the prefrontal cortex has been reported in both post-mortem studies (197) and in the prefrontal cortex, hippocampus and caudate nucleus in neuroimaging studies (193). More specifically, white matter microstructural changes have been observed (198-200), as has reduced grey matter volumes in the hippocampus, amygdala and prefrontal cortex (201-203). These are cortical areas responsible for executive functions and emotional processing (204, 205). Brain-derived neurotrophic factor (BDNF) is a neuronal growth factor that has been found depleted in postmortem analyses of the cerebral cortex of patients with MDD (206). On the other hand, reductions in hippocampal volume is not associated with MDD severity (207), and plasma concentrations of BDNF does not correlate with MDD symptoms (208).

Neuroendocrine hyperactivity

Hypercortisolism has been observed in MDD (209, 210), and is believed to be caused by adrenal hypertrophy (211) due to hypersensitivity to adrenocorticotrophic hormonal stimulation (212, 213). Elevated hypothalamus-pituitary-adrenal gland axis stimulation and subsequent secretion of cortisol is considered a hallmark of both stress and MDD (214). The hypothalamus responsible for secretion of corticotropin-releasing hormone is subject to GABAergic inhibitory control by the hippocampus (215). Prolonged stress, and thereby release of cortisol, is suggested to result in loss of hippocampal GABAergic interneurons (216). This could explain hippocampal volume reductions in MDD (203), and link hippocampal neuropathology and neurotransmitter deficiency to hyperactivity in the hypothalamus-pituitary-adrenal

gland. Additionally, cortisol signaling is immunosuppressive (217), but prolonged endogenous glucocorticoid production have been found to promote pro-inflammatory responses (218).

Neuroimmune deficits

Inflammatory processes in MDD are interpreted as a dysfunctional response to psychosocial stressors devoid of actual pathogenic infection, also known as the 'pathogen host defense' (219). Chronic stress leads to prolonged secretion of cortisol resulting in downregulation of glucocorticoid receptors on white blood cells. This makes them less responsive to anti-inflammatory signals and leads to upregulation of pro-inflammatory processes (220). There may be sex-specific responses to increases in pro-inflammatory signals, as women demonstrate increased depressed mood in response to LPS exposure (221), highlighting the observed increased risk of MDD in women (222). The concentration of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are elevated in the plasma of patients with MDD according to meta-analyses (223, 224). Furthermore, the concentration of the cytokines IL-1β and TNF-α hasB been associated with MDD severity in treatment-naïve patients (225). Increased pro-inflammatory cytokines in plasma has been shown to alter metabolism of serotonin and dopamine, as well as stimulate cortisol secretion (226), effectively coupling neuroendocrine and neuroimmune alterations in MDD. In addition, overproduction of pro-inflammatory cytokines inhibits BDNF production (227), furthermore linking neuroplasticity deficits with neuroimmune changes in MDD.

1.2.3 COMMON TREATMENT OPTIONS

At present time, there is no cure for MDD, and treatment is therefore primarily applied to provide symptom relief and lower the risk of recurrent episodes. Response to treatment is therefore defined as noticeable improvement in symptoms (often 50% reductions in symptoms), while remission is the near absence of symptoms (228). In this study, patients initially received a first course of pharmacological treatment, where approx.. 30% of patients reach remission, while approx.. 50% responded to the treatment (229), which is below a satisfactory level. Additionally, there is a high degree of spontaneous remission in untreated patients with MDD (230), but recurrence of depressive episodes is common (231). Pharmacological treatment is often combined with cognitive therapy (232), which can also be effective on its own (233). Nevertheless, literally hundreds of randomized clinical trials have proven the efficacy of antidepressants over placebo (234). Despite this, a moderate placebo-effect is commonly observed in patients with MDD, which can account for upwards of 67% of the treatment response (235). However, treatment-response has proven to be complex. For example, there is a temporal discrepancy between the molecular effect on monoamine availability (immediate) and the subsequent therapeutic effect (days, weeks) (236, 237), while genetic polymorphisms can predict treatment response and side effects (238).

The main function of many antidepressants used today is through monoamine signaling due to the discovery that drugs which increase extracellular availability of serotonin and norepinephrine have antidepressant effects (176, 177). In order to increase the availability of monoamines, antidepressants either limit monoamine breakdown in the synaptic cleft (239) or block their reuptake into the presynaptic neuron (240), hereby prolonging their neuroactive potential. The most widely used antidepressants are the selective serotonin reuptake inhibitors (SSRIs) and the selective serotonin/norepinephrine reuptake inhibitors (SNRIs) , as they have the most potent efficacy with the least side effects (241). See *Box 2* for a non-exhaustive overview of antidepressant classes, their trade names and mechanisms of action, which are used in Denmark.

Antidepressant class	Trade names	Mechanisms of action
Monoamine oxidase inhibitors	Moclobemide, selegiline	Inhibition of monoamine oxidase enzyme
Tricyclic antidepressants	Amytryptiline, desimipramine, imipramine	Blocking the serotonin and norepinephrine transporters. Serotonin, NMDA and histamine receptors antagonist
Selective serotonin reuptake inhibitors	Citalopram, fluoxetine, sertraline	Blocking the serotonin transporters
Selective norepinephrine reuptake inhibitors	Reboxetin	Blocking norepinephrine transporters
Selective serotonin-norepinephrine reuptake inhibitors	Duloxetine, venlafaxine	Blocking the serotonin and norepinephrine transporters
Noradrenergic and specific serotonergic antidepressants	Mirtazapine, mianserin	Serotonin antagonist, norepinephrine antagonist
Melatonergic antidepressants	Agomelatine	Melatonin receptor agonist, serotonin receptor antagonist

***Box 2* – An overview of antidepressant pharmaceutic treatment options.**

In addition to alterations in neurotransmitter signaling, antidepressants affect several brain areas (242) by stimulating neuronal proliferation (243) possibly mediated by BDNF production (244) or brain region-specific metabolic changes (242). This not only improves immediate neural circuit connectivity (245), but also induces secondary long-term transcriptional and translational changes that modulate cellular plasticity (246). Surprisingly, antidepressants have been found to contain antibacterial properties (247), which could influence the human gut microbiota. Given the high degree of heterogenic disease presentation and manifestation amongst patients with MDD, and the highly individual gut microbiota composition, an association between gut microbiota variations and MDD etiology and/or pathology has been postulated.

1.3 ASSOCIATION BETWEEN GUT MICROBIOTA AND DEPRESSION

There are several indications that the gut microbiota is important for governing human well-being. As mentioned previously, alterations in gut microbiota composition has been observed in several somatic (17, 128-138, 140-142) and psychiatric diseases (104-127) compared to the non-diseased population. In patients with MDD, gastrointestinal disturbances are reported with higher frequency than in the general population (248-250), which highlights an important association between gastrointestinal inhabitants and MDD. How, and why, alterations of the gut microbiota may be involved in MDD have been explored in several preclinical and clinical studies.

1.3.1 PRECLINICAL ASSESSMENT AND INVESTIGATIONS

Animal studies have explored how gut microbiota alterations and behavior are associated. This can be performed either through a "bottom-up" or a "top-down" approach. The "bottom-up" approach is the manipulation of the gut microbiota, leading to behavioral changes. The "top-down" approach is the provocation of depressive-like behavior in an animal with subsequent gut microbiota alterations.

Many animal models of depression have been explored for gut microbiota variations in such a "**top-down**" approach. The Flinders Sensitive Line (FSL) rat model of depression is a selectively bred, *genetic* rat model of depression originally derived from the Sprague-Dawley rat. The FSL displays depressive-like features when compared to its control counterpart, the Flinders Resistant Line (FRL) rat (corresponding to the original Sprague-Dawley rat) (251-253). In spite of being maintained in completely identical environments, the gut microbiota of the FSL rat is significantly different from the FRL rat. The FSL rat has a lower bacterial richness and bacterial taxa negatively associated with depressive-like behavior (254). In *environmentally*-induced depression, such as the chronic unpredictable mild stress (CUMS) model, significant changes in gut microbiota composition were found (255, 256). Additionally, these changes were associated with alterations in cerebral serotonin (257) and the cerebral serotonin transporter (256). Gut microbiota alterations have likewise been observed in other environmental "top-down" models, such as the olfactory bulbectomy model (258), the maternal separation model (259) and the social defeat stress model (260, 261).

In the "**bottom-up**" approach of associating the microbial community with a behavioral output, one of the first studies compared the completely germ-free (GF) mouse with the standard specific-pathogen free mouse. Here, it was found that the axenic GF mice, bred and raised in a sterile environment, had an exaggerated corticosterone response to a stressful stimuli as well as decreased expression of BDNF in the hippocampus (262), mirroring observations reported in patients with MDD. On the other hand, the behavioral assessments of the GF mouse showed less depressive-like behavior compared to the specific-pathogen free mouse (263), suggesting that

lack of bacteria is not the primary driving factor of developing MDD. Environmental animal models include the high-fat diet animal model, where microbial alterations and a depressive-like phenotype are found in combination with metabolic modulations in GABA signaling in the brain (264). The importance of the gut microbiota in MDD is especially highlighted in a study that showed that sustained antibiotic treatment induced depressive-like behaviors (265). Other interesting potential interactions of the gut microbiome and MDD has been demonstrated by *fecal microbiota transplantation* (FMT), which can also be interpreted as an environmental, or externally-induced, model of MDD. For example, transfer of fecal content from the FSL rat to the FRL rat transmitted the depressive-like phenotype to the FRL rat (254). A similar experiment performed FMT from CUMS-treated mice into recipient control mice, whom developed depressive-like behavior and neuroinflammation (266). The FMT models of depression have been replicated, using fecal content from patients with MDD into recipient animals. In two studies, the GF mouse was utilized (106, 267), while in two others, a GF rat (268) or an antibiotics-blasted rat (108) was used. All four studies established some degree of anxious and depressive-like behavior in the FMT-recipient animals, suggesting a causal link between a specific composition of the gut microbiota and the development of depressive features.

1.3.2 THE LEAKY GUT THEORY

Both direct and indirect mechanisms of actions can facilitate how an altered gut microbiota can affect clinical and biochemical features associated with MDD. One such indirect mechanism is the transfer of bacteria or metabolites from the intestinal lumen to the systemic circulation, which may affect susceptible organs in the host organism. Such a state is termed “leaky gut”, and has been widely discussed in the scientific community as an entity developed from a dysbiotic microbial structure (269). Under normal circumstances, bacteria are separated from direct interaction with the apical intestinal surface by two mucus layers (269), whereas a dysbiotic microbial community is believed to cause a breakdown of this separation, leading to local inflammatory changes (*Figure 5*).

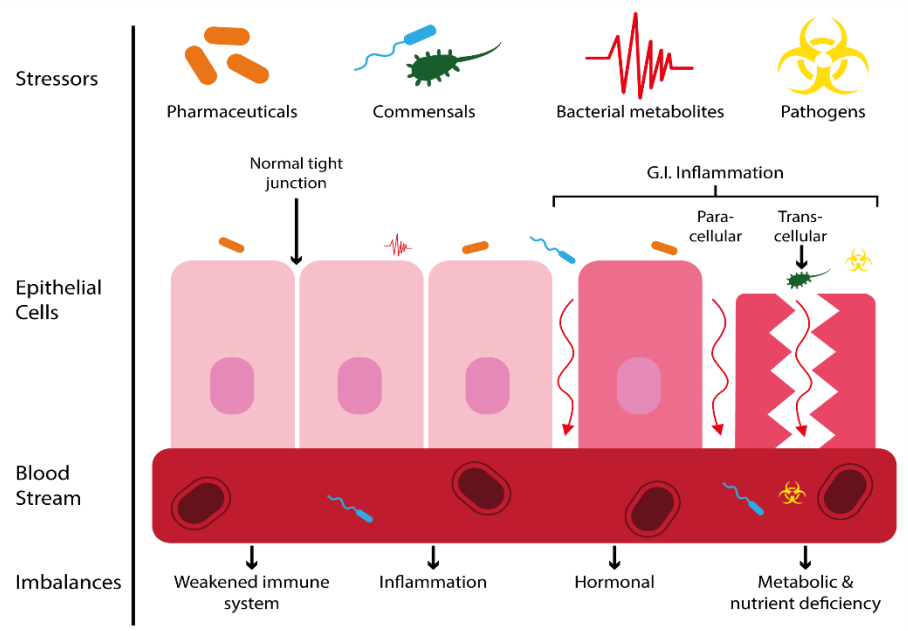

***Figure 5* – Display of the healthy intestinal barrier compared to leaky gut.** Left: Eubiotic state with intact intestinal barrier. Right: Increased dysbiotic features leading to breakdown of intestinal integrity and increased paracellular and transcellular transfer of bacteria and metabolites.

From the apical to the basal domain of the enterocytes, several types of TJPs ensure that the intestinal barrier is tightly shut against translocation of intestinal bacteria or their components (270). Beneficial commensals have been found to upregulate the expression of these TJPs (271), suggesting that a dysbiotic gut microbiota and loss of TJP expression may lead to leaky gut. This term covers the association between loss of TJPs and increased permeability of the intestinal barrier (272, 273). Indeed, loss of TJPs has been reported in animal models of depression, both at gene (274, 275) and protein (276-279) expression levels, as well as in functional assessments using dye that normally cannot penetrate the intestinal barrier (280, 281). Results in animal studies have mirrored clinical assessments of patients with MDD, where a lower plasma level of TJPs has been observed (282). However, this relationship is complex, as this finding is not consistently reported in clinical studies. In fact, some studies have linked a dysbiotic intestinal community with elevated plasma tight junction proteins and increased LPS (283). One suggested mechanism is loss of fecal SCFAs, which has been confirmed lower in patients with MDD (284). SCFAs upregulate the expression of tight junction proteins (83), whereby a loss of this stimuli can result in a more permeable barrier. TJP expression and endocytosis is not only regulated independently by intestinal commensals, but also by components of the inflammatory response (285).

1.3.3 SYSTEMIC INFLAMMATION

An increased permeability in the intestinal barrier can lead to elevated transfer of pro-inflammatory mediators to extra-intestinal circulation and organs. Biologically active substances, such as LPS, can initiate a cascade of events leading to systemic inflammation. They are first recognized by innate immune cells via pattern recognition receptors, such as the Toll-like receptors (286), which then activate the inflammasome (287). The inflammasome is a large, self-assembling protein complex that initiates production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, all major components of the innate immune system. The inflammasome has long been linked to MDD (219), and the gut microbiota has been researched in several preclinical and clinical studies to affect the inflammasome as exemplified below.

Preclinical studies support the vital role of the inflammasome in MDD, such as studies linking peripheral administration of LPS to the development of depressive-like behaviors in mice (288). In support of these results, mice with knockout of inflammasome-associated genes demonstrated decreased depressive-like behavior (289). Pro-inflammatory cytokines have also been found elevated in animal stress models of MDD, including the CUMS model (290-292). Additionally, FMT from patients with MDD into recipient rats increased rat plasma levels of C-reactive protein (108), IL-1, IL-6 and TNF-α, and decreased anti-inflammatory cytokines IL-4 and IL-10 (268).

Expression of the Toll-like receptor-4, which recognizes LPS, has been found increased in patients with MDD prior to cognitive therapy (293). Likewise, stimulation of peripheral blood mononuclear cells derived from patients with MDD with LPS resulted in altered cytokine responses compared to cell responses from healthy controls (294). Patients with MDD show elevated immune responses, such as increased IgM and IgA antibodies against enteric pathogens such as *Hafnia alvei* and *Pseudomonas aeruginosa* (295). Furthermore, patients with MDD have depleted fecal SCFA (284), which can result in loss of the anti-inflammatory properties provided by SCFAs (296, 297), exacerbating the underlying inflammatory pathways.

1.3.4 GUT-BRAIN AXIS

An altered gut microbiota with dysbiotic characteristics can lead to increased intestinal permeability and decreased function of the enteric barrier. The result is low-grade systemic inflammation that plausibly affects the brain and leads to neuroinflammatory disorders, such as MDD. These are the core components of the newer hypotheses of MDD and is based on the gut-brain axis, a bidirectional communication channel between the central nervous system and the intestinal system, and along with it the gut microbiota (298). It is believed that not only does the brain influence the enteric milieu, but also that the microbiota communicates with the brain through different mechanisms (see *Figure 6*).

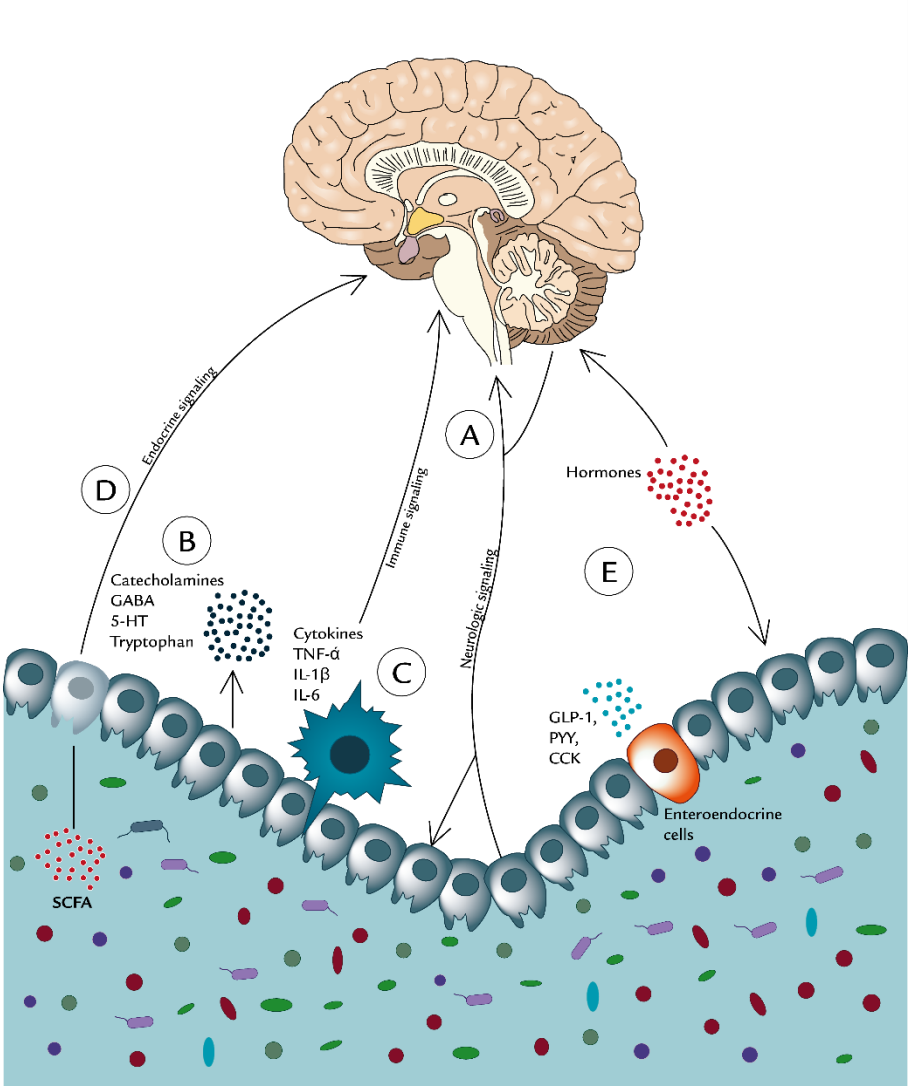

***Figure 6* – Signaling mechanisms in the microbiota-gut-brain axis.** (A) Direct neuronal signaling. (B) Production of neurotransmitters. (C) Activation and maturation of the immune system. (D) Endocrine signaling through metabolites. (E) Hormonal stimulation.

Direct neuronal stimulation of the enteric nervous system by enteric commensals have been found to induce behavioral changes in animal studies. Here, specific *Lactobacillus* strains induced depressive-like behavior in mice, an effect which was lost after vagotomy (299). The vagus nerve, which innervates the digestive wall, can also be activated by SCFAs and produce afferent nerve responses (300). Several

enteric bacteria can produce neurotransmitters such as catecholamines, serotonin, dopamine, and GABA locally in the gastrointestinal tract (301), and inoculation of animals with some of these strains can lead to an increase in these neurotransmitters in the brain (299, 302).

Systemic low-grade inflammation has also been found to affect the brain. In animal models of MDD, an apparent disruption of TJP expression in the blood-brain barrier of rats (279) lead to neuroinflammation (303, 304). Such neuroinflammation has likewise been observed in patients with MDD (305, 306). Stimulation of the nervous system can also occur through bacterial metabolites, such as SCFAs, which have been found to evoke extracellular action potentials in sympathetic nervous fibers *in vitro* (307). Additionally, the gram-positive cell wall component peptidoglycan can also penetrate the blood-brain barrier and interestingly, knockout of the rat peptidoglycan receptor resulted in increased sociability (308). The LPS-induced depressive-like phenotype has been coupled with elevated pro-inflammatory cytokines in the brain (309), and Toll-like receptor-4-mediated signaling is involved in depressive-like behaviors (310). Endocrine signaling can occur through several pathways; increased cortisol generated by CUMS can induce leaky gut by downregulating TJP expression (274), leading to the downstream effects involved in the depressive phenotype. Furthermore, gastrointestinal bacteria can stimulate release of gut hormones such as neuropeptide Y, peptide YY and cholecystokinin, which have been suggested to be involved in the development of neuropsychiatric disorders (311).

1.3.5 ANTIDEPRESSANTS AND THE GUT MICROBIOTA

It is well-established how antidepressants affect the cerebral neurocircuit and that they demonstrate anti-depressant relief superior to placebo. More recently it has been found that they have beneficial effects distinct from their main mechanism of action. Antidepressants have anti-inflammatory properties and may in fact, directly impose effects onto the gut microbiota. SSRIs have been found to promote an anti-inflammatory response both *in vitro* (312), in preclinical assessments (313) and in patients with MDD (314). As an example, fluoxetine and sertraline were found to reduce LPS-induced TNF-α production by microglia *in vitro* (312). Additionally, a high variety of oral therapeutics, including antidepressants, have been found to present antibacterial properties (50). Many commonly used antidepressants, such as desipramine and citalopram have been tested against intestinal commensals and demonstrate both bacteriostatic and bactericidal effects against certain bacterial strains. For example, growth of *Akkermansia muciniphila* was completely inhibited with 75 μg/mL desipramine (247). Also, *Ruminococcus flavefaciens* was found to modulate the antidepressant effect of duloxetine (315). This suggests that not only is the gut microbiota involved in depression, but the effect of antidepressants may be regulated by the human gut microbiota.

CHAPTER 2. AIMS AND STUDY PARTS

As given from the background, there appears to be an association between depression and the gut microbiota composition and function. Research on this area is still in its early stage. It is therefore very difficult to discern if there are any functional effects of the gut microbiota that may be involved in the development and severity of depression, as well as in the treatment response.

The hypothesis of the thesis is that there exists a significantly different composition of gut microbiota in antidepressant treatment-naïve patients with MDD. To be able to determine if there are a significant altered microbial community in patients with MDD, we wanted initially to explore into previous studies of the gut microbiota research in a MDD context (STUDY I).
Furthermore, we hypothesized that the microbial community from patients with MDD could induce a depressive-like behavior in rats upon fecal microbiota transplantation (STUDY II).
We then aimed at investigating how antidepressant treatment may affect the gut microbiota in an antidepressant treatment-naïve cohort over time, as compared to a healthy control group (STUDY III).

The main hypotheses for the project are therefore as follows, which have given rise to three articles based on their results;

1) *Overall, previous studies have been able to differentiate between patients with MDD and healthy individuals based on the gut microbiota composition.*
2) *FMT from patients with MDD into recipient rats can elicit depressive-like behavior compared to FMT from the healthy control group.*
3) *Antidepressant treatment-naïve patients with MDD have a significantly different gut microbiota composition compared to healthy individuals prior to initiation of treatment. Furthermore, their gut microbiota changes during the antidepressant therapy.*

It is expected that this project will contribute to not only clarify the previously mentioned hypotheses, but also provide insight and knowledge to build additional future studies. It is paramount to further explore how the gut microbiota affects MDD, and if it is possible to manipulate the gut microbiota in the treatment of MDD.

CHAPTER 3. METHODS

A short overview of the experimental setup of each study is presented below, followed by the rationale and a critical evaluation of the chosen methods for each study. The methods are also explained in detail in the included articles.

3.1 STUDY I – DESIGN OVERVIEW AND OUTCOMES

We wanted to perform a systematic review to determine if there were significant differences in gut microbiota between patients with MDD and healthy individuals. The databases PubMed, Embase (Ovid) and PsycINFO (Ovid) were searched using the search strategy described in *Appendix B* up until November 13th, 2020. The inclusion criteria for the studies were as follows: Clinical studies including patients with MDD diagnosed according to ICD or DSM criteria; gut microbiota characterization using both non-targeted and targeted approaches; inclusion of a non-depressed control group. The exclusion criteria for the studies were as follows: Inclusion of patients with known comorbidities, such as type 2 diabetes; evaluation of the effect of intervention with pre-, pro-, syn- or antibiotics on patients with MDD with no baseline measurement of the gut microbiota composition.

Primary outcome measures:

α- and β-diversity between patients with MDD and healthy individuals
Significantly different taxa between patients with MDD and healthy individuals

Secondary outcome measures:

Demographic and clinical data
Methods of gut microbiota characterization

3.2 STUDY I – CRITICAL EVALUATION OF METHODS

To generate an overview of the literature, we sought to answer the question whether there were differences in gut microbiota composition between patients with MDD and healthy individuals. We decided to perform a systematic reviews to answer this question, in an attempt to summarize the most current literature of the best evidence-based research in a systematic and unbiased way (316). Evidence-based medicine is the use and application of the current knowledge within a field in decision-making about the care of patients (317). Here, it is necessary to follow five steps (318) to conduct a thorough systematic review:

1. *Framing questions for a review*; In our case, "is the gut microbiota of patients with MDD significantly different from healthy individuals" was the question asked prior to the literature search.

2. *Identifying relevant work*; we included PubMed, Embase and psycINFO in our search, but naturally, we are limited to the articles published through these databases. Since the majority of articles published include primarily

positive findings, it cannot be excluded that publication biases affected our results (319). We included English-only articles, but this has been shown to impose a bias effect (320).

3. *Assessing the quality of studies;* The inclusion criteria in our methods were designed to allow for several methods of gut microbiota characterization to be employed. We did not employ any checklists to examine the quality of the studies, which may have led to inclusion of studies with poor reporting quality. On the other hand, quality assessment using scales such as the Newcastle-Ottawa Scale are often used for analysis of the quality of studies entering into meta-analyses (321), which was not performed here. Additionally, there have been observed discrepancy between authors and reviewers in quality assessment (322), whereby we would risk excluding articles that did not provide sufficient details in their articles.

4. *Summarizing the evidence;* During the critical evaluation of the different article designs, it became clear that it was not possible to conduct a meta-analysis on the included studies. The rationale behind this is covered under the "**Gut Microbiota Characterization**" chapter in the Methods section. Instead, the results were summarized in a series of tables to provide a general overview of the current knowledge.

5. *Interpreting the findings;* The heterogeneity between studies was assessed by comparing demographic and clinical data and methods used to determine the gut microbiota composition. This was then applied to the interpretation of discrepancy arising between studies.

The "Preferred Reporting Items for Systematic Reviews and Meta-Analyses" guidelines (323) contains a checklist that covers these five steps towards conducting a thorough systematic review, and this was used in the design of the study, and the formulation of the results section.

3.3 STUDY II – DESIGN OVERVIEW AND OUTCOMES

This study aimed at exploring how FMT from patients with MDD or healthy individuals into rats would affect their behavioral phenotype. The study was reported according to the "Animal Research: Reporting of *In Vivo* Experiments" guidelines (324). Here, we collected fecal samples from five treatment-naïve patients with MDD and five healthy individuals. These were pooled into fecal solutions representing gut microbiota in MDD versus in healthy individuals. We used two different rat models, the FSL and FRL rats. The schematic overview on the groups and treatments, as well as the timeline of the treatments, is displayed in *Figure 7*.

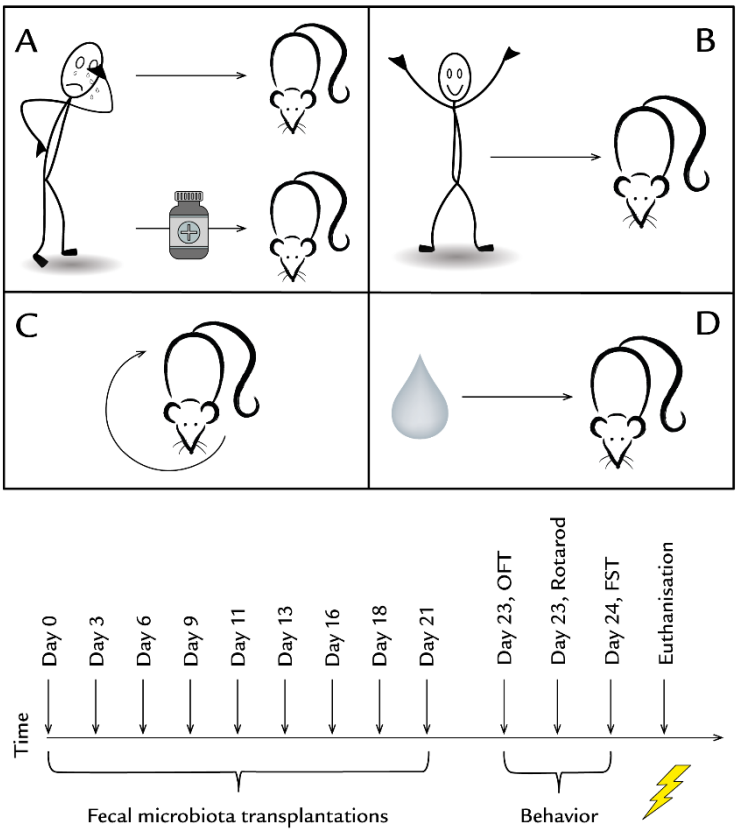

***Figure 7* – Treatment and timeline overview of FSL and FRL rats.** All procedures were performed by oral gavage. (A) FSL and FRL rats were given FMT from patients with MDD. One group of FRL rats additionally received daily treatment with sertraline, a SSRI. (B) FSL and FRL rats were given FMT from healthy individuals. (C) FSL and FRL rats received auto-transplantation with fecal matter collected from each individual cage. (D) FSL and FRL rats received water every time each other group received FMT (E) Timeline of treatments, behavioral analyses and euthanization. OFT: Open field test. FST: Forced swim test.

Behavioral tests included the open field test for locomotor activity, the rotarod test for motor coordination and time-to-exhaustion and the forced swim test for depressive-like behavior. Fecal samples were collected prior to treatments and subsequently after euthanization for gut microbiota characterization. Additionally, tissue samples were collected from the rat caecum to measure the expression of TJPs to determine the effect of the treatments on their gene expression.

Primary outcome measures:

Depressive-like behavior
α- and β-diversity between groups and before and after FMT
Significantly different taxa between groups and before and after FMT

Secondary outcome measures:

Locomotor and rotarod activity
Gene expression of TJPs in cecal tissue

3.4 STUDY II – CRITICAL EVALUATION OF METHODS

3.4.1 ANIMAL MODELS

Choosing the best suited animal model is imperative for the reliability of the obtained results. To assess how suitable an animal model is, it is often evaluated according to three main criteria, namely face-, construct- and predictive validity:

- *Face validity;* the model mirrors depressive aspects specific to MDD with features observed clinically.
- *Construct validity;* the model behavior conceptually reflects the disease cause of MDD.
- *Predictive validity;* the model correctly identifies diverse pharmacological antidepressant treatments and with effects in the model correlating with clinical potency (325).

The two Flinders lines were originally inbred from the Sprague-Dawley rat strain into either a phenotype *sensitive* towards the anticholinesterase diisopropyl fluorophosphate (FSL; Flinders Sensitive Line), or as the control rat remaining *resistant* towards chronic treatment (FRL; Flinders Resistant Line) (326, 327). Thus, the original FRL rat resembled Sprague-Dawley rats. Other lines of research have demonstrated that patients with MDD display sensitivity towards cholinergic agonists (328, 329), and those lines of research later converged and the FSL rat was proposed as an animal model of MDD. Compared to its control, the FSL rat model of depression fulfils the three abovementioned criteria to varying degrees.

The FSL rat robustly displays depressive-like behavior in the forced swim test, reduced appetite and weight gain during their growth period, increased rapid eye movement sleep, and psychomotor retardation compared to the FRL rat (251, 252,

330). However, they do not express all the typical behavioral traits associated with MDD in other animal models of depression, such as cognitive dysfunction or inherent anhedonia (253). Thus, the FSL rat model can be interpreted as containing moderate face validity (251, 252). In the context of Study II, this model was chosen to be able to combine the "top-down" and "bottom-up" approaches, by using a genetic animal model and expose it to external 'environmental' factors (the human gut microbiota) to explore how the FSL and FRL rats responded behaviorally.

Due to the complicated etiology of MDD, and the highly individual symptoms patients present with (147), it can be difficult to tangent full constructive validity in an animal model of depression. On the other hand, the FSL rat displays altered cerebral neurotransmitters (331, 332), neurotransmitter receptor expression (333) and plasma cytokines (334), all biochemical characteristics recognized to be altered in MDD. Interestingly, the FSL rat appears to have a significantly different composition of gut microbiota compared to the FRL rat (254), supporting the notion that they mirror biochemical, behavioral and microbiological aspects of MDD. Therefore, the FSL rat can be considered to contain particularly good construct validity for the current study.

In predictive validity, the FSL rat mirrors patients with MDD, as it is primarily chronic treatment rather than acute that has antidepressant effects (253, 335, 336). The tricyclic antidepressants, such as imipramine, and SSRIs, such as sertraline and citalopram have nevertheless been found to induce little effect on the FRL rat (335, 337). SSRIs have been found to contain antibacterial properties (247). Therefore, this class of antidepressants was chosen to treat FRL rats receiving FMT from patients with MDD. This was to examine if induced behavioral changes from the FMT could be countered by antidepressants based on their antibacterial properties rather than its inherent antidepressant characteristics. FRL rats were given daily antidepressant treatment for three consecutive weeks during the same time they were administered FMT. Pharmaceutical treatment was given at a minimum of two hours after FMT to enhance bacterial transfer from the stomach to the cecum. Sertraline hydrochloride was given by oral gavage at a dose of 16.7 mg/kg/day to mimic a clinically relevant administration and dose (338).

Rats were between 6-8 weeks of age, corresponding to the age of the human adolescents. As the gut microbiota varies during the human life time (33-35), so does the gut microbiota of common laboratory rats with a considerable change from three weeks of age to twelve weeks of age (339). Therefore, it was important to match the animals in development and behavior closely to that of the human donors (340).

3.4.2 FECAL MICROBIOTA TRANSPLANTATION

The hypothesis of Study II involves two conceptual counterparts: 1) the induction of depressive-like behavior in the FRL rat by transplanting fecal matter from patients with MDD and 2) the reversal of depressive-like behavior in the FSL rat by introducing gut microbiota from healthy individuals.

Several routes of administration have been explored in clinical studies of FMT, such as the upper route by nasoenteric or nasogastric infusion, or the lower route, such as enemas or colonoscopy (341). FMT performed with fecal specimens from either rat (342) or human donors (343) has previously been observed to colonize the recipient animal with long-term stability. Therefore, due to the less invasive nature, and the success of previous publications using oral gavage as the route of administration (106, 108, 267, 268), this method was chosen.

Fecal specimens were collected from five sex- and age-matched individuals, either antidepressant treatment-naïve patients with MDD, or from healthy individuals and pooled. Chosen dose for FMT was based on clinical guidelines to treat recurrent *C. difficile* infections (The European Consensus Conference on FMT), which recommend a minimum of 30g fecal matter deposited into the colon for optimal curative effect (344). Assuming the average patient weighs ~75kg, the FMT would have to supply 0.4g of fecal matter/kg bodyweight. Our fecal matter for FMT had a concentration of 0.16g per mL solution, and we assume the rats on average weigh 350g. Therefore 0.9mL solution was given to each rat to achieve ~0.4g fecal matter/kg rat bodyweight. The water control group likewise received 0.9mL demineralized water.

3.4.3 BEHAVIOR

All animal experiments and handling were performed by the same experimenter to ensure that handling was performed identically to all animals, and that scoring of behavioral outputs likewise was identically performed. Three different behavioral tests were employed to determine the effect of the treatments on the FSL and FRL rats. All animals underwent three tests on the same day in the order: 1) open field test, 2) rotarod test, 3) training-session forced swim test. The following day, the test-session forced swim test was performed, to limit carry-over effects from the open field test and rotarod tests to the forced swim test. Rats were moved from the open field test to the rotarod test immediately. After the rotarod test, animals were resting for a minimum of 15 minutes prior to the training-session forced swim test.

The **open field test** is a simple assay where an animal is introduced to an open arena and its activity recorded over a defined period. The test is largely used to assess gross locomotor activity. As rats cannot communicate directly with us, we are restricted to assessing behavioral outcomes that are based on the motoric behavior of the animals in an assay. In such cases, the open field test is indispensable as a control; by conducting the open field test prior to the forced swim test it can be revealed whether treatments induce a change in general activity level and reveal false-positive/negative findings in the forced swim test. The primary measurements in the open field test are the locomotor activity, namely the distance travelled over time, as well as anxiety-like behavior (345, 346). Here, only locomotor activity was measured in Study II by placing the rat in the center of a square and measuring its activity for 10 minutes.

The **rotarod test** additionally assess motor performance, but in this assay the activity is forced by a rotating cylinder. The rotarod is elevated slightly, and it is the natural behavior of rodents to try to avoid falling off the cylinder. Thus, this test evaluates endurance (time-to-exhaustion), as well as the motor coordination required for the rat to grip, balance and stay on the moving rotarod. It has been suggested that the forced swim test may be an adaptive response to exertion to preserve energy, rather than a display of depressive-like behavior. The rotarod test was included to evaluate whether treatments have imposed a difference in the rat's energy levels or time-to-exhaustion, which could also come across as false-negative/positives in the forced swim test.

The **forced swim test** is used to evaluate behavioral despair upon exposure to a stressful, inescapable situation. The test was originally developed by Porsolt and the animal is immersed in a water tank from which escape is impossible (347). Initially the rat will actively try to escape, but over time it will start to maintain an immobile posture and float, which is interpreted as a sign of behavioral despair. The initial escape-oriented behavior (active coping) has been suggested a parameter antonymous to the immobile posture (passive coping). The test was validated as antidepressant treatment decreases behavioral despair (time spent being immobile), whereas anxiolytics have no effect (347, 348). While its initial use was for antidepressant properties, it can also be used to determine inert depressive-like behavior in a rodent (349, 350). Three types of behavior were determined; struggling, swimming and immobility. Struggling included active coping such as attempting to climb the cylinder wall and diving to explore escape options. Immobility was scored as passive coping when the animal displayed passive behavior with only slight movements of front and hindlegs to remain afloat. Total time spent being immobile was used as outcome for depressive-like behavior and total time spent struggling as anti-depressive-like behavior. A training-session was included 24 hours prior to the behavioral test-session, as previous studies have found that inclusion of a training-session reduce latency to adoption of the immobility behavior (351).

3.4.4 MARKERS OF ALTERED INTESTINAL PERMEABILITY

As mentioned in the introduction, altered intestinal permeability is a common finding in MDD and animal models of depression (258). Especially intestinal TJP expression has been reported to differ between animal models of depression and their respective controls (274-279). It was therefore imperative that we also explored whether FMT from MDD or healthy individuals into rats would introduce any changes in rat intestinal TJP expression. Cecal tissue was collected from the rats after the experiment, RNA isolated for qRT-PCR and expression of following genes investigated; *ocln*, *cldn3* and *gapdh*. Cecal tissue was collected, as this organ resembles the human colon in microbial density and fermentation (352). We chose to examine occludin (*ocln*), as it is a transcellular protein, whereby less expression could be interpreted as leading to increased paracellular permeability (353). Claudin-3 (*cldn3*) is highly expressed throughout the murine gut and has been found to mediate barrier functions (354). These two targets were investigated as proxies for changes in the intestinal barrier permeability.

3.5 STUDY III – DESIGN OVERVIEW AND OUTCOMES

In this study, we wanted to examine the gut microbiota in antidepressant treatment-naïve patients with MDD compared to healthy individuals and assess how the gut microbiota changed over time during antidepressant treatment. Reporting of the results was performed according to "Strengthening the Reporting of Observational Studies in Epidemiology" guidelines. Young adults aged 18-24, both ages included, were diagnosed with MDD according to ICD-10 criteria by an experienced psychiatrist and were antidepressant treatment-naïve, including both pharmacological and cognitive therapy, at inclusion. Exclusion criteria were known comorbidities with neurological disorders, gastrointestinal disorders, endocrine, or metabolic diseases, pro- or antibiotic intake three months prior to inclusion, specific dietary habits such as vegetarianism, or pregnancy. Healthy controls aged 18-30 were recruited in the same time period and had to meet the same exclusion criteria. In addition, they could not previously have been diagnosed with, or received treatment for, MDD. The timeline of the study is presented in *Figure 8*.

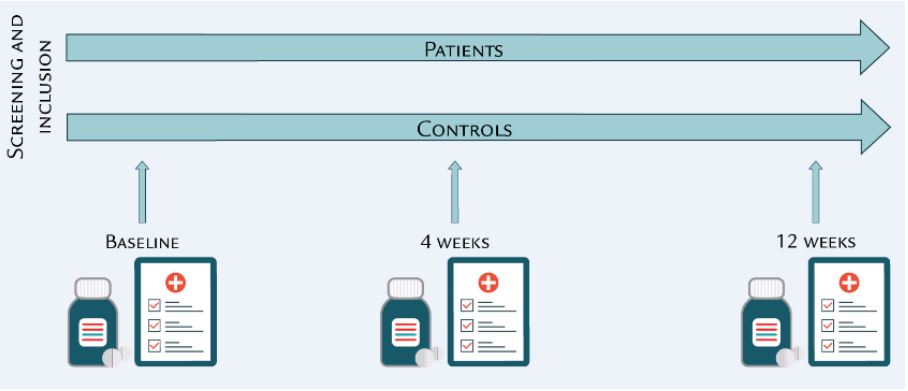

***Figure 8* – Timeline overview of sample and questionnaire collection.** After inclusion into the study, participants delivered fecal samples, as well as answered questions regarding their health. Patients then commenced their pharmacological and/or cognitive antidepressant treatment. Samples were then collected and the questions repeated at four and twelve weeks follow-up to the baseline measurement.

The severity of MDD was evaluated using the self-reported Major Depressive Inventory (MDI) (355). Furthermore, participants were asked about their eating and toilet habits, and gastrointestinal distress. Fecal samples were collected from each participant. Bacterial DNA was purified using the QIAamp PowerFecal kit (Qiagen). Gut microbiota was then characterized by gene sequencing of the hypervariable V4 region of the 16S rRNA gene using the primers 515FB and 806RB primers (356, 357) on the Illumina MiSeq platform. Here, sequences were used to build amplicon sequence variants (ASVs) which were aligned with MAFFT (358) and assigned taxonomy through the SILVA database (359) with results analyzed in RStudio IDE.

Primary outcome measures:

Compositional and bacterial differences between patients with MDD and healthy individuals in baseline samples

Compositional and bacterial differences between baseline, four and twelve weeks follow-up in patients with MDD after commencement of pharmacological and/or cognitive antidepressant treatment

Secondary outcome measures:

Changes in MDI score in patients with MDD

Gastrointestinal distress in patients with MDD

3.6 STUDY III – CRITICAL EVALUATION OF METHODS

3.6.1 RECRUITMENT OF PARTICIPANTS

Adults aged 18 - 24 years were screened and diagnosed by an experienced psychiatrist for unipolar depression according to ICD-10 criteria. Healthy individuals aged 18 - 30 years were included through social media posts and from the North Denmark Regional Hospital internal personnel page. The original goal was to include 50 participants in each group. This narrow age group for the patient group was chosen, due to legal changes to pharmacological antidepressant treatment of patients aged 18 – 24 in Denmark. See "Vejledning om behandling af voksne med antidepressive lægemidler", stk, 4.6, VEJ nr. 9899 af 11/11/20214 https://www.retsinformation.dk/eli/retsinfo/2014/9899 for specification. This age group would therefore have to be referred to the psychiatric departments, increasing the success rate of reaching 50 patients with MDD in the study. Additionally, as the gut microbiota have been found to be associated with many diseases (17, 104-138, 140-142), a narrow age group limited the risk of patients and/or controls suffering from comorbid disorders.

Patients had to be evaluated for clinical depression by an experienced psychiatrist, as previous studies have shown that general practitioners lack sensitivity in recognizing patients with MDD (360). Furthermore, as we wished to conduct a longitudinal study, and the antidepressant treatment therefore was a necessary factor in the assessment of the gut microbiota, it was paramount that patients were carefully monitored. Primary care physicians do not necessarily escalate antidepressant treatment or augment with supplementary drugs as needed (361), thereby risking a prolonged depressive episode. Patients risk discontinuation of medical treatment during the first three months (362), and adherence to medical treatment in patients is low in general in the primary care setting (363). It was necessary to ensure as much adherence as possible by recruiting primarily through the psychiatric departments. Danish national guidelines on treatment of patients with MDD in the hospital setting recommends supportive treatment with cognitive therapy no matter the severity of MDD. Therefore, it was speculated that patients would be more regularly monitored by the medical staff at the outpatient clinics, increasing the chance of medical adherence.

Finally, it was also a question of feasibility. The Danish psychiatric departments, given their nature and size, receives many more referrals for diagnosis of MDD than the individual general practitioner's office. Therefore, the successful inclusion of upwards of 50 patients with MDD was more likely by limiting the inclusion sites to those where the most individuals would be screened. Furthermore, it can be speculated that patients referred to the psychiatric departments would be moderate-to-severely depressed, whereas the patients with mild MDD would remain in the primary care setting.

3.6.2 – EVALUATION OF DEPRESSIVE AND GASTROINTESTINAL SYMPTOMS

The severity of clinical depression can be evaluated using a variety of different scales, such as the clinician administered Hamilton Depression Rating Scale (364), or the Montgomery-Åsberg Depression Rating Scale (365). Another choice is self-reporting questionnaires, such as the Beck's Depression Inventory (366). These offer a cost-efficient way of examining depressive features and their development over time, as the participant can rate themselves multiple times without the assistance of a professional. For this reason, the MDI was chosen as the method of analyzing participant severity of MDD. The MDI consists of a 10-item questionnaire which has been observed to have high validity in evaluating depressive symptoms based on ICD criteria (355), which is clinically used for diagnosis in Denmark. The MDI separates depressive severity into four categories; severe depression (31-50), moderate depression (26-30), mild depression (21-25) and no depression (0-21). The MDI was chosen to evaluate the depressive symptoms to reduce the stress of participating in the study. If the Hamilton Depression Rating Scale or the Montgomery-Åsberg Depression Rating Scales had been used instead, it would have required additional time and effort on behalf of the patient. As lack of motivation is one of the core symptoms of MDD (145, 146), it was necessary to reduce the stress of participating in the study to a minimum, while still being able to determine the effect of the antidepressant treatment.

Other than the MDI, participants were also required to answer questions regarding their health. They were asked these questions thrice; covering the two weeks prior to inclusion; covering the four weeks between the baseline sample and the four weeks follow-up; and covering the eight weeks between the four weeks follow-up and the twelve weeks follow-up. These questions primarily related to gastrointestinal symptoms, toilet habits, appetite, and dietary preferences. Additionally, a Bristol Stool Scale was used to evaluate the transit time of the fecal sample, as well as any gastrointestinal distress in the participant during the twelve weeks study period. Participants were instructed to answer the questions on the same day as the fecal sample was collected, to ensure that depressive severity reflected the gut microbiota composition. These questions were included into the study to determine if there were any dietary changes during the antidepressant treatment. Although the questions did

not include details about the exact diet of the participant, they could provide a basic overview of changes in appetite, caloric intake, or nutritional preferences. The answers were used to explore potential nutritional changes during the study. As diet highly impact the gut microbiota (43), potential changes could impose a bias of microbial differences being caused by dietary changes.

3.6.3 ANTIDEPRESSANT TREATMENT

After the delivery of the baseline fecal samples, patients were instructed to commence their antidepressant therapy. As this study was observational only, and not a clinical intervention study, patients initiated their antidepressant therapy according to the recommendations given by their respective psychiatrist. Patients were therefore not required to receive specific classes or types of antidepressant medication. In medicine, the patient care must be the first priority, and therefore the study did not limit participants to one type of antidepressant class or dose. This was primarily due to feasibility, as there was a high risk of discontinuation in the project, if it was necessary to change the medical regiment of the patient. As patients with MDD often require dose regulation, switching to another class of antidepressants or augmentation (229), the resulting increased dropout rate may have resulted in termination of the project. Therefore, the focus of the project was to evaluate the gut microbiota alterations during antidepressant therapy, rather than the antibacterial effect of antidepressant treatment on the gut microbiota.

3.7 METHODS COMMON FOR THE ANIMAL AND CLINICAL STUDIES

3.7.1 GUT MICROBIOTA CHARACTERIZATION

The gut microbiota can be characterized by using several different methods. *Figure 9* display an overview of the advantages and disadvantages of the methods used in Study II and Study III, while we here will present rationales for why each specific method.

We chose to use fecal samples in our study, as it is non-invasive, easy, and inexpensive. While this method of sampling can be repulsive for some, it would be ethically wrong to enforce an invasive procedure onto an already distressed patient with MDD. Nonetheless, fecal samples can only serve as a proxy of the gut microbiota, as early studies observed significant differences between gut microbiota sequenced from fecal samples compared to gut microbiota sequenced from mucosa samples (7). Bacterial organization depends on luminal or mucosal association. Closer to the mucosa, oxygen levels are higher, selectively providing better living conditions for facultative anaerobes and aerobes (42). Additionally, the loose mucus layer closely associated with the apical cell membrane is enriched in mucin-degrading bacteria (367). Fecal samples have been found to harbor distinct species, as well as a fraction of mucosa-associated taxa (7). This suggests that fecal samples represent a specific ecological niche, as well as accommodate subsets of mucosa-localized gut microbiota (6).

Method	Advantages	Disadvantages
Fecal sampling 	Non-invasive Cost-efficient Easy to sample	Not representative Anaerobes lost in storage process Sampling bias
DNA purification 	Bead-beating increases gram-positive taxa	Bead-beating decreases gram-negative taxa
16S rRNA gene sequencing 	High throughput Cost-efficient Non-targeted approach	Low sensitivity towards rare taxa Relative bacterial abundance, not absolute Low resolution at species level

***Figure 9* – Advantages and disadvantages of the chosen methods of gut microbiota characterization.**

Current bacterial DNA extraction methods recognize the fact that bacterial species variations in fecal samples are vast, and that extraction should attempt to be sensitive towards genetically and structurally diverse species. QIAamp has previously been observed to be highly effective and very sensitive towards detection of several gram-negative bacteria (368). The difference in cell wall structure between gram-negative and gram-positive bacteria does however often result in underrepresentation of gram-positive taxa in fecal samples, as chemical lysis does not always penetrate both cell wall barriers. Nonetheless, this can be countered by including mechanical lysis in the DNA purification setup (369), which was performed in Study II and III.

Attempts at characterizing the human gut microbiota have been made using targeted methods, such as qPCR (370) or fluorescence *in situ* hybridization (371), both of which are designed to target species-specific DNA or RNA strands. More semi-targeted methods have since been developed such as culturomics. Briefly, culturomics was developed as method to identify unknown bacterial species in the gut microbiota by *in vitro* propagation characterization by for example mass spectrometry (372). Cultures can be optimized for those species that are present in low amounts and therefore harder to detect by qPCR and *in situ* hybridization. However, cultoromics restricts the growth of species to a narrow set of living conditions, so it gives a lot of information about species living within these specific, distinct conditions (372-374). These methods often have the advantage of providing absolute numbers of bacterial

species, but are limited to the taxa they target, therefore not providing a nuanced picture of the complete gut microbiota composition.

The development of non-targeted analyses by next generation sequencing platforms such as 16S rRNA gene sequencing has led to higher resolutions of taxa at lower taxonomic orders, providing a more complex picture of the gut microbiota composition (375). This method is not without its own limitations. This sequencing method targets the hypervariable regions of the 16S rRNA gene. Construction of operational taxonomic units (OTUs), which was performed in Study II, results in clustering at 97%, whereby many different bacterial species can risk being clustered under the same genus (376). Also, due to the 97% clustering for OTUs used in Study II, it is most often not possible to detect bacteria at species or subspecies level (377), an issue which was resolved by using amplicon sequence variants (ASVs) (378) in Study III. Additionally, the 16S rRNA gene sequencing methods can only provide relative abundance of taxa, as the abundance of OTUs/ASVs is being measured against the total sum of OTUs/ASVs, which can be highly individual.

3.7.2 STATISTICAL AND BIOINFORMATICAL ANALYSES

As the animal study was performed before the clinical study, we there used the resulting sequences to cluster OTUs at 97% sequence alignment. The 97% percentage alignment was chosen based on a study which found that most bacterial strains had an average nucleotide identity at approx. 97% (379). Data was processed using the USEARCH workflow (380), implemented in QIIME (381). The MiDAS reference database (382) was used for taxonomic assignment of clustered OTUs as recommended by our collaborator DNASense during their assistance on the bioinformatical processing.

Afterwards, we found that the field had started to use ASVs instead, as these provide a much better resolution (378). ASVs were therefore built in the clinical study using the USEARCH workflow implemented in QIIME2 (383). Comparison of mock communities of amplicon data has revealed that the older version of QIIME had difficulty recognizing spurious OTUs, leading to inflated α-diversity measures (384), which is one of the reasons for switching both clustering method, and bioinformatic pipeline. In the clinical study, due to clustering in ASVs instead, and as many clinical studies use the SILVA database (108, 109, 117-119), we chose to use this database instead.

For bacterial β-diversity indices, Bray-Curtis dissimilarity, as well as weighted and unweighted UniFrac, were used in the clinical study (385). Several metrices were included, as they interpret findings differently depending on the properties of the intestinal community. Quantitative measures, as all three measures were, consider not only the presence of a taxa, but also its relative abundance. The Bray-Curtis is a widely used index because it takes both similarity and dissimilarity between gut microbiota communities into account, where regular distance measures such as Euclidian distance inflate dissimilarity values (386). However, it does not consider the relatedness of the

different taxa, which is why weighted and unweighted UniFrac were included (385). The unweighted UniFrac measures the evolutionary uniqueness of two different taxa and incorporates relatedness into the statistics, while weighted UniFrac additionally accounts for the differences in relative abundance of singular taxa (387). The unweighted version is more sensitive towards abundant taxa, while the weighted version is more sensitive towards rare taxa.

3.7.3 ETHICAL CONSIDERATIONS

The ethical animal approval was provided by the European Union Council Directive (ID Number: 2016-15-0201-01105). In animal research, there are substantial ethical considerations that must be undertaken before initiating any project and applying for ethical approval (388). This study aimed at upholding the Three Rs; reduce the number of animals used in the experiment, refine, or limit the pain and distress to which the animals are exposed and replace the use of animals with non-animal alternatives when possible. Animals underwent oral gavage, which may feel uncomfortable, but is a very quick and painless procedure with high translational value. This was only performed the number of times deemed necessary to ensure proper colonization of the rat intestinal tract with the donor gut microbiota. Rats are natural swimmers, but even so the forced swim test is considered a very stressful assessment of behavior. Nevertheless, the forced swim test is valued as one of the most efficient methods in assessing depressive-like behavior and therefore its pros outweigh its cons in a benefit-harm analysis. Furthermore, animals are sacrificed shortly after the conduction of the behavioral tests.

Use of human subjects was approved by the North Denmark Regional Ethical Committee (ID Number: N-20170056), registered at the Danish Data Protection agency and conducted in accordance with the Declaration of Helsinki. Written informed consent was given by all participants. There were very few other ethical considerations regarding the patients – the fecal sampling procedure may be unusual and slightly uncomfortable for the patient, but it is a very fast, efficient and non-invasive method that the participants were guided through in their written instructions.

CHAPTER 4. RESULTS

The following provides the hypothesis behind the studies, and a brief summary of the main results of the studies. A detailed presentation of all results and statistical data is provided within each individual manuscript.

STUDY I

Hypothesis: Overall, previous studies have been able to differentiate between patients with MDD and healthy individuals based on the gut microbiota composition.

Our systematic review included seventeen studies with a total of 738 patients with MDD and 782 healthy controls. These studies were found to vary highly in demographic and clinical information, resulting in heterogenous study populations. For example, only six of the studies excluded patients with known inflammatory bowel disorders (105, 108, 110, 113, 114, 120). Additionally, they utilized considerably different methods to evaluate and characterize the gut microbiota in their respective cohorts. As an example, very few of the studies applied the same primer set in their sequencing of the hypervariable regions of the 16S rRNA gene. Despite the heterogeneity in study designs, sixteen out of seventeen studies reported that they observed significant differences in the individual taxa of patients with MDD compared to their respective controls (see *Table 1*).

Overall, four out of seventeen studies found a reduction in α-diversity measures in patients with MDD using several diversity indices (108, 111, 113, 114). In β-diversity, the gut microbiota of patients with MDD were observed to cluster separately from the gut microbiota compositions of healthy individuals in different principal components analyses (104, 106, 109, 110, 112, 113, 115-117, 119, 120). In addition, sixteen out of seventeen studies found alterations in the relative abundance of specific taxa between patients with MDD and their respective control groups. In total, 5 phyla, 36 families and 78 genera of bacteria to be significantly altered in patients with MDD (The exhaustive list of these bacterial variations can be found in *Appendix C)*. At family level, *Bifidobacteriaceae* and *Coriobacteriaceae* were both consistently increased in relative abundance in patients with MDD in five (110-112, 116, 120) and four (106, 110, 111, 115) studies, respectively. The most consistent findings at genus level were an increase in relative abundance of *Eggerthella* in six studies (104, 110-112, 115, 116) and *Atopobium* (110, 111, 116) and *Bifidobacteria* (110-112, 116) in four study populations. On the other hand, *Faecalibacterium* was observed to be decreased in relative abundance in seven studies (105, 106, 110, 111, 115, 116, 119, 120). In general, studies were able to differentiate between patients and controls based on gut microbiota composition using either diversity indices and/or individual taxa.

Table 4 - Analyses Based on Next Generation Sequencing of Bacterial Taxa in Patients Diagnosed with MDD Compared to Healthy Controls	Naseribafrouei et al. (42)	Jiang et al. (43)		Zheng et al. (2016) (44)	Kelly et al. (46)	Lin et al. (47)	Chen et al. (2018) (48)		Rong et al. (49)	Chung et al. (50)	Huang et al. (51)	Vinberg et al. (52)	Chen et al. (2020) (53)		Lai et al. (54)	Liu et al. (55)	Mason et al. (56)	Stevens et al. (57)	Zheng et al. (2020) (58)	TOTAL OBSERVATIONS		
Bioinformatics analyses		*A-MDD*	*R-MDD*				*Males*	*Females*					*Young*	*MA*						↓	↑	**No diff.**
Observed OTUs/ASVs/species				No	*Yes* ↓			No		No		*Yes* ↓				No				2		5
Phylogenetic diversity				No	*Yes* ↓			No												1		2
Chao1 richness	No				*Yes* ↓	?			*Yes* ↓	No	*Yes* ↓		No	No					No	3		6
Shannon		*Yes* ↑		No	No	?		No		No	*Yes* ↓	No			No	No	No		No	1	1	10
Simpson	?	No		No	?	?		No	No (I)										No (I)			5
Unweighted UniFrac		No		*Yes*	*Yes*			*Yes*		No	No	No (G)				*Yes*				*Yes* = 4		**No = 4**
Weighted UniFrac				*Yes*	*Yes*	*Yes*				No	*Yes*						No			*Yes* = 4		**No = 3**
Relative abundance of phylum in MDD compared to HC																						
Actinobacteria		↓		↑				↑	↑	↑										4	1	
Bacteroidetes	↓	↑	↑	↓			↓		↓	↓									↑	3	5	
Relative abundance of family in MDD compared to HC																						
Actinobacteria - Bifidobacteriaceae								↑	↑	↑					↑				↑	5		
Actinobacteria - Coriobacteriaceae				↑				↑	↑					↑						4		
Bacteroidetes - Bacteroidaceae		↓	↑	↓										↓	↓				↑ and ↓	2	5	
Bacteroidetes - Prevotellaceae		↓			↓					↓				↓							4	
Firmicutes - Lachnospiraceae	↓	↓	↓	↑ and ↓			↑ and ↓	↑ and ↓		↑	↓			↑				↑ and ↓	↑	7	7	
Firmicutes - Ruminococcaceae		↓	↓	↑			↓	↑ and ↓	↑		↓		↓			↓		↓		3	8	
Relative abundance of genus in MDD compared to HC																						
Actinobacteria - Bifidobacteriaceae - Bifidobacterium								↑	↑	↑					↑					4		
Actinobacteria - Coriobacteriaceae - Atopobium							↑	↑	↑						↑					4		
Actinobacteria - Coriobacteriaceae - Eggerthella					↑			↑	↑	↑				↑	↑					6		
Bacteroidetes - Bacteroidaceae - Bacteroides		↓	↑				↑		↑					↓	↓			↓	↑	4	4	
Bacteroidetes - Porphyromonadaceae - Parabacteroides		↑	↑							↑								↑		4		
Bacteroidetes - Prevotellaceae - Prevotella		↓	↓		↓	↑			↑ and ↓	↓										2	5	
Firmicutes - Clostridiaceae - Faecalibacterium		↓	↓	↓				↑			↓			↓		↓		↓		1	7	
Firmicutes - Lachnospiraceae - Blautia		↑		↑				↑		↑	↓									4	1	
Firmicutes - Lachnospiraceae - Roseburia		↑	↑	↓				↑						↑				↓		4	2	
Firmicutes - Oscillospiraceae - Oscillibacter	↑	↑	↓						↑					↓	↑			↑ and ↓		5	3	
Firmicutes - Ruminococcaceae - Ruminococcus		↓	↓							↑						↓			↓	1	4	
Firmicutes - Streptococcaceae - Streptococcus						↑			↑ and ↓	↑				↑	↑					5	1	

Table 1 **- Bacterial taxa obtained from next generation sequencing of bacterial DNA comparing patients with MDD to their healthy controls.** Observations between the patient and control groups found to be statistically significant in the bioinformatical analyses are designated as either " *Yes*" or " No" . The direction of change is displayed with arrows and represent alterations in patients with MDD compared to the controls. Fields with a question mark correspond to studies, where the bioinformatical analysis was conducted according to the methods section, but no conclusions were reported in the results. Bacterial taxa are listed with their taxonomy as *phylum - family - genus*. If a study conducted analyses on several populations, such as dividing participants into young versus middle-aged, each observation reported was counted as an independent observation. Arrows here specify the increase (green) or decrease (red) in relative abundance of bacterial taxa if this was observed in four or more study populations of patients with MDD compared to healthy controls "Total observations" is the cumulative number of times, and taxa were included in this table if a specific direction of change was observed in four or more study populations. A-MDD: Treatment-resistant patients with MDD. R-MDD: Patients with MDD responding to antidepressant medical treatment. MA: Middle-aged. OTUs: Operational taxonomic Units. ASVs: Amplicon Sequence Variants. (I): Inverse Simpson. (G): Generalized UniFrac, a combination of weighted and unweighted UniFrac.

STUDY II

Hypothesis: FMT from patients with MDD into recipient rats can elicit depressive-like behavior compared to FMT from the healthy control group.

Our study followed two separate conceptual lines of research:

A) To examine whether gut microbiota from *patients with MDD* could introduce a *depressive-like phenotype* into healthy control rats, we performed several FMT into the **FRL** (*Figure 10A + 10B*). FRL rats received the following interventions:

1) FMT from patients with MDD (FMT-MDD)
2) FMT from healthy individuals (FMT-Healthy)
3) FMT-MDD and simultaneous treatment with sertraline (FMT-MDD-Ser)
4) FMT with their own gut microbiota collected fresh from the two cohoused animals (CON-Auto)
5) demineralized water (CON-H2O)

B) To examine whether gut microbiota from *healthy controls* would introduce an *antidepressant-like phenotype* into an animal model of depression, we performed FMT into the **FSL** (*Figure 10C + 10D*). FSL rats received the following interventions:

1) FMT from patients with MDD (FMT-MDD)
2) FMT from healthy individuals (FMT-Healthy)
3) FMT with their own gut microbiota collected fresh from the two cohoused animals (CON-Auto)
4) demineralized water (CON-H2O).

We used the forced swim test to assess depressive-like behavior, using both immobility time as the classical proxy for depressive-like behavior and struggling as assessment for engagement in active escape-oriented behavior interpreted as antidepressant-like behavior. The FSL rats clearly demonstrate higher overall immobility time in the forced swim test than FRL rats, (depressive-like behavior), which is the hallmark of this animal model (*Figure 10A + 10C*).

For part **A)**, the FMT-MDD group displayed a tendency towards higher immobility ($p = 0.088$) and significantly less struggling ($p = 0.013$) compared to the FMT-Healthy group (*Figure 10A*). However, while the FMT-MDD rats displayed depressive-like behavior compared to the FMT-Healthy rats, there was no difference in behavior compared to the neither the CON-Auto rats nor the CON-H2O rats (*Figure 10B*). The lack of a depressive-like phenotype in the FMT-MDD group compared to the two control groups suggests that the FMT-Healthy group presents an antidepressant phenotype, rather than an induced depressive phenotype in the FMT-MDD group.

For part **B)**, there was no difference in behavior in the FSL between treatment groups (*Figure 10C + 10D*). The remainder of the study therefore focused solely on the FRL rats.

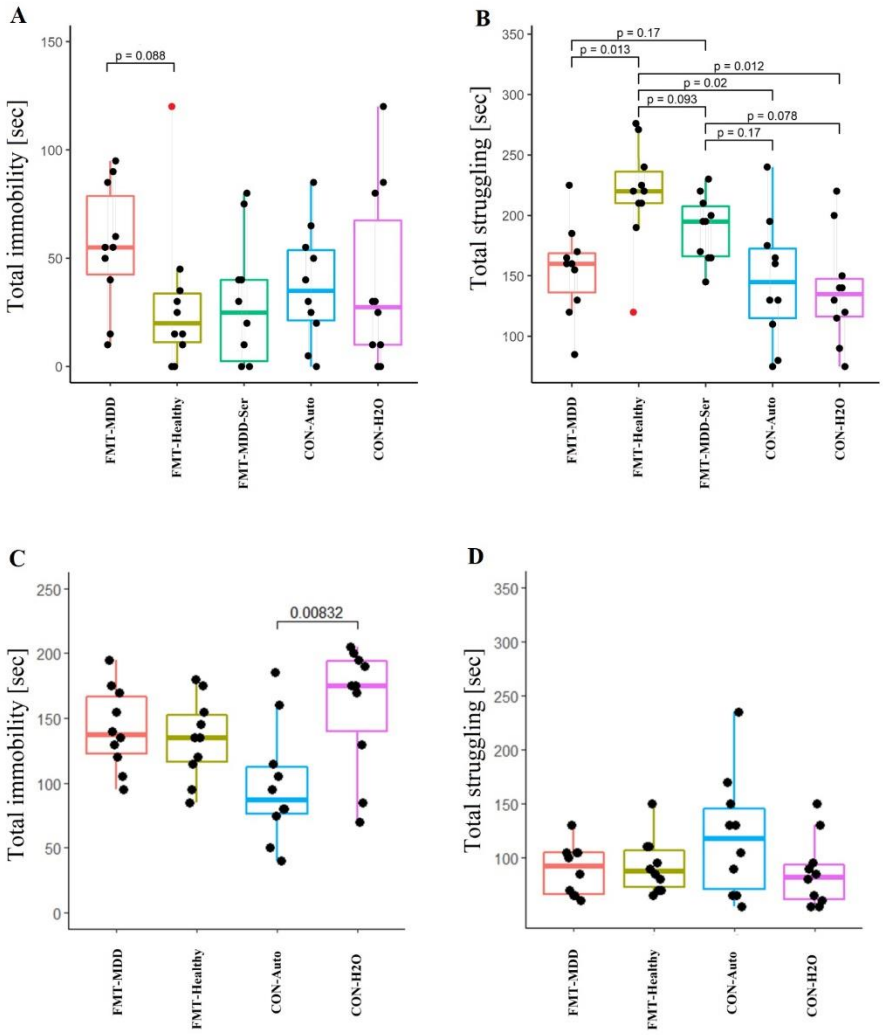

Figure 10 **- Immobility and struggling times in the forced swim test.** Total immobility time (sec) from each group based on recordings of the first 5 minutes in either FRL rats (A) or FSL rats (C). Total struggling time (sec) from each group based on recordings of the first 5 minutes in either FRL rats (B) or FSL rats (D).

After evaluation of the behavioral phenotypes, we determined how much of the donor material could be traced to the rat colonic system. 16S rRNA sequencing of fecal samples collected prior to the interventions and after interventions found that the human donor material accounted for 9.7 % ± 1.5 SD of the gut microbiota composition (*Figure 2* in Paper II). Even so, it was possible to identify individual taxa from the human donors in the rat fecal samples after FMT.

An MA plot was built to illustrate the significant changes observed in bacterial taxa over time, and here we found that seventeen taxa were overrepresented in FMT-MDD compared to FMT-Healthy, while eight were underrepresented (*Figure 11*). Of these, five could be identified from the donor material. Three genera belonging to the *Ruminococacceae* family and the genus *Lachnospira* were elevated in relative abundance in FMT-MDD rats, while the genus *Coprococcus* was found depleted in relative abundance in the FMT-MDD rats.

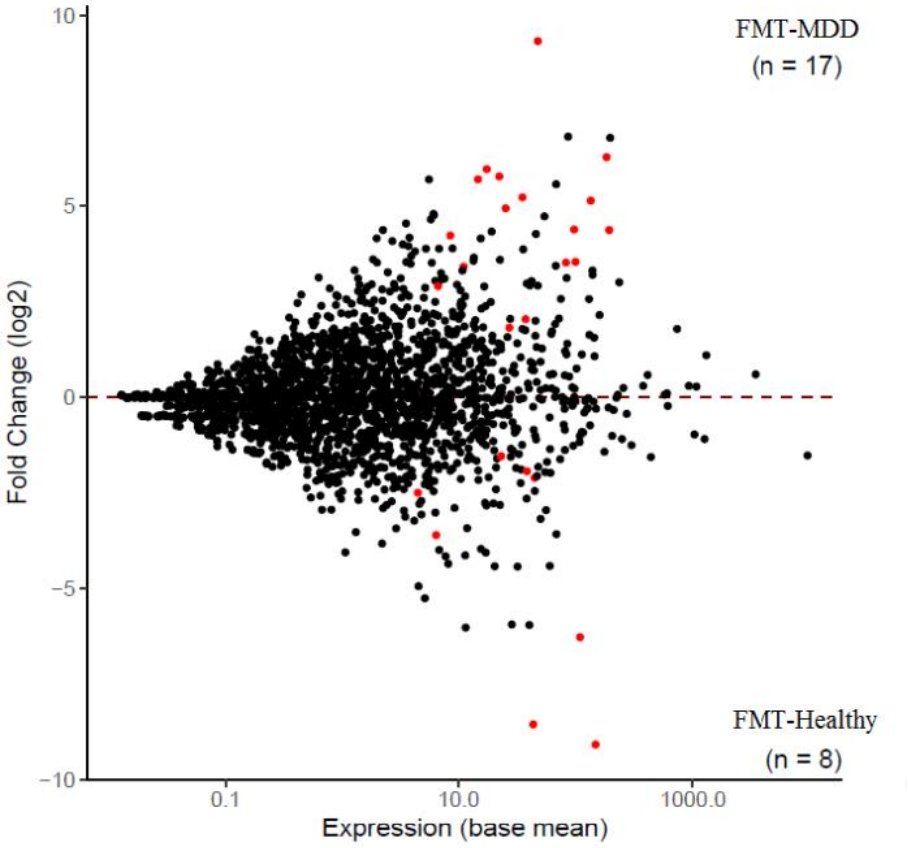

***Figure 11* - Fold change in OTU expression comparing the OTUs observed in samples taken after FMT to OTUs observed in samples taken before FMT.** OTU expression in rats receiving FMT from patients with MDD compared to rats receiving FMT from healthy individuals.

STUDY III

Hypothesis: Antidepressant treatment-naïve patients with MDD have a significantly different gut microbiota composition compared to healthy individuals prior to initiation of treatment. Furthermore, their gut microbiota changes during therapeutic course.

In total, 27 patients with MDD were recruited for the study. Of these, 21 delivered the baseline samples, 14 delivered the fecal sample at the four weeks follow-up and 12 completed the study with all three samples. For healthy controls, 32 were recruited and 30 completed the study with all three samples. The patients commenced their antidepressant therapy after delivery of the baseline sample, but two only received cognitive treatment and no pharmaceutical antidepressant treatment. The remaining ten patients with MDD received a variety of antidepressant medication, such as sertraline, quetiapine, escitalopram, citalopram, mirtazapine, and duloxetine. Patients went from having a mean MDI score of 40,9 at inclusion, to 35,5 at the four weeks follow-up and 27 at the twelve weeks follow-up. The MDI score of patients decreased significantly from inclusion to completion of the study ($p = 0.02$). Of all twelve patients that completed the study, two patients went into remission, two patients responded to the medicine with a 50% reduction in MDI score and four went from having severe MDD to having mild MDD. Patients with MDD reported a higher frequency of gastrointestinal distress throughout the study, such as nausea, diarrhea, constipation and stomach pain, compared to the healthy individuals. For the comparative analyses of the gut microbiota, we included both the patients on pharmaceutical and cognitive treatments.

We did not observe any differences in neither α- nor β-diversity indices explored between patients with MDD and healthy individuals at baseline. Additionally, we did not find any longitudinal differences in neither α- nor β-diversity indices in the gut microbiota of patients with MDD from the baseline to the twelve weeks follow-up. Nonetheless, we found significantly different taxa between patients with MDD and healthy individuals at baseline in the linear discriminant analysis – effect size (*Figure 12*). Furthermore, we found that individual bacteria changed significantly over time in patients with MDD from the baseline to the twelve weeks follow-up. We observed that the genera *Ruminococcus gnavus group*, *Ruminoccocus torques group* and *Intestinibacter* were increased in relative abundance, while the genera *Coprobacter*, *Bilophila*, *Desulfovibrio*, and *Lactobacillus* were decreased in relative abundance in patients with MDD compared to healthy individuals. In patients with MDD, the family *Ruminococcaceae* and the genus *Clostridium sensu stricto* were found to decrease in relative abundance from baseline to the twelve weeks follow-up.

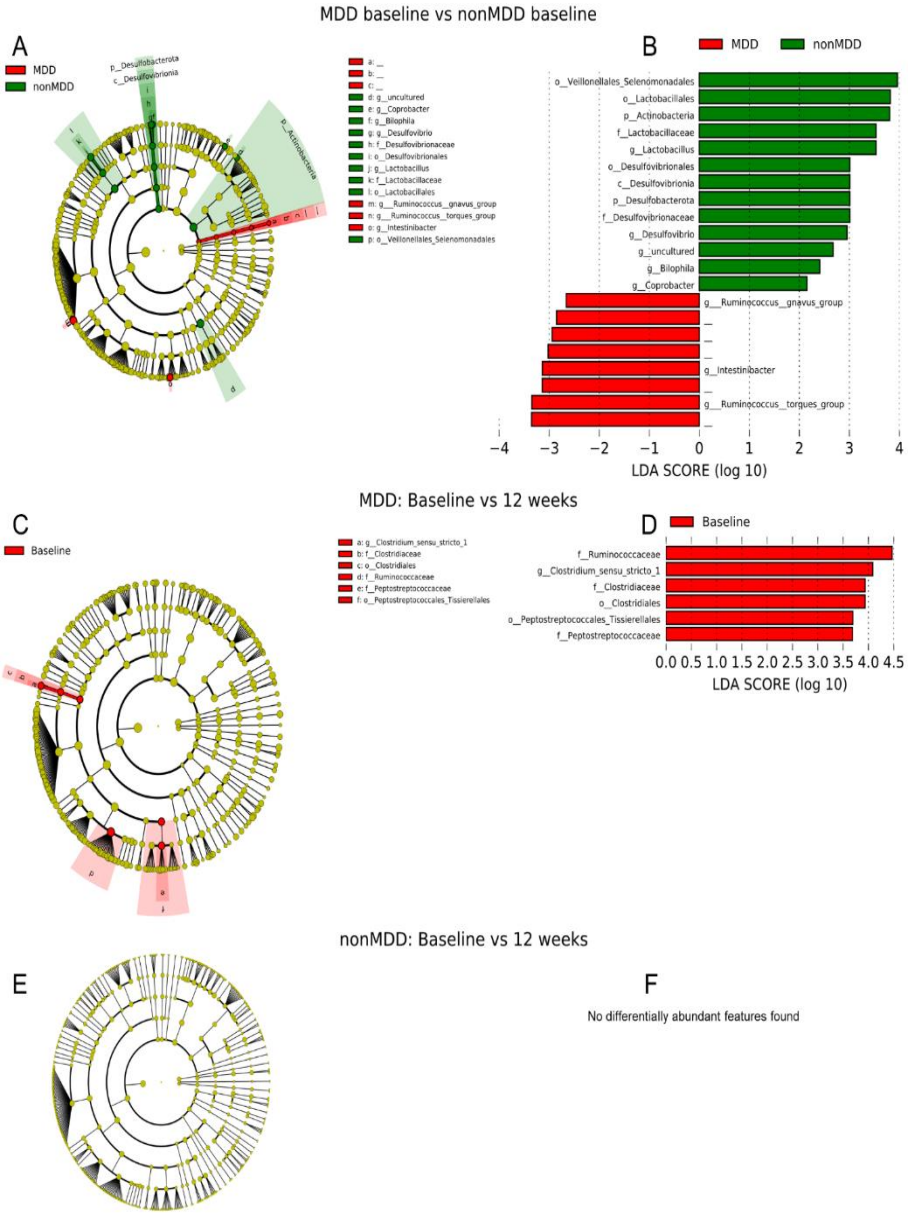

Figure 12 **– Linear discriminant analysis – effect size of changes in individual bacterial taxa.** Cladograms (A, C, E) display the internal relationship between bacteria significantly different. Barplots (B, D, F) display linear discriminant analysis scores of bacterial taxa in patients with MDD compared to healthy individuals (nonMDD) (A, B), bacterial taxa at baseline compared to twelve weeks follow-up in patients with MDD (C, D), and baseline compared to twelve weeks follow-up in

CHAPTER 5. DISCUSSION

In this project, new insight into the scientific field of gut microbiota involvement in depression through the three studies was gained:

- As demonstrated by our systematic review, the gut microbiota of patients with MDD can be separated from the gut microbiota of healthy individuals by either α- or β-diversity indices, or in individual taxa.

- FMT from healthy individuals into recipient FRL rats was able to elicit an antidepressant behavior when compared to FMT from patients with MDD. FMT from patients with MDD into FRL rats did not produce a depressive-like phenotype when compared to the CON-Auto or the CON-H2O FRL rats. Several bacterial taxa from the human donor material could be observed in the recipient rats.

- The separation of gut microbiota between patients with MDD and healthy individuals based on α- or β-diversity indices could not be replicated in our patient cohort. However, we did find distinct bacterial taxa that were significantly different between patients with MDD and healthy individuals Additionally, individual taxa did significantly change after initiation of pharmacological and/or cognitive antidepressant treatment.

The following sections will go through the three studies in detail, critically evaluating the results obtained here compared to previous publications, and furthermore attempt to highlight the clinical and translational value to provide future perspectives. For detailed discussions on each topic, we refer to Paper I, II and III.

5.1. THE GUT MICROBIOTA IN PATIENTS WITH MDD IS SIGNIFICANTLY DIFFERENT IN TAXA AND DIVERSITY

Several systematic reviews had been conducted prior to our addition to the scientific field (389-393). While these reviews were excellent, this field is rapidly expanding with many studies published a year, whereby an updated review is be necessary to establish a consensus on which bacterial species are of interest in patients with MDD. The more publications, the larger the combined sample size and the more confidence is gathered in which specific bacterial variations are predominant in patients with MDD. Furthermore, the previous reviews focused primarily on the potential depressive and/or antidepressant properties of the bacterial taxa altered in patients with MDD. We additionally attempted to be critical of which methods were applied to observe the taxa significantly different between patient and control cohorts.

Overall, the knowledge gained from Study I does imply that there are indeed an association between gut microbiota and MDD, as almost all included studies observed significant differences between their patients and control groups based on either α- or β-diversity. A loss of diversity, which was observed in four of the studies (108, 111, 113, 114), is a consistent finding of many modern diseases, especially associated with the western diet and lifestyle consisting of high caloric intake and low physical activity (394). Likewise, the gut microbiota of patients with MDD clustered separately from healthy individuals in eleven of the included studies (104, 106, 109, 110, 112, 113, 115-117, 119, 120), which has also been observed, primarily in non-communicable diseases (395). Like us, previous systematic reviews have reported a lack of consensus between studies in diversity indices. Nevertheless, while there was little overlap between the individual studies in which specific taxa that were predominantly altered in patients with MDD, seventeen out of the eighteen studies could observe significant differences between the patient and control groups. Furthermore, the reduced relative abundance of the anti-inflammatory genus *Faecalibacterium* (396) observed in Study I was confirmed in the meta-analysis of Sanada et al. (391). This suggests that this particular genus, or functional properties it contains, may be specific for gut microbiota alterations in patients with MDD in general despite methodological variations in gut microbiota characterization and population differences.

In recent systems biology publications, it is highlighted that the gut microbiota-host interactions is a complex system with many factors affecting the entire system (397). Instead of viewing the gut microbiota as a community consisting of separate taxa with specific effects on its host, it may be more clinically relevant to perceive it as a complex system, such as a holobiont consisting of all living entities in combination with the human host (398, 399). Therefore, we argue that the single bacteria alone does not affect the system as a whole, and thereby MDD. Rather it is the entire gut microbiota that is influenced, visible as alterations in single taxa, which may then exacerbate the depressive symptoms. Therefore, the individual bacteria observed to be significantly different in each of the included studies may rather represent species susceptible to the overall changes unique to MDD in that specific geographical and ethnic population.

The included studies focused primarily on assessing the functional potential of the gut microbiota through genetic analyses, rather than analyzing the bacterial end-products through for example metabolomics (400). Newer studies have attempted to integrate both in network analyses to determine which bacteria are associated with which metabolic markers (401, 402). Here, they have been able to generate models that with high sensitivity and specificity can predict the diagnosis of MDD in their cohorts, suggesting that the structural and functional composition of the gut microbiota has implications for MDD diagnosis and treatment in the future.

5.1.1. LIMITATIONS AND STRENGTHS

Meta-analyses provide the medical community with the best evidence-based summary of a great body of literature for optimization of patient care (403). We decided against performing a meta-analysis for several reasons.

Firstly, the methods used in each individual study were widely different. Variations in the methods applied to characterize the gut microbiota can impose several biases, such as differentially affecting which bacterial taxa can be observed in the fecal samples (404). For example, it is generally accepted that the primer pairs targeting the hypervariable region of the 16S rRNA gene has higher specificity for some bacterial taxa over others, whereby some taxa can appear over- or underestimated in relative abundance (369, 405, 406). Secondly, we recognize the differences in patient and control populations. Most of the sudies were conducted primarily in Asia, with the remaining studies performed in Europe or the United States. Geographical and dietary preferences may therefore have masked bacterial variations specifically associated with MDD and not with ethnic groups (47, 406). Thirdly, the studies conducted thus far have had low sample sizes, possibly leading to insecure results, in which a meta-analysis would have led to overestimation of the importance of individual bacteria (407).

Combined, we therefore decided that systematically extracting the data and presenting it as a broad representation of the gut microbiota composition in patients with MDD would give the medical community the most representative overview. Systematic reviews are often viewed as providing the most recent literature summary, and are therefore also used clinically in combination with meta-analyses to provide the best evidence for future study designs and practice guidelines (408). While all studies in the review fit the in- and exclusion criteria, systematic reviews are subject to publication bias, where only studies with positive results are published (319). We cannot rule the case of negative, unpublished results out, but as several of the studies did not observe any differences between patients and controls in diversity measures, we argue that it is unlikely here.

While there were many variations in the design of the included studies, many agreed upon specific bacterial variations in patients with MDD and healthy individuals. Therefore, we argue that the bacterial variations within patients with MDD are substantial enough in the microbial community, and in individual taxa, that it is observable through next generation sequencing assessments regardless of methodological variations. Therefore, our systematic review provides the most current knowledge in the field in an easily understood overview, which can inspire future studies in bacteria widely reported different in patients with MDD, such as *Faecalibacterium*. However, if systematic reviews and meta-analyses of gut microbiota in patients with MDD are to have an impact on future intervention studies, technical golden standards are necessary.

5.2 FMT FROM HEALTHY DONORS INCREASED STRUGGLING IN FRL RECIPIENT RATS

In our animal study, we found that FMT from patients with MDD or healthy individuals into FSL rats did not alter their depressive-like behavior. On the other hand, in the FRL rats, we observed a tendency towards lower immobility, and significantly more struggling in the FMT-Healthy group compared to the FMT-MDD in the forced swim test. Although, struggling is not the immediate proxy for depression-like behavior, it is a behavioral trait which suggests that the animal actively engages in escape-oriented behavior and therefore shows the opposite of behavioral despair.

Several studies of FMT from patients with MDD into rats have been performed (106, 108, 267, 268). All these studies showed depressive-like behavior in recipient animals of FMT from patients with MDD through either behavioral despair assessments like the forced swim test or the tail suspension test (106, 267, 268), or through evaluation of anhedonia in the sucrose preference test (108, 268). The inherent behavior of a rat or mouse depends on the strain used in the study (409, 410), which therefore naturally affects interventions with behavior as a primary outcome. Therefore, it is difficult to compare the behavioral effect of the FMT from patients with MDD between the studies, as these differ substantially in both animal species and strain. For example, the forced swim test has marked strain differences in both mice (411) and rats (412). The tail suspension test, where the rodent is suspended from the roof of a box in its tail only, mirrors the forced swim test in behavioral assessment. It cannot be applied to rats as they are too heavy to support their own weight (413). The depressive-like behaviors analyzed in mice with the tail suspension test can therefore be difficult to compare to the depressive-like behaviors observed in rats in the forced swim test. In the end, when we compare only the FMT-Healthy and FMT-MDD groups, we were able to replicate findings reported from previous studies that shows that depressive-like behavior can be induced by FMT from patients with MDD compared to FMT from healthy individuals.

When comparing the behavior of our FMT-MDD FRL rats to the CON-Auto or CON-H2O FRL rats, there was no difference in behavior. This suggests that the FMT-Healthy FRL rats received beneficial microbes, which may promote health and an antidepressant-like phenotype, rather than induced depressive-like behavior in the FMT-MDD FRL rats. Previously, only one study reported the inclusion of additional groups that did not receive FMT from human donors (268). Here, they were able to induce depressive-like behavior in their rats receiving FMT from patients with MDD compared to both FMT from healthy individuals, as well as the non-intervention control group. Our observed non-difference and their observed difference may have been due to model differences, as they utilized a GF Sprague-Dawley rat.

We would argue that our model has higher translational value than the previous study performed, as they utilized a model that does not represent a normal, healthy animal.

As our hypothesis is that the gut microbiota is involved in MDD, the animal model should resemble the human counterpart as much as possible. Complete lack of gut microbiota is not a natural human state, and therefore we believe that our model has higher translational value as a model of FMT-induced MDD.

None of the previous studies included animals who contained a natural, habiting microbiota. This may have resulted in induced behavioral differences between the altered animal, and its natural, microbiota-containing counterpart. This could have led to misinterpretation of behavioral analyses, especially when the lack of non-humanized control groups cannot reveal the natural behavior of the animal. Previous studies have primarily used the GF animal model in either mice or rats (106, 267, 268), or antibiotics-blasting of rats prior to FMT (108). There are a variety of behavioral differences between the GF mouse and its fully colonized control mouse, making it a less ideal model to use to assess the effects of FMT (414). Chronic antibiotics treatment been found to induce depressive-like behavior in mice (415), whereby separation between behavior induced by the FMT or by the antibiotic treatment is difficult. Furthermore, previous studies found that pre-treatment with antibiotics did not increase colonization efficiency in recipient animals (416), which was the rationale for choosing not to perform any colonic cleansing in Study II. Although our rats did contain a natural gut microbiota, and this may have limited the colonization effect, the FSL and FRL rats did not have behavioral differences induced prior to FMT, which may have been the case for the GF and antibiotics-treated animals. Therefore, our FMT model has higher translational value, although we did not confirm a depressive-like phenotype in the FMT-MDD group compared to the FMT-Healthy group.

In our study of FRL rats, it was possible to identify bacterial taxa in the recipient rats from the human donors, with increased relative abundance of three genera belonging to the *Ruminococcaceae* family and the genus *Lachnospira*, and decreased relative abundance of the genus *Coprococcus* in FMT-MDD compared to FMT-Healthy. In previous studies, it was assumed that the bacterial variations observed in recipient animals originated from the donor material due to their induced anxenic state prior to FMT. However, there was no consensus between our study and previous studies on which specific bacteria were altered in relative abundance. For example, we observed increased relative abundance of the family *Ruminococcaceae* in the FMT-MDD FRL rats. In the study by Zheng et al., they observed decreased relative abundance of the same family in rats receiving FMT from patients with MDD compared to FMT from healthy individuals (106). In spite of this, several of the bacteria observed increased or decreased in relative abundance in our FMT-MDD FRL rats has likewise been observed in clinical studies of patients with MDD. For example, the genus *Coprococcus* has been observed in three studies included in Study I (106, 113, 115), as well as the large population-based study by Valles-Colomer et al. (121). Here, they confirmed that *Coprococcus* was depleted in patients with MDD. They likewise confirmed an positive association between increased relative abundance of

Faecalibacterium and higher quality of life, strengthening the association of these two genera with MDD.

The article of Valles-Colomer et al. was rightfully titled "The neuroactive potential of the human gut microbiota in quality of life and depression" (121), as their main focus was on the functionality of the gut microbiota in patients with MDD. They specifically focused on the SCFA-producing properties of both *Coprococcus* and *Faecalibacterium,* which was similarly our main argument for a potential biological function related to MDD in Paper II. Loss of bacterial taxa with this property may lead to less SCFAs, and thereby a lack of their neuroprotective properties (84, 417), furthermore suggesting that it is the functional capacity of the gut microbiota that is involved in the pathology of MDD.

5.2.1 LIMITATIONS AND STRENGHTS

The strength of the FSL rat is that it displays a robust and strong increased immobility in the forced swim test compared to the FRL, thereby reducing the number of animals necessary to observe a significant difference between groups. Although, application of only one measure of depressive-like behavior can limit the confidence in both the validity of the results and the translational value in the context of MDD. However, FSL rats do not display anhedonia compared to the FRL rat unless a stress paradigm is applied (252, 418). As our original hypothesis is not based on a stress paradigm, it would therefore not make sense to include an additional test of depressive-like behavior such as anhedonia in the investigation of the depressive-like effect of FMT from patients with MDD. It could therefore be discussed whether the limitation of the behavioral battery of assessments for the FSL and FRL rats weakens the clinical relevance of our study, as the reproducibility across FMT studies is otherwise high using other animal models. Thus, it is possible that the study here more readily demonstrated an anti-depressant potential of the FMT from healthy individuals than the depressive-like effect of FMT from patients with MDD.

One of the strengths of the study is the choice not to eliminate the inhabiting gut microbiota of the rats. As discussed previously, antibiotics-blasting or GF models also contains biases regarding behavior, while in our study, there should be no induced behavioral alterations prior to FMT. Contrarily, the rats did harbor a living gut microbiota which would be in direct competition with the transplanted donor material. Nevertheless, it has previously been observed that the success of FMT highly depends on which species are naturally present in the host gut (419), whereby the exact bacteria from the human donors which survived the competition may have some evolutionary or competitive advantage. After all, it was possible to distinguish between the FMT-MDD and FMT-Healthy groups based on individual taxa despite the limited donor material detected. This adds to our hypothesis that the functional capacity of the gut microbiota is what drives potential depressive- and/or antidepressant-like behaviors.

5.3 CLINICAL RELEVANCE OF GUT MICROBIOTA IN PATIENTS WITH MDD

In our clinical Study III, we characterized the gut microbiota of patients with MDD prior to pharmacological and/or cognitive treatment and healthy individuals. The gut microbiota was then characterized again at four and twelve weeks follow-up after patients initiated treatment. Here, we found that there was no significant difference in neither α- nor β-diversity between patients with MDD and healthy individuals at baseline. On the other hand, individual taxa were significantly different between the two groups, with an increased relative abundance of the genera *Ruminococcus* and *Intestinibacter* and a decreased relative abundance of the genera *Coprobacter*, *Bilophila*, *Desulfovibrio*, and *Lactobacillus* in patients with MDD compared to healthy individuals.

As was observed in Study I, the majority of previous studies of patients with MDD only sampled their population once, and most of the patients were in active pharmacological treatment at the time of sampling (420). Despite differences in study design, several studies agreed with our observations regarding the lack of significant differences in both α- and β-diversity between patient and control groups. Additionally, several of the bacteria observed to differentiate patients from healthy individuals here were confirmed in other studies. In our patients, *Ruminococcus* was observed to be elevated in relative abundance in patients with MDD compared to healthy individuals at baseline, which has likewise been observed in previous studies (112, 401). In Paper III, we argued that this specific genus may be involved in tryptophan metabolism, limiting the bioavailability of this metabolite for serotonin production. In the study by Yang et al. (401), they found altered microbiota-associated enzyme-related genes for both tryptophan and GABA metabolism, as well as alterations in their metabolites, in patients with MDD compared to healthy individuals. This, combined with our confirmation of a significant increase in relative abundance of *Ruminococcus*, furthermore strengthens the hypothesis that it is not the individual bacterial taxa that are involved in MDD, but rather functions associated with these taxa that are disturbed. Tryptophan biosynthesis and metabolism was additionally disturbed in patients with MDD in the study by Lai et al. (116), while Valles-Colomer found altered GABA synthesis (121). This strongly suggests that the gut microbiota is involved in the regulation of neurotransmitters (421), although how much reaches the brain from the gut (301), and how it affects psychiatric diseases such as MDD is still unknown.

In our study, we also saw a loss of *Lactobacillus*, a genus commonly used in probiotic formulas due to its SCFA-producing characteristic (422). Species of *Lactobacillus* has also been observed depleted in previous studies of patients with MDD (111) and loss of this genus may result in lower production of SCFAs. Not only is these metabolites neuroprotective (84, 417), they also regulate the production of neurotransmitters (423). This ties together the results from Study II, where we observed depletion of *Coprococcus* in the FMT-MDD group of FRL rats compared to the FRL-Healthy FRL

rats. Furthermore, the genus *Faecalibacterium* reported depleted in relative abundance in patients with MDD in Study I also produces SCFAs (424). As these three genera are depleted in patients with MDD and produces SCFAs, this suggests that patients with MDD overall lack the bacteria-derived SCFAs and their regulation of neurotransmitter metabolism.

Theoretically, it is possible to imply that the elevation or depletion in the abundance of individual bacteria may have a functional consequence for the pathology of MDD. This has been exemplified in animal studies, such as the famed study by Sudo et al. (262). Here, inoculation of *Escherichia coli* in a GF mouse resulted in immunological and neurochemical alterations associated with MDD, which could be reversed by introduction of a strain of *Bifidobacterium* (262). Infection with *Toxoplasma gondii* in rats altered their fear response, making them more easily preyed upon by cats (425). While these studies illustrate how singular bacterial taxa can affect parameters associated with MDD, they involved animals devoid of a natural gut microbiota Therefore, they do not represent a natural system where introduction of a pathogen would meet colonization resistance (68). For that reason, the singular bacteria observed to be significantly different between patients with MDD and healthy individuals in Study III may alternatively be a symptom of how the intestinal system is affected in MDD, instead of these taxa representing infectious pathogens responsible for depressive features. This additionally ties in with what was observed in Study I, namely that studies could not agree upon which individual taxa were observed different between the two groups. We would argue that these alterations in bacteria are genuine differences between the two groups, despite the small sample size, as inter-individual variations far exceed intra-individual variations (426). However, we believe that these singular bacterial differences between patients with MDD and healthy controls are a manifestation of functional deficits arising from the gut microbiota as a whole, rather than diagnostically relevant biomarkers of MDD.

Patients were antidepressant treatment-naïve, both in pharmacological and cognitive therapy, prior to entry into the project and commenced antidepressant treatment after the delivery of baseline samples. Ultimately, patients received several types of antidepressants, primarily SSRIs; some received only one antidepressant; some switched to a different type; some had their initial treatment augmented with an additional drug; and some never received pharmaceutical treatment, but only cognitive therapy. Bacterial variations in patients with MDD due to the pharmacological antidepressant treatment can therefore only be speculated, as each class of antidepressants have specific antibacterial properties (247). During the twelve weeks, there were some alterations in bacterial taxa observed. In the twelve weeks follow-up, the family *Ruminococcaceae* was decreased in relative abundance compared to baseline. No previous study has included completely treatment-naïve patients, but some studies have attempted to evaluate gut microbiota changes during antidepressant treatment (427-429). One study in particular observed that increased relative abundance of the *Ruminococcus gnavus* group and decreased relative

abundance of *Coprococcus* was associated with treatment-resistance, and that remission correlated to the family *Ruminoccaceae*. The efficacy of treatment has likewise been associated with species of *Ruminococcus* in a preclinical study (315). This ties in well with the observations in our Study II and Study III, and can also explain why several of our patients did not achieve remission or responded well to medication. This suggests that these particular bacteria may be a proxy for treatment response.

While some of our results correlate well with previous observations, bacterial variations may have arisen in patients with MDD due to other factors than the antibacterial effect of the pharmacological antidepressant treatment. One of the somatic symptoms in MDD is altered appetite, most often resulting in decreased appetite and weight loss during their depressive episode (430). Antidepressants also have side effects, such as appetite alterations and subsequent weight gain (431). In our study, patients with MDD did not report substantial changes in neither appetite, caloric intake, or dietary preferences, but they may have been subject to self-report bias (432), as patients with MDD have previously been found to consume an unhealthier diet (433). Participants were asked to recall up to eight weeks of appetite and caloric intake. Combined with social desirability and recall bias, this may have compromised the validity of the self-reporting dietary intake (434). It is possible that patients with MDD may have changed their dietary habits during their antidepressant therapy. As dietary interventions have been observed to reduce depressive symptoms (435), the effect of antidepressants on the gut microbiota cannot be separated by the effect of potential dietary changes.

In the end, Study III did confirm bacterial alterations in patients with MDD as observed in previous publications. The clinical relevance is still difficult to determine. There are substantial disagreements between studies in which *specific* bacteria are associated with MDD, whereby it can be difficult to use these bacteria as biomarkers of depression. Likewise, it is therefore difficult to determine establish which bacteria are suitable targets for intervention. However, the more studies published, the more clear the pattern. It does appear as if the *Ruminococcaceae* family and the *Coprococcus* genus are involved in MDD and potentially can predict treatment response.

5.3.1 LIMITATIONS AND STRENGHTS

One of the major strengths of the clinical study is the design of the patient cohort. We limited the patient population greatly by choosing a stringent, homogenic group of young individuals. We excluded several comorbid disorders to reduce the impact of potential microbial ‘background noise’ generated by these diseases. We chose a narrow age group and had strict limitations on pharmacological treatments other than antidepressants and specific diets. These were all measures taken to ensure consistent patient and control populations and our study population is therefore one of the most homogenous group of patients with MDD whose gut microbiota has been

characterized when considering those included and screened in Study I. Here, most studies had very broad age groups (18 – 65 years) and in many cases did not exclude patients or controls with additional diseases.

Although, in such a study design, we will not capture the entire spectrum of MDD phenotypes, and our study may therefore have moderate external validity. As we age, the gut microbiota composition shifts, and some bacterial species increase while others decrease in relative abundance (436). Chen et al. exemplified this in patients with MDD, as they observed age-specific gut microbiota variations compared to healthy age-matched individuals (115). As the age group in our study is very narrow, the gut microbiota composition observed in Study III may therefore be a definitive representation of a 'depressed' microbiota only representing this specific age group. On the other hand, the prevalence of MDD is not higher amongst the elderly (437), so the narrow age group in our study population may not have limited the representativeness of the gut microbiota. One additional pitfall here may be that there is a risk that the MDD is misdiagnosed in a patient that actually has bipolar disorder but has not yet presented manic episodes (438). We however anticipated this risk and performed retrospective follow-up through the Danish Electronic Patient Journal at a minimum of one year after the initial diagnosis of MDD and found no new diagnosis of bipolar disorder.

One major limitation of selecting to conduct a study with such a narrow demographic also limits the possibility of recruiting large samples of participants within the timespan of our project. One of the biggest obstacles during the project was motivating participants to enter the study and ensuring retention in the project. The dropout rate before completion was 52% and the reasons reported were lack of productivity and motivation, which are recognized as symptoms of their underlying disorder (439, 440).

CHAPTER 6. CONCLUSIONS

Overall, the three studies combined have led to several important findings on the association between gut microbiota and MDD. We have broadened the knowledge of this field in new and exciting ways and laid the groundwork for additional work to explore how the gut microbiota as a whole, and the individual bacteria inhabiting our intestinal system, can be involved in the pathology of MDD.

In the systematic review, we found that, despite of the high heterogenicity between studies in the study design and methodological approach to gut microbiota characterization, it was possible to distinguish between patients with MDD and healthy individuals. Most of the studies were able to do so based on the respective α- and/or β-diversity of the two groups. Furthermore, several of the studies agreed upon specific bacterial changes such as an increase in relative abundance of the genera *Atopobium*, *Bifidobacterium* and *Eggerthella*, and a decrease in relative abundance of the genus *Faecalibacterium* in patients with MDD compared to healthy individuals.

In the animal study, we found in FRL rats that depressive-like behavior was higher in the FMT-MDD group compared to the FMT-Healthy group, but not to the CON-Auto or the CON-H2O group, indicating that the depressive phenotype of this animal model only functions in comparison between groups transplanted with human material. On the other hand, FSL rats receiving human donor material did not display behavior different from the CON-Auto or CON-H2O groups. In the FMT-MDD and FMT-Healthy FRL rats, we additionally observed bacterial taxa from the human donor material. Here, we found that, in the FMT-MDD animals, there was an increase in relative abundance in three genera belonging to the *Ruminococcaceae* family, and the genus *Lachnospira*, and a decrease in relative abundance in the genus *Coprococcus* compared to the FMT-Healthy animals.

In the clinical study, it was not possible to separate the healthy individuals from the patients with MDD prior to commencement of antidepressant treatment. Interestingly though, there were individual taxa significantly different between the two groups. At baseline, patients with MDD had an increase relative abundance of the genera *Intestinibacter*, *Ruminococcus gnavus* group and *Ruminococcus torques* group, and a decreased relative abundance of the genera *Bilophila*, *Coprobacter*, *Desulfovibrio* and *Lactobacillus* compared to healthy individuals. After twelve weeks of pharmacological and/or cognitive antidepressant treatment, patients with MDD had a decreased relative abundance of the family *Ruminococcaceae* and the genus *Clostridium sensu stricto* as compared to before antidepressant treatment.

In general, we aimed at establishing an association between the gut microbiota and MDD, and from the three studies conducted in this thesis, we can conclude that this correlation has been confirmed. The family *Ruminoccaceae* may be involved in response to pharmacological antidepressant treatment.

CHAPTER 7. PERSPECTIVES

During the analyses conducted in this thesis, it was clear that there are several ways to conduct such a study in the future, and several clinical settings in which the results can be applicable.

First, and most importantly of all, it is highly necessary to streamline the methods used to characterize the gut microbiota. As was mentioned in both the Methods and Discussion sections, the many variations in DNA purification, sequencing platforms, 16S rRNA hypervariable gene targets, as well as bioinformatical quality assessments and databases used to assign taxonomy, can influence the identity of the bacterial taxa observed in the samples. If we are to compare studies in the future and conduct meta-analyses which may provide researchers with the necessary bacterial targets for intervention studies, it is an absolute must that we as a scientific community agree upon a golden standard.

For preclinical studies, it is evident that there can be several pitfalls. The animal model used, and the pretreatment to eradicate or limit the inhabiting gut microbiota, can affect the inherent behavior of the animal. For example, as we observed in our study, successful colonization by donor material is possible without prior restriction of the intrinsic gut microbiota. Furthermore, the choice of animal model and strain must be considered carefully as different animal strains and models can lead to different behavioral outputs, making robust conclusions across publications difficult to make. Behavioral proxies for depression-like behavior vary within each animal model, so it is necessary to replicate studies to confirm whether findings are a biologically demonstrable phenomena or spurious false-positive findings. To promote translatability, future studies could therefore aim at developing humanized animal models that is suitable for behavioral analyses. Additionally, animal studies are indispensable in first line of research in exploring functional effects of the gut microbiota, which can be ethically impossible to conduct in humans. To be able to examine potential causal effects between the gut microbiota of patients with MDD and a depressive phenotype, future studies should also attempt to incorporate transcriptomics, proteomics and metabolomics in a network with genomic analyses to identify and explore potential medical targets. Therefore, future animal studies which aim at conducting FMT from human donors, especially in studies examining behavioral outputs.

In the clinical study, the narrow design of the study led to the difficulty in recruiting subjects. However, we believe that this is the proper way to restrict the amount of background noise arising in the observed gut microbiota from comorbid disorders, medication and/or age. In future studies, it is necessary though to characterize several age groups, as gut microbiota also changes with ages, and this study therefore potentially only can be characteristic of the age group we explored. It was also not possible to separate the effect of antidepressants and the potential dietary changes

which often follows antidepressant treatment, whereby future studies should include more detailed descriptions of diet, or even limit participants to a specific diet. If possible, future studies should also restrict the pharmacological treatment to one type of antidepressant only, so as to be able to build correlation between bacterial variations and pharmacological treatment. Lastly, it is of high importance that the diagnosis of MDD is valid, and not based on self-reported symptoms of MDD, to be able to define if there is an association between gut microbiota alterations and MDD.

Gut microbiota studies have explored during the last decade, and rightfully so – this is a field that has been underestimated in importance and influence. Based on the three studies, it is evident that there is an association between the gut microbiota and MDD. Therefore, characterization of the intestinal commensals should be developed further, should this method be an applicable model of MDD diagnosis in the future. This is evident from the obvious bacterial variations observed between the included studies in Study I, although we argue that these arise from ethnic, dietary and/or geographical variations. Therefore, for this market to have any future applicability, we must focus on determining if there exists population-specific gut microbiota, as was suggested in the study by Arumugam et al. (12).

The relevancy of gut microbiota research in MDD extends to interventions and treatments. Several probiotic studies have aimed at modifying the gut microbiota composition of patients with MDD, specifically using strains of *Lactobacillus* due to their SCFA-producing function, producing low-to-moderate antidepressant effects (441-444). On the other hand, several of the included studies in Study I found increased relative abundance of *Lactobacillus* in their patient cohorts, whereby this bacterial species may not be appropriate for this disorder.

A single case report found that an elderly woman with poor antidepressant treatment response received FMT from her six-year old, healthy grandchild, resulting in complete remission (445). This study provides an optimistic approach to how bacteriotherapy can be used in the future treatment of MDD, suggesting that the gut microbiota may be used for more than diagnostic and prognostic markers of MDD.

CHAPTER 8. REFERENCE LIST

1. Thursby E, Juge N. Introduction to the human gut microbiota. Biochem J. 2017;474(11):1823-36.
2. Marchesi JR, Ravel J. The vocabulary of microbiome research: a proposal. Microbiome. 2015;3:31.
3. Allaband C, McDonald D, Vazquez-Baeza Y, Minich JJ, Tripathi A, Brenner DA, et al. Microbiome 101: Studying, Analyzing, and Interpreting Gut Microbiome Data for Clinicians. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association. 2019;17(2):218-30.
4. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLoS Biol. 2016;14(8):e1002533.
5. Hillman ET, Lu H, Yao T, Nakatsu CH. Microbial Ecology along the Gastrointestinal Tract. Microbes Environ. 2017;32(4):300-13.
6. Tang Q, Jin G, Wang G, Liu T, Liu X, Wang B, et al. Current Sampling Methods for Gut Microbiota: A Call for More Precise Devices. Front Cell Infect Microbiol. 2020;10:151.
7. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. Science. 2005;308(5728):1635-8.
8. Human Microbiome Project C. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486(7402):207-14.
9. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59-65.
10. Almeida A, Mitchell AL, Boland M, Forster SC, Gloor GB, Tarkowska A, et al. A new genomic blueprint of the human gut microbiota. Nature. 2019;568(7753):499-504.
11. Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, et al. Human genetics shape the gut microbiome. Cell. 2014;159(4):789-99.
12. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. Nature. 2011;473(7346):174-80.
13. Huse SM, Ye Y, Zhou Y, Fodor AA. A core human microbiome as viewed through 16S rRNA sequence clusters. PLoS One. 2012;7(6):e34242.
14. Tap J, Mondot S, Levenez F, Pelletier E, Caron C, Furet JP, et al. Towards the human intestinal microbiota phylogenetic core. Environmental microbiology. 2009;11(10):2574-84.
15. Falony G, Joossens M, Vieira-Silva S, Wang J, Darzi Y, Faust K, et al. Population-level analysis of gut microbiome variation. Science. 2016;352(6285):560-4.
16. Aguirre de Carcer D. The human gut pan-microbiome presents a compositional core formed by discrete phylogenetic units. Sci Rep. 2018;8(1):14069.

17. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature. 2009;457(7228):480-4.
18. Murphy K, CA OS, Ryan CA, Dempsey EM, PW OT, Stanton C, et al. The gut microbiota composition in dichorionic triplet sets suggests a role for host genetic factors. PLoS One. 2015;10(4):e0122561.
19. Ferretti P, Pasolli E, Tett A, Asnicar F, Gorfer V, Fedi S, et al. Mother-to-Infant Microbial Transmission from Different Body Sites Shapes the Developing Infant Gut Microbiome. Cell Host Microbe. 2018;24(1):133-45 e5.
20. Perez-Munoz ME, Arrieta MC, Ramer-Tait AE, Walter J. A critical assessment of the "sterile womb" and "in utero colonization" hypotheses: implications for research on the pioneer infant microbiome. Microbiome. 2017;5(1):48.
21. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. Sci Transl Med. 2014;6(237):237ra65.
22. Steel JH, Malatos S, Kennea N, Edwards AD, Miles L, Duggan P, et al. Bacteria and inflammatory cells in fetal membranes do not always cause preterm labor. Pediatr Res. 2005;57(3):404-11.
23. Jimenez E, Fernandez L, Marin ML, Martin R, Odriozola JM, Nueno-Palop C, et al. Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. Curr Microbiol. 2005;51(4):270-4.
24. Olomu IN, Pena-Cortes LC, Long RA, Vyas A, Krichevskiy O, Luellwitz R, et al. Elimination of "kitome" and "splashome" contamination results in lack of detection of a unique placental microbiome. BMC Microbiol. 2020;20(1):157.
25. Hill CJ, Lynch DB, Murphy K, Ulaszewska M, Jeffery IB, O'Shea CA, et al. Evolution of gut microbiota composition from birth to 24 weeks in the INFANTMET Cohort. Microbiome. 2017;5(1):4.
26. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A. 2010;107(26):11971-5.
27. MacIntyre DA, Chandiramani M, Lee YS, Kindinger L, Smith A, Angelopoulos N, et al. The vaginal microbiome during pregnancy and the postpartum period in a European population. Sci Rep. 2015;5:8988.
28. Valles Y, Gosalbes MJ, de Vries LE, Abellan JJ, Francino MP. Metagenomics and development of the gut microbiota in infants. Clin Microbiol Infect. 2012;18 Suppl 4:21-6.
29. Valles Y, Artacho A, Pascual-Garcia A, Ferrus ML, Gosalbes MJ, Abellan JJ, et al. Microbial succession in the gut: directional trends of taxonomic and functional change in a birth cohort of Spanish infants. PLoS Genet. 2014;10(6):e1004406.
30. Marcobal A, Barboza M, Froehlich JW, Block DE, German JB, Lebrilla CB, et al. Consumption of human milk oligosaccharides by gut-related microbes. Journal of agricultural and food chemistry. 2010;58(9):5334-40.

31. Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. PLoS Biol. 2007;5(7):e177.
32. Fallani M, Amarri S, Uusijarvi A, Adam R, Khanna S, Aguilera M, et al. Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres. Microbiology (Reading). 2011;157(Pt 5):1385-92.
33. Backhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, et al. Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. Cell Host Microbe. 2015;17(6):852.
34. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature. 2012;486(7402):222-7.
35. Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL, et al. The long-term stability of the human gut microbiota. Science. 2013;341(6141):1237439.
36. Cremer J, Arnoldini M, Hwa T. Effect of water flow and chemical environment on microbiota growth and composition in the human colon. Proc Natl Acad Sci U S A. 2017;114(25):6438-43.
37. Cremer J, Segota I, Yang CY, Arnoldini M, Sauls JT, Zhang Z, et al. Effect of flow and peristaltic mixing on bacterial growth in a gut-like channel. Proc Natl Acad Sci U S A. 2016;113(41):11414-9.
38. Stephen AM, Cummings JH. The microbial contribution to human faecal mass. J Med Microbiol. 1980;13(1):45-56.
39. Duncan SH, Louis P, Thomson JM, Flint HJ. The role of pH in determining the species composition of the human colonic microbiota. Environmental microbiology. 2009;11(8):2112-22.
40. Saad RJ, Rao SS, Koch KL, Kuo B, Parkman HP, McCallum RW, et al. Do stool form and frequency correlate with whole-gut and colonic transit? Results from a multicenter study in constipated individuals and healthy controls. Am J Gastroenterol. 2010;105(2):403-11.
41. Vandeputte D, Falony G, Vieira-Silva S, Tito RY, Joossens M, Raes J. Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. Gut. 2016;65(1):57-62.
42. Albenberg L, Esipova TV, Judge CP, Bittinger K, Chen J, Laughlin A, et al. Correlation between intraluminal oxygen gradient and radial partitioning of intestinal microbiota. Gastroenterology. 2014;147(5):1055-63 e8.
43. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012;489(7415):220-30.
44. Vangay P, Johnson AJ, Ward TL, Al-Ghalith GA, Shields-Cutler RR, Hillmann BM, et al. US Immigration Westernizes the Human Gut Microbiome. Cell. 2018;175(4):962-72 e10.
45. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota revealed by a comparative

study in children from Europe and rural Africa. Proc Natl Acad Sci U S A. 2010;107(33):14691-6.
46. Schnorr SL, Candela M, Rampelli S, Centanni M, Consolandi C, Basaglia G, et al. Gut microbiome of the Hadza hunter-gatherers. Nat Commun. 2014;5:3654.
47. Khine WWT, Zhang Y, Goie GJY, Wong MS, Liong M, Lee YY, et al. Gut microbiome of pre-adolescent children of two ethnicities residing in three distant cities. Sci Rep. 2019;9(1):7831.
48. Clarke SF, Murphy EF, Nilaweera K, Ross PR, Shanahan F, O'Toole PW, et al. The gut microbiota and its relationship to diet and obesity: new insights. Gut Microbes. 2012;3(3):186-202.
49. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science. 2011;334(6052):105-8.
50. Maier L, Pruteanu M, Kuhn M, Zeller G, Telzerow A, Anderson EE, et al. Extensive impact of non-antibiotic drugs on human gut bacteria. Nature. 2018;555(7698):623-8.
51. Jernberg C, Lofmark S, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. The ISME journal. 2007;1(1):56-66.
52. Dethlefsen L, Huse S, Sogin ML, Relman DA. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol. 2008;6(11):e280.
53. Jakobsson HE, Jernberg C, Andersson AF, Sjolund-Karlsson M, Jansson JK, Engstrand L. Short-Term Antibiotic Treatment Has Differing Long-Term Impacts on the Human Throat and Gut Microbiome. Plos One. 2010;5(3).
54. Ng KM, Ferreyra JA, Higginbottom SK, Lynch JB, Kashyap PC, Gopinath S, et al. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. Nature. 2013;502(7469):96-9.
55. Ge X, Ding C, Zhao W, Xu L, Tian H, Gong J, et al. Antibiotics-induced depletion of mice microbiota induces changes in host serotonin biosynthesis and intestinal motility. J Transl Med. 2017;15(1):13.
56. Maurice CF, Haiser HJ, Turnbaugh PJ. Xenobiotics shape the physiology and gene expression of the active human gut microbiome. Cell. 2013;152(1-2):39-50.
57. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. 2006;124(4):837-48.
58. Tian L, Wang XW, Wu AK, Fan Y, Friedman J, Dahlin A, et al. Deciphering functional redundancy in the human microbiome. Nat Commun. 2020;11(1):6217.
59. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. Cell. 2014;157(1):121-41.

60. Bouskra D, Brezillon C, Berard M, Werts C, Varona R, Boneca IG, et al. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature. 2008;456(7221):507-10.
61. Cash HL, Whitham CV, Behrendt CL, Hooper LV. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science. 2006;313(5790):1126-30.
62. Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science. 2004;303(5664):1662-5.
63. Bollinger RR, Everett ML, Palestrant D, Love SD, Lin SS, Parker W. Human secretory immunoglobulin A may contribute to biofilm formation in the gut. Immunology. 2003;109(4):580-7.
64. Lotz M, Gutle D, Walther S, Menard S, Bogdan C, Hornef MW. Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. J Exp Med. 2006;203(4):973-84.
65. Kollmann TR, Levy O, Montgomery RR, Goriely S. Innate immune function by Toll-like receptors: distinct responses in newborns and the elderly. Immunity. 2012;37(5):771-83.
66. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell. 2005;122(1):107-18.
67. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A. 2008;105(43):16731-6.
68. Buffie CG, Pamer EG. Microbiota-mediated colonization resistance against intestinal pathogens. Nat Rev Immunol. 2013;13(11):790-801.
69. Lee SM, Donaldson GP, Mikulski Z, Boyajian S, Ley K, Mazmanian SK. Bacterial colonization factors control specificity and stability of the gut microbiota. Nature. 2013;501(7467):426-9.
70. Deriu E, Liu JZ, Pezeshki M, Edwards RA, Ochoa RJ, Contreras H, et al. Probiotic bacteria reduce salmonella typhimurium intestinal colonization by competing for iron. Cell Host Microbe. 2013;14(1):26-37.
71. Pacheco AR, Curtis MM, Ritchie JM, Munera D, Waldor MK, Moreira CG, et al. Fucose sensing regulates bacterial intestinal colonization. Nature. 2012;492(7427):113-7.
72. Abdelsalam NA, Ramadan AT, ElRakaiby MT, Aziz RK. Toxicomicrobiomics: The Human Microbiome vs. Pharmaceutical, Dietary, and Environmental Xenobiotics. Frontiers in pharmacology. 2020;11:390.
73. Sannasiddappa TH, Lund PA, Clarke SR. In Vitro Antibacterial Activity of Unconjugated and Conjugated Bile Salts on Staphylococcus aureus. Front Microbiol. 2017;8:1581.
74. Kang DJ, Ridlon JM, Moore DR, 2nd, Barnes S, Hylemon PB. Clostridium scindens baiCD and baiH genes encode stereo-specific 7alpha/7beta-

hydroxy-3-oxo-delta4-cholenoic acid oxidoreductases. Biochim Biophys Acta. 2008;1781(1-2):16-25.
75. Rea MC, Sit CS, Clayton E, O'Connor PM, Whittal RM, Zheng J, et al. Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against Clostridium difficile. Proc Natl Acad Sci U S A. 2010;107(20):9352-7.
76. Hasper HE, Kramer NE, Smith JL, Hillman JD, Zachariah C, Kuipers OP, et al. An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. Science. 2006;313(5793):1636-7.
77. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science. 2005;307(5717):1915-20.
78. Miller TL, Wolin MJ. Pathways of acetate, propionate, and butyrate formation by the human fecal microbial flora. Appl Environ Microbiol. 1996;62(5):1589-92.
79. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J Lipid Res. 2013;54(9):2325-40.
80. Boets E, Gomand SV, Deroover L, Preston T, Vermeulen K, De Preter V, et al. Systemic availability and metabolism of colonic-derived short-chain fatty acids in healthy subjects: a stable isotope study. J Physiol. 2017;595(2):541-55.
81. Rossi M, Corradini C, Amaretti A, Nicolini M, Pompei A, Zanoni S, et al. Fermentation of fructooligosaccharides and inulin by bifidobacteria: a comparative study of pure and fecal cultures. Appl Environ Microbiol. 2005;71(10):6150-8.
82. Willemsen LE, Koetsier MA, van Deventer SJ, van Tol EA. Short chain fatty acids stimulate epithelial mucin 2 expression through differential effects on prostaglandin E(1) and E(2) production by intestinal myofibroblasts. Gut. 2003;52(10):1442-7.
83. Peng L, Li ZR, Green RS, Holzman IR, Lin J. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. J Nutr. 2009;139(9):1619-25.
84. Vinolo MA, Rodrigues HG, Nachbar RT, Curi R. Regulation of inflammation by short chain fatty acids. Nutrients. 2011;3(10):858-76.
85. Gantois I, Ducatelle R, Pasmans F, Haesebrouck F, Hautefort I, Thompson A, et al. Butyrate specifically down-regulates salmonella pathogenicity island 1 gene expression. Appl Environ Microbiol. 2006;72(1):946-9.
86. Metges CC, El-Khoury AE, Henneman L, Petzke KJ, Grant I, Bedri S, et al. Availability of intestinal microbial lysine for whole body lysine homeostasis in human subjects. Am J Physiol. 1999;277(4):E597-607.
87. Strozzi GP, Mogna L. Quantification of folic acid in human feces after administration of Bifidobacterium probiotic strains. Journal of clinical gastroenterology. 2008;42 Suppl 3 Pt 2:S179-84.

88. Taranto MP, Vera JL, Hugenholtz J, De Valdez GF, Sesma F. Lactobacillus reuteri CRL1098 produces cobalamin. J Bacteriol. 2003;185(18):5643-7.
89. Hooks KB, O'Malley MA. Dysbiosis and Its Discontents. mBio. 2017;8(5).
90. Haenel H. Some Rules in Ecology of Intestinal Microflora of Man. Journal of Applied Bacteriology. 1961;24(3):242-&.
91. Lee YK, Mazmanian SK. Has the Microbiota Played a Critical Role in the Evolution of the Adaptive Immune System? Science. 2010;330(6012):1768-73.
92. Omenetti S, Pizarro TT. The Treg/Th17 Axis: A Dynamic Balance Regulated by the Gut Microbiome. Front Immunol. 2015;6:639.
93. Petersen C, Round JL. Defining dysbiosis and its influence on host immunity and disease. Cell Microbiol. 2014;16(7):1024-33.
94. Olesen SW, Alm EJ. Dysbiosis is not an answer. Nature Microbiology. 2016;1(12).
95. Sevelsted A, Stokholm J, Bonnelykke K, Bisgaard H. Cesarean section and chronic immune disorders. Pediatrics. 2015;135(1):e92-8.
96. Francino MP. Antibiotics and the Human Gut Microbiome: Dysbioses and Accumulation of Resistances. Front Microbiol. 2015;6:1543.
97. Lavebratt C, Yang LL, Giacobini M, Forsell Y, Schalling M, Partonen T, et al. Early exposure to antibiotic drugs and risk for psychiatric disorders: a population-based study. Translational psychiatry. 2019;9.
98. Horton DB, Scott FI, Haynes K, Putt ME, Rose CD, Lewis JD, et al. Antibiotic Exposure and Juvenile Idiopathic Arthritis: A Case-Control Study. Pediatrics. 2015;136(2):e333-43.
99. Zhang CH, Zhang MH, Wang SY, Han RJ, Cao YF, Hua WY, et al. Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. Isme Journal. 2010;4(2):232-41.
100. Singh RK, Chang HW, Yan D, Lee KM, Ucmak D, Wong K, et al. Influence of diet on the gut microbiome and implications for human health. J Transl Med. 2017;15(1):73.
101. Jantchou P, Morois S, Clavel-Chapelon F, Boutron-Ruault MC, Carbonnel F. Animal protein intake and risk of inflammatory bowel disease: The E3N prospective study. Am J Gastroenterol. 2010;105(10):2195-201.
102. Brown K, DeCoffe D, Molcan E, Gibson DL. Diet-induced dysbiosis of the intestinal microbiota and the effects on immunity and disease. Nutrients. 2012;4(8):1095-119.
103. Wilkins LJ, Monga M, Miller AW. Defining Dysbiosis for a Cluster of Chronic Diseases. Sci Rep. 2019;9(1):12918.
104. Naseribafrouei A, Hestad K, Avershina E, Sekelja M, Linlokken A, Wilson R, et al. Correlation between the human fecal microbiota and depression. Neurogastroenterol Motil. 2014;26(8):1155-62.

105. Jiang H, Ling Z, Zhang Y, Mao H, Ma Z, Yin Y, et al. Altered fecal microbiota composition in patients with major depressive disorder. Brain, Behavior, and Immunity. 2015;48:186-94.
106. Zheng P, Zeng B, Zhou C, Liu M, Fang Z, Xu X, et al. Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. Molecular Psychiatry. 2016;21(6):786-96.
107. Aizawa E, Tsuji H, Asahara T, Takahashi T, Teraishi T, Yoshida S, et al. Possible association of Bifidobacterium and Lactobacillus in the gut microbiota of patients with major depressive disorder. Journal of Affective Disorders. 2016;202:254-7.
108. Kelly JR, Borre Y, C OB, Patterson E, El Aidy S, Deane J, et al. Transferring the blues: Depression-associated gut microbiota induces neurobehavioural changes in the rat. J Psychiatr Res. 2016;82:109-18.
109. Lin P, Ding B, Feng C, Yin S, Zhang T, Qi X, et al. Prevotella and Klebsiella proportions in fecal microbial communities are potential characteristic parameters for patients with major depressive disorder. Journal of Affective Disorders. 2017;207:300-4.
110. Chen JJ, Zheng P, Liu YY, Zhong XG, Wang HY, Guo YJ, et al. Sex differences in gut microbiota in patients with major depressive disorder. Neuropsychiatr Dis Treat. 2018;14:647-55.
111. Rong H, Xie XH, Zhao J, Lai WT, Wang MB, Xu D, et al. Similarly in depression, nuances of gut microbiota: Evidences from a shotgun metagenomics sequencing study on major depressive disorder versus bipolar disorder with current major depressive episode patients. J Psychiatr Res. 2019;113:90-9.
112. Chung Y-CE, Chen H-C, Chou H-CL, Chen IM, Lee M-S, Chuang L-C, et al. Exploration of microbiota targets for major depressive disorder and mood related traits. Journal of Psychiatric Research. 2019;111:74-82.
113. Huang Y, Li Z, Shen Y, Shi X, Wang L, Li G, et al. Possible association of firmicutes in the gut microbiota of patients with major depressive disorder. Neuropsychiatric Disease and Treatment. 2018;14:3329-37.
114. Vinberg M, Ottesen NM, Meluken I, Sorensen N, Pedersen O, Kessing LV, et al. Remitted affective disorders and high familial risk of affective disorders associate with aberrant intestinal microbiota. Acta psychiatrica Scandinavica. 2019;139(2):174-84.
115. Chen JJ, He S, Fang L, Wang B, Bai SJ, Xie J, et al. Age-specific differential changes on gut microbiota composition in patients with major depressive disorder. Aging (Albany NY). 2020;12(3):2764-76.
116. Lai WT, Deng WF, Xu SX, Zhao J, Xu D, Liu YH, et al. Shotgun metagenomics reveals both taxonomic and tryptophan pathway differences of gut microbiota in major depressive disorder patients. Psychol Med. 2019:1-12.
117. Liu RT, Rowan-Nash AD, Sheehan AE, Walsh RFL, Sanzari CM, Korry BJ, et al. Reductions in anti-inflammatory gut bacteria are associated with depression in a sample of young adults. Brain Behav Immun. 2020;88:308-24.

118. Mason BL, Li Q, Minhajuddin A, Czysz AH, Coughlin LA, Hussain SK, et al. Reduced anti-inflammatory gut microbiota are associated with depression and anhedonia. J Affect Disord. 2020;266:394-401.
119. Stevens BR, Roesch L, Thiago P, Russell JT, Pepine CJ, Holbert RC, et al. Depression phenotype identified by using single nucleotide exact amplicon sequence variants of the human gut microbiome. Mol Psychiatry. 2020.
120. Zheng P, Yang J, Li Y, Wu J, Liang W, Yin B, et al. Gut Microbial Signatures Can Discriminate Unipolar from Bipolar Depression. Advanced science (Weinheim, Baden-Wurttemberg, Germany). 2020;7(7):1902862.
121. Valles-Colomer M, Falony G, Darzi Y, Tigchelaar EF, Wang J, Tito RY, et al. The neuroactive potential of the human gut microbiota in quality of life and depression. Nat Microbiol. 2019;4(4):623-32.
122. Zhu F, Ju Y, Wang W, Wang Q, Guo R, Ma Q, et al. Metagenome-wide association of gut microbiome features for schizophrenia. Nat Commun. 2020;11(1):1612.
123. Xu MY, Xu XF, Li JJ, Li F. Association Between Gut Microbiota and Autism Spectrum Disorder: A Systematic Review and Meta-Analysis. Frontiers in psychiatry. 2019;10.
124. Szopinska-Tokov J, Dam S, Naaijen J, Konstanti P, Rommelse N, Belzer C, et al. Investigating the Gut Microbiota Composition of Individuals with Attention-Deficit/Hyperactivity Disorder and Association with Symptoms. Microorganisms. 2020;8(3).
125. Wang LJ, Yang CY, Chou WJ, Lee MJ, Chou MC, Kuo HC, et al. Gut microbiota and dietary patterns in children with attention-deficit/hyperactivity disorder. European child & adolescent psychiatry. 2020;29(3):287-97.
126. Lu QQ, Lai JB, Lu HF, Ng C, Huang TT, Zhang H, et al. Gut Microbiota in Bipolar Depression and Its Relationship to Brain Function: An Advanced Exploration. Frontiers in psychiatry. 2019;10.
127. Evans SJ, Bassis CM, Hein R, Assari S, Flowers SA, Kelly MB, et al. The gut microbiome composition associates with bipolar disorder and illness severity. J Psychiatr Res. 2017;87:23-9.
128. Walker AW, Sanderson JD, Churcher C, Parkes GC, Hudspith BN, Rayment N, et al. High-throughput clone library analysis of the mucosa-associated microbiota reveals dysbiosis and differences between inflamed and non-inflamed regions of the intestine in inflammatory bowel disease. Bmc Microbiology. 2011;11.
129. Hedin CR, McCarthy NE, Louis P, Farquharson FM, McCartney S, Taylor K, et al. Altered intestinal microbiota and blood T cell phenotype are shared by patients with Crohn's disease and their unaffected siblings. Gut. 2014;63(10):1578-86.
130. Joossens M, Huys G, Cnockaert M, De Preter V, Verbeke K, Rutgeerts P, et al. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. Gut. 2011;60(5):631-7.

131. Zhang X, Zhang D, Jia H, Feng Q, Wang D, Liang D, et al. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. Nat Med. 2015;21(8):895-905.
132. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology - Human gut microbes associated with obesity. Nature. 2006;444(7122):1022-3.
133. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006;444(7122):1027-31.
134. Le Chatelier E, Nielsen T, Qin JJ, Prifti E, Hildebrand F, Falony G, et al. Richness of human gut microbiome correlates with metabolic markers. Nature. 2013;500(7464):541-+.
135. Zhang F, Wang M, Yang J, Xu Q, Liang C, Chen B, et al. Response of gut microbiota in type 2 diabetes to hypoglycemic agents. Endocrine. 2019;66(3):485-93.
136. Li T, Li H, Li W, Chen S, Feng T, Jiao W, et al. Interleukin-37 sensitize the elderly type 2 diabetic patients to insulin therapy through suppressing the gut microbiota dysbiosis. Mol Immunol. 2019;112:322-9.
137. de Goffau MC, Luopajarvi K, Knip M, Ilonen J, Ruohtula T, Harkonen T, et al. Fecal Microbiota Composition Differs Between Children With beta-Cell Autoimmunity and Those Without. Diabetes. 2013;62(4):1238-44.
138. Murri M, Leiva I, Gomez-Zumaquero JM, Tinahones FJ, Cardona F, Soriguer F, et al. Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. Bmc Medicine. 2013;11.
139. Tang WH, Kitai T, Hazen SL. Gut Microbiota in Cardiovascular Health and Disease. Circ Res. 2017;120(7):1183-96.
140. Jin M, Li J, Liu F, Lyu N, Wang K, Wang L, et al. Analysis of the Gut Microflora in Patients With Parkinson's Disease. Front Neurosci. 2019;13:1184.
141. Unger MM, Spiegel J, Dillmann KU, Grundmann D, Philippeit H, Burmann J, et al. Short chain fatty acids and gut microbiota differ between patients with Parkinson's disease and age-matched controls. Parkinsonism & Related Disorders. 2016;32:66-72.
142. Miyake S, Kim S, Suda W, Oshima K, Nakamura M, Matsuoka T, et al. Dysbiosis in the Gut Microbiota of Patients with Multiple Sclerosis, with a Striking Depletion of Species Belonging to Clostridia XIVa and IV Clusters. PLoS One. 2015;10(9):e0137429.
143. American Psychiatric Association., American Psychiatric Association. Task Force on DSM-IV. Diagnostic and statistical manual of mental disorders : DSM-IV. 4th ed. Washington, DC: American Psychiatric Association; 1994. xxvii, 886 p. p.
144. Bramer GR. International statistical classification of diseases and related health problems. Tenth revision. World Health Stat Q. 1988;41(1):32-6.
145. Rock PL, Roiser JP, Riedel WJ, Blackwell AD. Cognitive impairment in depression: a systematic review and meta-analysis. Psychol Med. 2014;44(10):2029-40.

146. Simon GE, VonKorff M, Piccinelli M, Fullerton C, Ormel J. An international study of the relation between somatic symptoms and depression. New Engl J Med. 1999;341(18):1329-35.
147. Fried EI, Nesse RM. Depression is not a consistent syndrome: An investigation of unique symptom patterns in the STAR*D study. J Affect Disord. 2015;172:96-102.
148. Kessler RC, Berglund P, Demler O, Jin R, Koretz D, Merikangas KR, et al. The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R). JAMA. 2003;289(23):3095-105.
149. Bromet E, Andrade LH, Hwang I, Sampson NA, Alonso J, de Girolamo G, et al. Cross-national epidemiology of DSM-IV major depressive episode. BMC Med. 2011;9:90.
150. Sullivan PF, Neale MC, Kendler KS. Genetic epidemiology of major depression: review and meta-analysis. Am J Psychiatry. 2000;157(10):1552-62.
151. Santini ZI, Jose PE, York Cornwell E, Koyanagi A, Nielsen L, Hinrichsen C, et al. Social disconnectedness, perceived isolation, and symptoms of depression and anxiety among older Americans (NSHAP): a longitudinal mediation analysis. Lancet Public Health. 2020;5(1):e62-e70.
152. Tomita A, Manuel JI. Evidence on the Association Between Cigarette Smoking and Incident Depression From the South African National Income Dynamics Study 2008-2015: Mental Health Implications for a Resource-Limited Setting. Nicotine Tob Res. 2020;22(1):118-23.
153. Schuch FB, Vancampfort D, Firth J, Rosenbaum S, Ward PB, Silva ES, et al. Physical Activity and Incident Depression: A Meta-Analysis of Prospective Cohort Studies. Am J Psychiatry. 2018;175(7):631-48.
154. Luppino FS, de Wit LM, Bouvy PF, Stijnen T, Cuijpers P, Penninx BW, et al. Overweight, obesity, and depression: a systematic review and meta-analysis of longitudinal studies. Arch Gen Psychiatry. 2010;67(3):220-9.
155. Jacka FN, Pasco JA, Mykletun A, Williams LJ, Hodge AM, O'Reilly SL, et al. Association of Western and traditional diets with depression and anxiety in women. Am J Psychiatry. 2010;167(3):305-11.
156. Lye MS, Tey YY, Tor YS, Shahabudin AF, Ibrahim N, Ling KH, et al. Predictors of recurrence of major depressive disorder. PLoS One. 2020;15(3):e0230363.
157. Vuorilehto M, Melartin T, Isometsa E. Depressive disorders in primary care: recurrent, chronic, and co-morbid. Psychol Med. 2005;35(5):673-82.
158. Warden D, Rush AJ, Trivedi MH, Fava M, Wisniewski SR. The STAR*D Project results: a comprehensive review of findings. Current psychiatry reports. 2007;9(6):449-59.
159. Weissman MM, Bland RC, Canino GJ, Faravelli C, Greenwald S, Hwu HG, et al. Cross-national epidemiology of major depression and bipolar disorder. JAMA. 1996;276(4):293-9.

160. Vestergaard SV, Rasmussen TB, Stallknecht S, Olsen J, Skipper N, Sorensen HT, et al. Occurrence, mortality and cost of brain disorders in Denmark: a population-based cohort study. BMJ open. 2020;10(11):e037564.
161. Olsen LR, Mortensen EL, Bech P. Prevalence of major depression and stress indicators in the Danish general population. Acta psychiatrica Scandinavica. 2004;109(2):96-103.
162. Gustavsson A, Svensson M, Jacobi F, Allgulander C, Alonso J, Beghi E, et al. Cost of disorders of the brain in Europe 2010. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology. 2011;21(10):718-79.
163. Murray CJ, Lopez AD. Evidence-based health policy--lessons from the Global Burden of Disease Study. Science. 1996;274(5288):740-3.
164. Brenes GA. Anxiety, depression, and quality of life in primary care patients. Prim Care Companion J Clin Psychiatry. 2007;9(6):437-43.
165. Murray CJ, Vos T, Lozano R, Naghavi M, Flaxman AD, Michaud C, et al. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet. 2012;380(9859):2197-223.
166. Moussavi S, Chatterji S, Verdes E, Tandon A, Patel V, Ustun B. Depression, chronic diseases, and decrements in health: results from the World Health Surveys. Lancet. 2007;370(9590):851-8.
167. Chisholm D, Sanderson K, Ayuso-Mateos JL, Saxena S. Reducing the global burden of depression: population-level analysis of intervention cost-effectiveness in 14 world regions. Br J Psychiatry. 2004;184:393-403.
168. Otte C, Gold SM, Penninx BW, Pariante CM, Etkin A, Fava M, et al. Major depressive disorder. Nature Reviews Disease Primers. 2016;2.
169. Wray NR, Ripke S, Mattheisen M, Trzaskowski M, Byrne EM, Abdellaoui A, et al. Genome-wide association analyses identify 44 risk variants and refine the genetic architecture of major depression. Nat Genet. 2018;50(5):668-81.
170. Abkevich V, Camp NJ, Hensel CH, Neff CD, Russell DL, Hughes DC, et al. Predisposition locus for major depression at chromosome 12q22-12q23.2. Am J Hum Genet. 2003;73(6):1271-81.
171. Kendler KS, Karkowski LM, Prescott CA. Causal relationship between stressful life events and the onset of major depression. American Journal of Psychiatry. 1999;156(6):837-41.
172. Risch N, Herrell R, Lehner T, Liang KY, Eaves L, Hoh J, et al. Interaction Between the Serotonin Transporter Gene (5-HTTLPR), Stressful Life Events, and Risk of Depression A Meta-analysis. Jama-J Am Med Assoc. 2009;301(23):2462-71.
173. Border R, Johnson EC, Evans LM, Smolen A, Berley N, Sullivan PF, et al. No Support for Historical Candidate Gene or Candidate Gene-by-Interaction Hypotheses for Major Depression Across Multiple Large Samples. American Journal of Psychiatry. 2019;176(5):376-87.

174. Williams LM. Precision psychiatry: a neural circuit taxonomy for depression and anxiety. Lancet Psychiat. 2016;3(5):472-80.
175. Nutt DJ. Relationship of neurotransmitters to the symptoms of major depressive disorder. J Clin Psychiatry. 2008;69 Suppl E1:4-7.
176. Schildkraut JJ. The catecholamine hypothesis of affective disorders: a review of supporting evidence. Am J Psychiatry. 1965;122(5):509-22.
177. Coppen A. The biochemistry of affective disorders. Br J Psychiatry. 1967;113(504):1237-64.
178. Reivich M, Amsterdam JD, Brunswick DJ, Shiue CY. PET brain imaging with [C-11](+)McN5652 shows increased serotonin transporter availability in major depression. Journal of Affective Disorders. 2004;82(2):321-7.
179. Drevets WC, Frank E, Price JC, Kupfer DJ, Holt D, Greer PJ, et al. PET imaging of serotonin 1A receptor binding in depression. Biol Psychiatry. 1999;46(10):1375-87.
180. Ordway GA, Schenk J, Stockmeier CA, May W, Klimek V. Elevated agonist binding to alpha2-adrenoceptors in the locus coeruleus in major depression. Biol Psychiatry. 2003;53(4):315-23.
181. Klimek V, Stockmeier C, Overholser J, Meltzer HY, Kalka S, Dilley G, et al. Reduced levels of norepinephrine transporters in the locus coeruleus in major depression. J Neurosci. 1997;17(21):8451-8.
182. Der-Avakian A, Markou A. The neurobiology of anhedonia and other reward-related deficits. Trends Neurosci. 2012;35(1):68-77.
183. Forbes EE, Hariri AR, Martin SL, Silk JS, Moyles DL, Fisher PM, et al. Altered striatal activation predicting real-world positive affect in adolescent major depressive disorder. Am J Psychiatry. 2009;166(1):64-73.
184. Meyer JH, Kruger S, Wilson AK, Christensen BK, Goulding VS, Schaffer A, et al. Lower dopamine transporter binding potential in striatum during depression. Neuroreport. 2001;12(18):4121-5.
185. Gordon I, Weizman R, Rehavi M. Modulatory effect of agents active in the presynaptic dopaminergic system on the striatal dopamine transporter. Eur J Pharmacol. 1996;298(1):27-30.
186. Tozuka Y, Fukuda S, Namba T, Seki T, Hisatsune T. GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. Neuron. 2005;47(6):803-15.
187. Ge S, Goh EL, Sailor KA, Kitabatake Y, Ming GL, Song H. GABA regulates synaptic integration of newly generated neurons in the adult brain. Nature. 2006;439(7076):589-93.
188. Earnheart JC, Schweizer C, Crestani F, Iwasato T, Itohara S, Mohler H, et al. GABAergic control of adult hippocampal neurogenesis in relation to behavior indicative of trait anxiety and depression states. J Neurosci. 2007;27(14):3845-54.
189. Rajkowska G, O'Dwyer G, Teleki Z, Stockmeier CA, Miguel-Hidalgo JJ. GABAergic neurons immunoreactive for calcium binding proteins are reduced in the prefrontal cortex in major depression. Neuropsychopharmacology. 2007;32(2):471-82.

190. Sanacora G, Gueorguieva R, Epperson CN, Wu YT, Appel M, Rothman DL, et al. Subtype-specific alterations of gamma-aminobutyric acid and glutamate in patients with major depression. Arch Gen Psychiatry. 2004;61(7):705-13.
191. Sanacora G, Treccani G, Popoli M. Towards a glutamate hypothesis of depression: an emerging frontier of neuropsychopharmacology for mood disorders. Neuropharmacology. 2012;62(1):63-77.
192. Pessoa L. On the relationship between emotion and cognition. Nature Reviews Neuroscience. 2008;9(2):148-58.
193. Koolschijn PC, van Haren NE, Lensvelt-Mulders GJ, Hulshoff Pol HE, Kahn RS. Brain volume abnormalities in major depressive disorder: a meta-analysis of magnetic resonance imaging studies. Hum Brain Mapp. 2009;30(11):3719-35.
194. Sequeira A, Mamdani F, Ernst C, Vawter MP, Bunney WE, Lebel V, et al. Global Brain Gene Expression Analysis Links Glutamatergic and GABAergic Alterations to Suicide and Major Depression. Plos One. 2009;4(8).
195. Bernard R, Kerman IA, Thompson RC, Jones EG, Bunney WE, Barchas JD, et al. Altered expression of glutamate signaling, growth factor, and glia genes in the locus coeruleus of patients with major depression. Mol Psychiatry. 2011;16(6):634-46.
196. Choudary PV, Molnar M, Evans SJ, Tomita H, Li JZ, Vawter MP, et al. Altered cortical glutamatergic and GABAergic signal transmission with glial involvement in depression. Proc Natl Acad Sci U S A. 2005;102(43):15653-8.
197. Rajkowska G, Miguel-Hidalgo JJ, Wei JR, Dilley G, Pittman SD, Meltzer HY, et al. Morphometric evidence for neuronal and glial prefrontal cell pathology in major depression. Biological Psychiatry. 1999;45(9):1085-98.
198. Jiang J, Zhao YJ, Hu XY, Du MY, Chen ZQ, Wu M, et al. Microstructural brain abnormalities in medication-free patients with major depressive disorder: a systematic review and meta-analysis of diffusion tensor imaging. J Psychiatry Neurosci. 2017;42(3):150-63.
199. Drevets WC, Videen TO, Price JL, Preskorn SH, Carmichael ST, Raichle ME. A functional anatomical study of unipolar depression. J Neurosci. 1992;12(9):3628-41.
200. Ongur D, Drevets WC, Price JL. Glial reduction in the subgenual prefrontal cortex in mood disorders. Proc Natl Acad Sci U S A. 1998;95(22):13290-5.
201. Wise T, Radua J, Via E, Cardoner N, Abe O, Adams TM, et al. Common and distinct patterns of grey-matter volume alteration in major depression and bipolar disorder: evidence from voxel-based meta-analysis. Mol Psychiatry. 2017;22(10):1455-63.
202. Grieve SM, Korgaonkar MS, Koslow SH, Gordon E, Williams LM. Widespread reductions in gray matter volume in depression. Neuroimage Clin. 2013;3:332-9.

203. Hastings RS, Parsey RV, Oquendo MA, Arango V, Mann JJ. Volumetric analysis of the prefrontal cortex, amygdala, and hippocampus in major depression. Neuropsychopharmacology. 2004;29(5):952-9.
204. Yuan P, Raz N. Prefrontal cortex and executive functions in healthy adults: a meta-analysis of structural neuroimaging studies. Neurosci Biobehav Rev. 2014;42:180-92.
205. Lindquist KA, Wager TD, Kober H, Bliss-Moreau E, Barrett LF. The brain basis of emotion: A meta-analytic review. Behavioral and Brain Sciences. 2012;35(3):121-43.
206. Karege F, Vaudan G, Schwald M, Perroud N, La Harpe R. Neurotrophin levels in postmortem brains of suicide victims and the effects of antemortem diagnosis and psychotropic drugs. Brain Res Mol Brain Res. 2005;136(1-2):29-37.
207. Nifosi F, Toffanin T, Follador H, Zonta F, Padovan G, Pigato G, et al. Reduced right posterior hippocampal volume in women with recurrent familial pure depressive disorder. Psychiatry Res. 2010;184(1):23-8.
208. Bus BA, Molendijk ML, Penninx BW, Buitelaar JK, Prickaerts J, Elzinga BM, et al. Low serum BDNF levels in depressed patients cannot be attributed to individual depressive symptoms or symptom cluster. World J Biol Psychiatry. 2014;15(7):561-9.
209. Burke HM, Davis MC, Otte C, Mohr DC. Depression and cortisol responses to psychological stress: a meta-analysis. Psychoneuroendocrinology. 2005;30(9):846-56.
210. Kennis M, Gerritsen L, van Dalen M, Williams A, Cuijpers P, Bockting C. Prospective biomarkers of major depressive disorder: a systematic review and meta-analysis. Mol Psychiatry. 2020;25(2):321-38.
211. Rubin RT, Phillips JJ, McCracken JT, Sadow TF. Adrenal gland volume in major depression: relationship to basal and stimulated pituitary-adrenal cortical axis function. Biol Psychiatry. 1996;40(2):89-97.
212. Amsterdam JD, Winokur A, Abelman E, Lucki I, Rickels K. Cosyntropin (ACTH alpha 1-24) stimulation test in depressed patients and healthy subjects. Am J Psychiatry. 1983;140(7):907-9.
213. Young EA, Watson SJ, Kotun J, Haskett RF, Grunhaus L, Murphy-Weinberg V, et al. Beta-lipotropin-beta-endorphin response to low-dose ovine corticotropin releasing factor in endogenous depression. Preliminary studies. Arch Gen Psychiatry. 1990;47(5):449-57.
214. Mello AF, Mello MF, Carpenter LL, Price LH. Update on stress and depression: the role of the hypothalamic-pituitary-adrenal (HPA) axis. Braz J Psychiatry. 2003;25(4):231-8.
215. Cullinan WE, Herman JP, Watson SJ. Ventral subicular interaction with the hypothalamic paraventricular nucleus: evidence for a relay in the bed nucleus of the stria terminalis. J Comp Neurol. 1993;332(1):1-20.
216. Hu W, Zhang M, Czeh B, Flugge G, Zhang W. Stress impairs GABAergic network function in the hippocampus by activating nongenomic

glucocorticoid receptors and affecting the integrity of the parvalbumin-expressing neuronal network. Neuropsychopharmacology. 2010;35(8):1693-707.
217. Coutinho AE, Chapman KE. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Mol Cell Endocrinol. 2011;335(1):2-13.
218. Tian R, Hou G, Li D, Yuan TF. A possible change process of inflammatory cytokines in the prolonged chronic stress and its ultimate implications for health. ScientificWorldJournal. 2014;2014:780616.
219. Miller AH, Raison CL. The role of inflammation in depression: from evolutionary imperative to modern treatment target. Nat Rev Immunol. 2016;16(1):22-34.
220. Miller GE, Cohen S, Ritchey AK. Chronic psychological stress and the regulation of pro-inflammatory cytokines: a glucocorticoid-resistance model. Health Psychol. 2002;21(6):531-41.
221. Moieni M, Irwin MR, Jevtic I, Olmstead R, Breen EC, Eisenberger NI. Sex differences in depressive and socioemotional responses to an inflammatory challenge: implications for sex differences in depression. Neuropsychopharmacology. 2015;40(7):1709-16.
222. Salk RH, Hyde JS, Abramson LY. Gender differences in depression in representative national samples: Meta-analyses of diagnoses and symptoms. Psychol Bull. 2017;143(8):783-822.
223. Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, et al. A meta-analysis of cytokines in major depression. Biol Psychiatry. 2010;67(5):446-57.
224. Osimo EF, Pillinger T, Rodriguez IM, Khandaker GM, Pariante CM, Howes OD. Inflammatory markers in depression: A meta-analysis of mean differences and variability in 5,166 patients and 5,083 controls. Brain Behav Immun. 2020;87:901-9.
225. Zou W, Feng R, Yang Y. Changes in the serum levels of inflammatory cytokines in antidepressant drug-naive patients with major depression. PLoS One. 2018;13(6):e0197267.
226. Dunn AJ, Wang J, Ando T. Effects of cytokines on cerebral neurotransmission. Comparison with the effects of stress. Advances in experimental medicine and biology. 1999;461:117-27.
227. Lu T, Pan Y, Kao SY, Li C, Kohane I, Chan J, et al. Gene regulation and DNA damage in the ageing human brain. Nature. 2004;429(6994):883-91.
228. Frank E, Prien RF, Jarrett RB, Keller MB, Kupfer DJ, Lavori PW, et al. Conceptualization and rationale for consensus definitions of terms in major depressive disorder. Remission, recovery, relapse, and recurrence. Arch Gen Psychiatry. 1991;48(9):851-5.
229. Sinyor M, Schaffer A, Levitt A. The Sequenced Treatment Alternatives to Relieve Depression (STAR*D) Trial: A Review. Can J Psychiat. 2010;55(3):126-35.

230. Whiteford HA, Harris MG, McKeon G, Baxter A, Pennell C, Barendregt JJ, et al. Estimating remission from untreated major depression: a systematic review and meta-analysis. Psychol Med. 2013;43(8):1569-85.
231. Burcusa SL, Iacono WG. Risk for recurrence in depression. Clin Psychol Rev. 2007;27(8):959-85.
232. DeRubeis RJ, Hollon SD, Amsterdam JD, Shelton RC, Young PR, Salomon RM, et al. Cognitive therapy vs medications in the treatment of moderate to severe depression. Arch Gen Psychiatry. 2005;62(4):409-16.
233. DeRubeis RJ, Gelfand LA, Tang TZ, Simons AD. Medications versus cognitive behavior therapy for severely depressed outpatients: mega-analysis of four randomized comparisons. Am J Psychiatry. 1999;156(7):1007-13.
234. Cipriani A, Furukawa TA, Salanti G, Chaimani A, Atkinson LZ, Ogawa Y, et al. Comparative efficacy and acceptability of 21 antidepressant drugs for the acute treatment of adults with major depressive disorder: a systematic review and network meta-analysis. Lancet. 2018;391(10128):1357-66.
235. Marchesi C, De Panfilis C, Tonna M, Ossola P. Is placebo useful in the treatment of major depression in clinical practice? Neuropsychiatric Disease and Treatment. 2013;9:915-20.
236. Hyman SE, Nestler EJ. Initiation and adaptation: a paradigm for understanding psychotropic drug action. Am J Psychiatry. 1996;153(2):151-62.
237. Racagni G, Popoli M. Cellular and molecular mechanisms in the long-term action of antidepressants. Dialogues Clin Neurosci. 2008;10(4):385-400.
238. Horstmann S, Lucae S, Menke A, Hennings JM, Ising M, Roeske D, et al. Polymorphisms in GRIK4, HTR2A, and FKBP5 Show Interactive Effects in Predicting Remission to Antidepressant Treatment. Neuropsychopharmacology. 2010;35(3):727-40.
239. Finberg JP, Rabey JM. Inhibitors of MAO-A and MAO-B in Psychiatry and Neurology. Frontiers in pharmacology. 2016;7:340.
240. Gillman PK. Tricyclic antidepressant pharmacology and therapeutic drug interactions updated. Br J Pharmacol. 2007;151(6):737-48.
241. Anderson IM. SSRIS versus tricyclic antidepressants in depressed inpatients: a meta-analysis of efficacy and tolerability. Depress Anxiety. 1998;7 Suppl 1:11-7.
242. Drevets WC, Bogers W, Raichle ME. Functional anatomical correlates of antidepressant drug treatment assessed using PET measures of regional glucose metabolism. European Neuropsychopharmacology. 2002;12(6):527-44.
243. Boldrini M, Underwood MD, Hen R, Rosoklija GB, Dwork AJ, John Mann J, et al. Antidepressants increase neural progenitor cells in the human hippocampus. Neuropsychopharmacology. 2009;34(11):2376-89.
244. Chen B, Dowlatshahi D, MacQueen GM, Wang JF, Young LT. Increased hippocampal BDNF immunoreactivity in subjects treated with antidepressant medication. Biol Psychiatry. 2001;50(4):260-5.

245. Chen CH, Suckling J, Ooi C, Fu CH, Williams SCR, Walsh ND, et al. Functional coupling of the amygdala in depressed patients treated with antidepressant medication. Neuropsychopharmacology. 2008;33(8):1909-18.
246. Svenningsson P, Chergui K, Rachleff I, Flajolet M, Zhang X, El Yacoubi M, et al. Alterations in 5-HT1B receptor function by p11 in depression-like states. Science. 2006;311(5757):77-80.
247. Ait Chait Y, Mottawea W, Tompkins TA, Hammami R. Unravelling the antimicrobial action of antidepressants on gut commensal microbes. Sci Rep. 2020;10(1):17878.
248. Lee SY, Ryu HS, Choi SC, Jang SH. A Study of Psychological Factors Associated with Functional Gastrointestinal Disorders and Use of Health Care. Clinical Psychopharmacology and Neuroscience. 2020;18(4):580-6.
249. Mayey EA, Craske M, Naliboff BD. Depression, anxiety, and the gastrointestinal system. Journal of Clinical Psychiatry. 2001;62:28-37.
250. Huang J, Cai Y, Su Y, Zhang M, Shi Y, Zhu N, et al. Gastrointestinal Symptoms During Depressive Episodes in 3256 Patients with Major Depressive Disorders: Findings from the NSSD. J Affect Disord. 2021;286:27-32.
251. Overstreet DH. The Flinders sensitive line rats: a genetic animal model of depression. Neurosci Biobehav Rev. 1993;17(1):51-68.
252. Overstreet DH, Friedman E, Mathe AA, Yadid G. The Flinders Sensitive Line rat: a selectively bred putative animal model of depression. Neurosci Biobehav Rev. 2005;29(4-5):739-59.
253. Overstreet DH, Wegener G. The flinders sensitive line rat model of depression--25 years and still producing. Pharmacol Rev. 2013;65(1):143-55.
254. Tillmann S, Abildgaard A, Winther G, Wegener G. Altered fecal microbiota composition in the Flinders sensitive line rat model of depression. Psychopharmacology. 2019;236(5):1445-57.
255. Sun L, Zhang H, Cao Y, Wang C, Zhao C, Wang H, et al. Fluoxetine ameliorates dysbiosis in a depression model induced by chronic unpredicted mild stress in mice. Int J Med Sci. 2019;16(9):1260-70.
256. Li H, Wang P, Huang L, Li P, Zhang D. Effects of regulating gut microbiota on the serotonin metabolism in the chronic unpredictable mild stress rat model. Neurogastroenterol Motil. 2019;31(10):e13677.
257. Jianguo L, Xueyang J, Cui W, Changxin W, Xuemei Q. Altered gut metabolome contributes to depression-like behaviors in rats exposed to chronic unpredictable mild stress. Translational psychiatry. 2019;9(1):40.
258. Park AJ, Collins J, Blennerhassett PA, Ghia JE, Verdu EF, Bercik P, et al. Altered colonic function and microbiota profile in a mouse model of chronic depression. Neurogastroenterol Motil. 2013;25(9):733-e575.
259. O'Mahony SM, Marchesi JR, Scully P, Codling C, Ceolho AM, Quigley EM, et al. Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. Biol Psychiatry. 2009;65(3):263-7.

260. Aoki-Yoshida A, Aoki R, Moriya N, Goto T, Kubota Y, Toyoda A, et al. Omics Studies of the Murine Intestinal Ecosystem Exposed to Subchronic and Mild Social Defeat Stress. Journal of proteome research. 2016;15(9):3126-38.
261. Yang C, Fujita Y, Ren Q, Ma M, Dong C, Hashimoto K. Bifidobacterium in the gut microbiota confer resilience to chronic social defeat stress in mice. Sci Rep. 2017;7:45942.
262. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, et al. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J Physiol. 2004;558(Pt 1):263-75.
263. Lukic I, Getselter D, Koren O, Elliott E. Role of Tryptophan in Microbiota-Induced Depressive-Like Behavior: Evidence From Tryptophan Depletion Study. Frontiers in behavioral neuroscience. 2019;13:123.
264. Hassan AM, Mancano G, Kashofer K, Frohlich EE, Matak A, Mayerhofer R, et al. High-fat diet induces depression-like behaviour in mice associated with changes in microbiome, neuropeptide Y, and brain metabolome. Nutritional neuroscience. 2019;22(12):877-93.
265. Hoban AE, Moloney RD, Golubeva AV, McVey Neufeld KA, O'Sullivan O, Patterson E, et al. Behavioural and neurochemical consequences of chronic gut microbiota depletion during adulthood in the rat. Neuroscience. 2016;339:463-77.
266. Li N, Wang Q, Wang Y, Sun A, Lin Y, Jin Y, et al. Fecal microbiota transplantation from chronic unpredictable mild stress mice donors affects anxiety-like and depression-like behavior in recipient mice via the gut microbiota-inflammation-brain axis. Stress (Amsterdam, Netherlands). 2019;22(5):592-602.
267. Luo Y, Zeng B, Zeng L, Du X, Li B, Huo R, et al. Gut microbiota regulates mouse behaviors through glucocorticoid receptor pathway genes in the hippocampus. Translational psychiatry. 2018;8(1):187.
268. Liu S, Guo R, Liu F, Yuan Q, Yu Y, Ren F. Gut Microbiota Regulates Depression-Like Behavior in Rats Through the Neuroendocrine-Immune-Mitochondrial Pathway. Neuropsychiatr Dis Treat. 2020;16:859-69.
269. Camilleri M. Leaky gut: mechanisms, measurement and clinical implications in humans. Gut. 2019;68(8):1516-26.
270. Gonzalez-Mariscal L, Betanzos A, Nava P, Jaramillo BE. Tight junction proteins. Prog Biophys Mol Biol. 2003;81(1):1-44.
271. Ulluwishewa D, Anderson RC, McNabb WC, Moughan PJ, Wells JM, Roy NC. Regulation of tight junction permeability by intestinal bacteria and dietary components. J Nutr. 2011;141(5):769-76.
272. Arrieta MC, Bistritz L, Meddings JB. Alterations in intestinal permeability. Gut. 2006;55(10):1512-20.
273. Shen L, Weber CR, Raleigh DR, Yu D, Tumer JR. Tight, Junction Pore and Leak Pathways: A Dynamic Duo. Annu Rev Physiol. 2011;73:283-309.
274. Zheng G, Victor Fon G, Meixner W, Creekmore A, Zong Y, M KD, et al. Chronic stress and intestinal barrier dysfunction: Glucocorticoid receptor and

transcription repressor HES1 regulate tight junction protein Claudin-1 promoter. Sci Rep. 2017;7(1):4502.
275. Demaude J, Salvador-Cartier C, Fioramonti J, Ferrier L, Bueno L. Phenotypic changes in colonocytes following acute stress or activation of mast cells in mice: implications for delayed epithelial barrier dysfunction. Gut. 2006;55(5):655-61.
276. Shen W, Wolf PG, Carbonero F, Zhong W, Reid T, Gaskins HR, et al. Intestinal and Systemic Inflammatory Responses Are Positively Associated with Sulfidogenic Bacteria Abundance in High-Fat-Fed Male C57BL/6J Mice. Journal of Nutrition. 2014;144(8):1181-7.
277. Martin-Hernandez D, Caso JR, Bris AG, Maus SR, Madrigal JL, Garcia-Bueno B, et al. Bacterial translocation affects intracellular neuroinflammatory pathways in a depression-like model in rats. Neuropharmacology. 2016;103:122-33.
278. Wei L, Li Y, Tang W, Sun Q, Chen L, Wang X, et al. Chronic Unpredictable Mild Stress in Rats Induces Colonic Inflammation. Frontiers in physiology. 2019;10:1228.
279. Geng S, Yang L, Cheng F, Zhang Z, Li J, Liu W, et al. Gut Microbiota Are Associated With Psychological Stress-Induced Defections in Intestinal and Blood-Brain Barriers. Front Microbiol. 2019;10:3067.
280. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell. 2013;155(7):1451-63.
281. Feng Y, Huang Y, Wang Y, Wang P, Song H, Wang F. Antibiotics induced intestinal tight junction barrier dysfunction is associated with microbiota dysbiosis, activated NLRP3 inflammasome and autophagy. PLoS One. 2019;14(6):e0218384.
282. Ohlsson L, Gustafsson A, Lavant E, Suneson K, Brundin L, Westrin A, et al. Leaky gut biomarkers in depression and suicidal behavior. Acta psychiatrica Scandinavica. 2019;139(2):185-93.
283. Stevens BR, Goel R, Seungbum K, Richards EM, Holbert RC, Pepine CJ, et al. Increased human intestinal barrier permeability plasma biomarkers zonulin and FABP2 correlated with plasma LPS and altered gut microbiome in anxiety or depression. Gut. 2017.
284. Skonieczna-zydecka K, Grochans E, Maciejewska D, Szkup M, Schneider-Matyka D, Jurczak A, et al. Faecal short chain fatty acids profile is changed in Polish depressive women. Nutrients. 2018;10(12):1939.
285. Luissint AC, Parkos CA, Nusrat A. Inflammation and the Intestinal Barrier: Leukocyte-Epithelial Cell Interactions, Cell Junction Remodeling, and Mucosal Repair. Gastroenterology. 2016;151(4):616-32.
286. Kawai T, Akira S. The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immunol. 2009;21(4):317-37.
287. Zhang Y, Liu L, Peng YL, Liu YZ, Wu TY, Shen XL, et al. Involvement of inflammasome activation in lipopolysaccharide-induced mice depressive-like behaviors. CNS neuroscience & therapeutics. 2014;20(2):119-24.

288. O'Connor JC, Lawson MA, Andre C, Moreau M, Lestage J, Castanon N, et al. Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice. Mol Psychiatry. 2009;14(5):511-22.
289. Wong ML, Inserra A, Lewis MD, Mastronardi CA, Leong L, Choo J, et al. Inflammasome signaling affects anxiety- and depressive-like behavior and gut microbiome composition. Mol Psychiatry. 2016;21(6):797-805.
290. Yan X, Zeng D, Zhu H, Zhang Y, Shi Y, Wu Y, et al. MiRNA-532-5p Regulates CUMS-Induced Depression-Like Behaviors and Modulates LPS-Induced Proinflammatory Cytokine Signaling by Targeting STAT3. Neuropsychiatr Dis Treat. 2020;16:2753-64.
291. Grippo AJ, Francis J, Beltz TG, Felder RB, Johnson AK. Neuroendocrine and cytokine profile of chronic mild stress-induced anhedonia. Physiology & behavior. 2005;84(5):697-706.
292. Hodes GE, Pfau ML, Leboeuf M, Golden SA, Christoffel DJ, Bregman D, et al. Individual differences in the peripheral immune system promote resilience versus susceptibility to social stress. Proc Natl Acad Sci U S A. 2014;111(45):16136-41.
293. Keri S, Szabo C, Kelemen O. Expression of Toll-Like Receptors in peripheral blood mononuclear cells and response to cognitive-behavioral therapy in major depressive disorder. Brain Behavior and Immunity. 2014;40:235-43.
294. Lin P, Ding B, Wu Y, Dong K, Li Q. Mitogen-stimulated cell proliferation and cytokine production in major depressive disorder patients. BMC Psychiatry. 2018;18(1):330.
295. Maes M, Kubera M, Leunis JC, Berk M. Increased IgA and IgM responses against gut commensals in chronic depression: further evidence for increased bacterial translocation or leaky gut. J Affect Disord. 2012;141(1):55-62.
296. Asarat M, Apostolopoulos V, Vasiljevic T, Donkor O. Short-Chain Fatty Acids Regulate Cytokines and Th17/Treg Cells in Human Peripheral Blood Mononuclear Cells in vitro. Immunol Invest. 2016;45(3):205-22.
297. Cox MA, Jackson J, Stanton M, Rojas-Triana A, Bober L, Laverty M, et al. Short-chain fatty acids act as antiinflammatory mediators by regulating prostaglandin E(2) and cytokines. World J Gastroenterol. 2009;15(44):5549-57.
298. Carabotti M, Scirocco A, Maselli MA, Severi C. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. Ann Gastroenterol. 2015;28(2):203-9.
299. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(38):16050-5.
300. Lal S, Kirkup AJ, Brunsden AM, Thompson DG, Grundy D. Vagal afferent responses to fatty acids of different chain length in the rat. Am J Physiol-Gastr L. 2001;281(4):G907-G15.

301. Strandwitz P. Neurotransmitter modulation by the gut microbiota. Brain research. 2018;1693(Pt B):128-33.
302. Janik R, Thomason LA, Stanisz AM, Forsythe P, Bienenstock J, Stanisz GJ. Magnetic resonance spectroscopy reveals oral Lactobacillus promotion of increases in brain GABA, N-acetyl aspartate and glutamate. Neuroimage. 2016;125:988-95.
303. Alshammari TK, Alghamdi HM, Alduhailan HE, Saja MF, Alrasheed NM, Alshammari MA. Examining the central effects of chronic stressful social isolation on rats. Biomed Rep. 2020;13(6):56.
304. Menard C, Pfau ML, Hodes GE, Kana V, Wang VX, Bouchard S, et al. Social stress induces neurovascular pathology promoting depression. Nat Neurosci. 2017;20(12):1752-60.
305. Greene C, Hanley N, Campbell M. Blood-brain barrier associated tight junction disruption is a hallmark feature of major psychiatric disorders. Translational psychiatry. 2020;10(1).
306. Enache D, Pariante CM, Mondelli V. Markers of central inflammation in major depressive disorder: A systematic review and meta-analysis of studies examining cerebrospinal fluid, positron emission tomography and post-mortem brain tissue. Brain Behav Immun. 2019;81:24-40.
307. Kimura I, Inoue D, Maeda T, Hara T, Ichimura A, Miyauchi S, et al. Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). Proc Natl Acad Sci U S A. 2011;108(19):8030-5.
308. Arentsen T, Qian Y, Gkotzis S, Femenia T, Wang T, Udekwu K, et al. The bacterial peptidoglycan-sensing molecule Pglyrp2 modulates brain development and behavior. Mol Psychiatry. 2017;22(2):257-66.
309. Qiu J, Liu RJ, Ma YY, Li Y, Chen Z, He HY, et al. Lipopolysaccharide-Induced Depression-Like Behaviors Is Ameliorated by Sodium Butyrate via Inhibiting Neuroinflammation and Oxido-Nitrosative Stress. Pharmacology. 2020;105(9-10):550-60.
310. Wang Y, Xu J, Liu Y, Li Z, Li X. TLR4-NF-kappaB Signal Involved in Depressive-Like Behaviors and Cytokine Expression of Frontal Cortex and Hippocampus in Stressed C57BL/6 and ob/ob Mice. Neural Plast. 2018;2018:7254016.
311. Lach G, Schellekens H, Dinan TG, Cryan JF. Anxiety, Depression, and the Microbiome: A Role for Gut Peptides. Neurotherapeutics. 2018;15(1):36-59.
312. Tynan RJ, Weidenhofer J, Hinwood M, Cairns MJ, Day TA, Walker FR. A comparative examination of the anti-inflammatory effects of SSRI and SNRI antidepressants on LPS stimulated microglia. Brain Behav Immun. 2012;26(3):469-79.
313. Lu Y, Xu X, Jiang T, Jin L, Zhao XD, Cheng JH, et al. Sertraline ameliorates inflammation in CUMS mice and inhibits TNF-alpha-induced inflammation in microglia cells. Int Immunopharmacol. 2019;67:119-28.

314. Sluzewska A, Rybakowski JK, Laciak M, Mackiewicz A, Sobieska M, Wiktorowicz K. Interleukin-6 serum levels in depressed patients before and after treatment with fluoxetine. Ann Ny Acad Sci. 1995;762:474-6.
315. Lukic I, Getselter D, Ziv O, Oron O, Reuveni E, Koren O, et al. Antidepressants affect gut microbiota and Ruminococcus flavefaciens is able to abolish their effects on depressive-like behavior. Translational psychiatry. 2019;9(1):133.
316. Gopalakrishnan S, Ganeshkumar P. Systematic Reviews and Meta-analysis: Understanding the Best Evidence in Primary Healthcare. J Family Med Prim Care. 2013;2(1):9-14.
317. Sackett DL, Rosenberg WM, Gray JA, Haynes RB, Richardson WS. Evidence based medicine: what it is and what it isn't. BMJ. 1996;312(7023):71-2.
318. Khan KS, Kunz R, Kleijnen J, Antes G. Five steps to conducting a systematic review. J R Soc Med. 2003;96(3):118-21.
319. Joober R, Schmitz N, Annable L, Boksa P. Publication bias: what are the challenges and can they be overcome? J Psychiatry Neurosci. 2012;37(3):149-52.
320. Morrison A, Polisena J, Husereau D, Moulton K, Clark M, Fiander M, et al. The effect of English-language restriction on systematic review-based meta-analyses: a systematic review of empirical studies. Int J Technol Assess Health Care. 2012;28(2):138-44.
321. Stang A. Critical evaluation of the Newcastle-Ottawa scale for the assessment of the quality of nonrandomized studies in meta-analyses. Eur J Epidemiol. 2010;25(9):603-5.
322. Lo CK, Mertz D, Loeb M. Newcastle-Ottawa Scale: comparing reviewers' to authors' assessments. BMC Med Res Methodol. 2014;14:45.
323. Moher D, Liberati A, Tetzlaff J, Altman DG, Group P. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. BMJ. 2009;339:b2535.
324. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol. 2020;18(7):e3000410.
325. Willner P. The validity of animal models of depression. Psychopharmacology (Berl). 1984;83(1):1-16.
326. Overstreet DH, Russell RW, Helps SC, Messenger M. Selective breeding for sensitivity to the anticholinesterase DFP. Psychopharmacology (Berl). 1979;65(1):15-20.
327. Russell RW, Overstreet DH, Messenger M, Helps SC. Selective breeding for sensitivity to DFP: generalization of effects beyond criterion variables. Pharmacology, biochemistry, and behavior. 1982;17(5):885-91.
328. Furey ML, Khanna A, Hoffman EM, Drevets WC. Scopolamine Produces Larger Antidepressant and Antianxiety Effects in Women Than in Men. Neuropsychopharmacology. 2010;35(12):2479-88.

329. Philip NS, Carpenter LL, Tyrka AR, Price LH. Nicotinic acetylcholine receptors and depression: a review of the preclinical and clinical literature. Psychopharmacology (Berl). 2010;212(1):1-12.
330. Abildgaard A, Solskov L, Volke V, Harvey BH, Lund S, Wegener G. A high-fat diet exacerbates depressive-like behavior in the Flinders Sensitive Line (FSL) rat, a genetic model of depression. Psychoneuroendocrinology. 2011;36(5):623-33.
331. Hasegawa S, Nishi K, Watanabe A, Overstreet DH, Diksic M. Brain 5-HT synthesis in the Flinders Sensitive Line rat model of depression: an autoradiographic study. Neurochem Int. 2006;48(5):358-66.
332. Voget M, Rummel J, Avchalumov Y, Sohr R, Haumesser JK, Rea E, et al. Altered local field potential activity and serotonergic neurotransmission are further characteristics of the Flinders sensitive line rat model of depression. Behav Brain Res. 2015;291:299-305.
333. Nishi K, Kanemaru K, Diksic M. A genetic rat model of depression, Flinders sensitive line, has a lower density of 5-HT(1A) receptors, but a higher density of 5-HT(1B) receptors, compared to control rats. Neurochem Int. 2009;54(5-6):299-307.
334. Carboni L, Becchi S, Piubelli C, Mallei A, Giambelli R, Razzoli M, et al. Early-life stress and antidepressants modulate peripheral biomarkers in a gene-environment rat model of depression. Progress in neuro-psychopharmacology & biological psychiatry. 2010;34(6):1037-48.
335. Overstreet DH, Keeney A, Hogg S. Antidepressant effects of citalopram and CRF receptor antagonist CP-154,526 in a rat model of depression. Eur J Pharmacol. 2004;492(2-3):195-201.
336. Pucilowski O, Overstreet DH. Effect of chronic antidepressant treatment on responses to apomorphine in selectively bred rat strains. Brain research bulletin. 1993;32(5):471-5.
337. Overstreet DH, Pucilowski O, Rezvani AH, Janowsky DS. Administration of antidepressants, diazepam and psychomotor stimulants further confirms the utility of Flinders Sensitive Line rats as an animal model of depression. Psychopharmacology (Berl). 1995;121(1):27-37.
338. Melis V, Usach I, Peris JE. Determination of sertraline in rat plasma by HPLC and fluorescence detection and its application to in vivo pharmacokinetic studies. J Sep Sci. 2012;35(23):3302-7.
339. Flemer B, Gaci N, Borrel G, Sanderson IR, Chaudhary PP, Tottey W, et al. Fecal microbiota variation across the lifespan of the healthy laboratory rat. Gut Microbes. 2017;8(5):428-39.
340. Sengupta P. The Laboratory Rat: Relating Its Age With Human's. Int J Prev Med. 2013;4(6):624-30.
341. Brandt LJ, Aroniadis OC. An overview of fecal microbiota transplantation: Techniques, indications, and outcomes. Gastrointestinal Endoscopy. 2013;78(2):240-9.

342. Wang JH, Kim BS, Han K, Kim H. Ephedra-Treated Donor-Derived Gut Microbiota Transplantation Ameliorates High Fat Diet-Induced Obesity in Rats. International journal of environmental research and public health. 2017;14(6).
343. Alpert C, Sczesny S, Gruhl B, Blaut M. Long-term stability of the human gut microbiota in two different rat strains. Curr Issues Mol Biol. 2008;10(1-2):17-24.
344. Cammarota G, Ianiro G, Tilg H, Rajilic-Stojanovic M, Kump P, Satokari R, et al. European consensus conference on faecal microbiota transplantation in clinical practice. Gut. 2017;66(4):569-80.
345. Walsh RN, Cummins RA. The Open-Field Test: a critical review. Psychol Bull. 1976;83(3):482-504.
346. Seibenhener ML, Wooten MC. Use of the Open Field Maze to measure locomotor and anxiety-like behavior in mice. J Vis Exp. 2015(96):e52434.
347. Porsolt RD, Bertin A, Jalfre M. Behavioral despair in mice: a primary screening test for antidepressants. Arch Int Pharmacodyn Ther. 1977;229(2):327-36.
348. Porsolt RD, Le Pichon M, Jalfre M. Depression: a new animal model sensitive to antidepressant treatments. Nature. 1977;266(5604):730-2.
349. Slattery DA, Cryan JF. Using the rat forced swim test to assess antidepressant-like activity in rodents. Nat Protoc. 2012;7(6):1009-14.
350. Yankelevitch-Yahav R, Franko M, Huly A, Doron R. The forced swim test as a model of depressive-like behavior. J Vis Exp. 2015(97).
351. Bogdanova OV, Kanekar S, D'Anci KE, Renshaw PF. Factors influencing behavior in the forced swim test. Physiology & behavior. 2013;118:227-39.
352. Vdoviakova K, Petrovova E, Maloveska M, Kresakova L, Teleky J, Elias MZ, et al. Surgical Anatomy of the Gastrointestinal Tract and Its Vasculature in the Laboratory Rat. Gastroenterol Res Pract. 2016;2016:2632368.
353. Balda MS, Flores-Maldonado C, Cereijido M, Matter K. Multiple domains of occludin are involved in the regulation of paracellular permeability. Journal of cellular biochemistry. 2000;78(1):85-96.
354. Patel RM, Myers LS, Kurundkar AR, Maheshwari A, Nusrat A, Lin PW. Probiotic bacteria induce maturation of intestinal claudin 3 expression and barrier function. Am J Pathol. 2012;180(2):626-35.
355. Bech P, Rasmussen NA, Olsen LR, Noerholm V, Abildgaard W. The sensitivity and specificity of the Major Depression Inventory, using the Present State Examination as the index of diagnostic validity. J Affect Disord. 2001;66(2-3):159-64.
356. Walters W, Hyde ER, Berg-Lyons D, Ackermann G, Humphrey G, Parada A, et al. Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Surveys. mSystems. 2016;1(1).
357. Parada AE, Needham DM, Fuhrman JA. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock

communities, time series and global field samples. Environmental microbiology. 2016;18(5):1403-14.
358. Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 2002;30(14):3059-66.
359. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41(Database issue):D590-6.
360. Egede LE. Failure to recognize depression in primary care: issues and challenges. J Gen Intern Med. 2007;22(5):701-3.
361. Weilburg JB, O'Leary KM, Meigs JB, Hennen J, Stafford RS. Evaluation of the adequacy of outpatient antidepressant treatment. Psychiatr Serv. 2003;54(9):1233-9.
362. Demyttenaere K, Enzlin P, Dewe W, Boulanger B, De Bie J, De Troyer W, et al. Compliance with antidepressants in a primary care setting, 1: Beyond lack of efficacy and adverse events. J Clin Psychiatry. 2001;62 Suppl 22:30-3.
363. Hunot VM, Horne R, Leese MN, Churchill RC. A cohort study of adherence to antidepressants in primary care: the influence of antidepressant concerns and treatment preferences. Prim Care Companion J Clin Psychiatry. 2007;9(2):91-9.
364. Hamilton M. A rating scale for depression. J Neurol Neurosurg Psychiatry. 1960;23:56-62.
365. Montgomery SA, Asberg M. A new depression scale designed to be sensitive to change. Br J Psychiatry. 1979;134:382-9.
366. Beck AT, Ward CH, Mendelson M, Mock J, Erbaugh J. An inventory for measuring depression. Arch Gen Psychiatry. 1961;4:561-71.
367. Berry D, Stecher B, Schintlmeister A, Reichert J, Brugiroux S, Wild B, et al. Host-compound foraging by intestinal microbiota revealed by single-cell stable isotope probing. Proc Natl Acad Sci U S A. 2013;110(12):4720-5.
368. McOrist AL, Jackson M, Bird AR. A comparison of five methods for extraction of bacterial DNA from human faecal samples. J Microbiol Methods. 2002;50(2):131-9.
369. Albertsen M, Karst SM, Ziegler AS, Kirkegaard RH, Nielsen PH. Back to Basics--The Influence of DNA Extraction and Primer Choice on Phylogenetic Analysis of Activated Sludge Communities. PLoS One. 2015;10(7):e0132783.
370. Jian C, Luukkonen P, Yki-Jarvinen H, Salonen A, Korpela K. Quantitative PCR provides a simple and accessible method for quantitative microbiota profiling. PLoS One. 2020;15(1):e0227285.
371. Valm AM, Mark Welch JL, Rieken CW, Hasegawa Y, Sogin ML, Oldenbourg R, et al. Systems-level analysis of microbial community organization through combinatorial labeling and spectral imaging. Proc Natl Acad Sci U S A. 2011;108(10):4152-7.
372. Lagier JC, Dubourg G, Million M, Cadoret F, Bilen M, Fenollar F, et al. Culturing the human microbiota and culturomics. Nat Rev Microbiol. 2018;16:540-50.

373. Browne HP, Forster SC, Anonye BO, Kumar N, Neville BA, Stares MD, et al. Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. Nature. 2016;533(7604):543-6.
374. Lagier JC, Khelaifia S, Alou MT, Ndongo S, Dione N, Hugon P, et al. Culture of previously uncultured members of the human gut microbiota by culturomics. Nat Microbiol. 2016;1:16203.
375. Jovel J, Patterson J, Wang W, Hotte N, O'Keefe S, Mitchel T, et al. Characterization of the Gut Microbiome Using 16S or Shotgun Metagenomics. Front Microbiol. 2016;7:459.
376. Vetrovsky T, Baldrian P. The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. PLoS One. 2013;8(2):e57923.
377. Janda JM, Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. J Clin Microbiol. 2007;45(9):2761-4.
378. Callahan BJ, McMurdie PJ, Holmes SP. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. The ISME journal. 2017;11(12):2639-43.
379. Konstantinidis KT, Tiedje JM. Genomic insights that advance the species definition for prokaryotes. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(7):2567-72.
380. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26(19):2460-1.
381. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(5):335-6.
382. McIlroy SJ, Kirkegaard RH, McIlroy B, Nierychlo M, Kristensen JM, Karst SM, et al. MiDAS 2.0: an ecosystem-specific taxonomy and online database for the organisms of wastewater treatment systems expanded for anaerobic digester groups. Database (Oxford). 2017;2017(1).
383. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37(8):852-7.
384. Prodan A, Tremaroli V, Brolin H, Zwinderman AH, Nieuwdorp M, Levin E. Comparing bioinformatic pipelines for microbial 16S rRNA amplicon sequencing. PLoS One. 2020;15(1):e0227434.
385. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol. 2005;71(12):8228-35.
386. Ricotta C, Podani J. On some properties of the Bray-Curtis dissimilarity and their ecological meaning. Ecol Complex. 2017;31:201-5.
387. Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R. UniFrac: an effective distance metric for microbial community comparison. The ISME journal. 2011;5(2):169-72.

388. Ferdowsian HR, Beck N. Ethical and Scientific Considerations Regarding Animal Testing and Research. Plos One. 2011;6(9).
389. Cheung SG, Goldenthal AR, Uhlemann AC, Mann JJ, Miller JM, Sublette ME. Systematic Review of Gut Microbiota and Major Depression. Frontiers in psychiatry. 2019;10:34.
390. Barandouzi ZA, Starkweather AR, Henderson WA, Gyamfi A, Cong XS. Altered Composition of Gut Microbiota in Depression: A Systematic Review. Frontiers in psychiatry. 2020;11:541.
391. Sanada K, Nakajima S, Kurokawa S, Barcelo-Soler A, Ikuse D, Hirata A, et al. Gut microbiota and major depressive disorder: A systematic review and meta-analysis. J Affect Disord. 2020;266:1-13.
392. Vindegaard N, Speyer H, Nordentoft M, Rasmussen S, Benros ME. Gut microbial changes of patients with psychotic and affective disorders: A systematic review. Schizophr Res. 2020.
393. Simpson CA, Diaz-Arteche C, Eliby D, Schwartz OS, Simmons JG, Cowan CSM. The gut microbiota in anxiety and depression - A systematic review. Clin Psychol Rev. 2021;83:101943.
394. Mosca A, Leclerc M, Hugot JP. Gut Microbiota Diversity and Human Diseases: Should We Reintroduce Key Predators in Our Ecosystem? Frontiers in Microbiology. 2016;7.
395. Lynch SV, Pedersen O. The Human Intestinal Microbiome in Health and Disease. N Engl J Med. 2016;375(24):2369-79.
396. Lopez-Siles M, Duncan SH, Garcia-Gil LJ, Martinez-Medina M. Faecalibacterium prausnitzii: from microbiology to diagnostics and prognostics. The ISME journal. 2017;11(4):841-52.
397. Wu H, Tremaroli V, Backhed F. Linking Microbiota to Human Diseases: A Systems Biology Perspective. Trends Endocrinol Metab. 2015;26(12):758-70.
398. Rosenberg E, Zilber-Rosenberg I. Microbes Drive Evolution of Animals and Plants: the Hologenome Concept. mBio. 2016;7(2):e01395.
399. Bordenstein SR, Theis KR. Host Biology in Light of the Microbiome: Ten Principles of Holobionts and Hologenomes. PLoS Biol. 2015;13(8):e1002226.
400. Averina OV, Zorkina YA, Yunes RA, Kovtun AS, Ushakova VM, Morozova AY, et al. Bacterial Metabolites of Human Gut Microbiota Correlating with Depression. Int J Mol Sci. 2020;21(23).
401. Yang J, Zheng P, Li YF, Wu J, Tan XM, Zhou JJ, et al. Landscapes of bacterial and metabolic signatures and their interaction in major depressive disorders. Science Advances. 2020;6(49).
402. Kurokawa S, Tomizawa Y, Miyaho K, Ishii D, Takamiya A, Ishii C, et al. Fecal Microbial and Metabolomic Change during treatment course for depression: An Observational Study. J Psychiatr Res. 2021;140:45-52.
403. Haidich AB. Meta-analysis in medical research. Hippokratia. 2010;14(1):29-37.

404. Goodrich JK, Di Rienzi SC, Poole AC, Koren O, Walters WA, Caporaso JG, et al. Conducting a microbiome study. Cell. 2014;158(2):250-62.
405. Hamady M, Knight R. Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. Genome Res. 2009;19(7):1141-52.
406. Lozupone CA, Stombaugh J, Gonzalez A, Ackermann G, Wendel D, Vazquez-Baeza Y, et al. Meta-analyses of studies of the human microbiota. Genome Res. 2013;23(10):1704-14.
407. Button KS, Ioannidis JP, Mokrysz C, Nosek BA, Flint J, Robinson ES, et al. Power failure: why small sample size undermines the reliability of neuroscience. Nat Rev Neurosci. 2013;14(5):365-76.
408. Moller MH, Ioannidis JPA, Darmon M. Are systematic reviews and meta-analyses still useful research? We are not sure. Intensive Care Med. 2018;44(4):518-20.
409. Rex A, Sondern U, Voigt JP, Franck S, Fink H. Strain differences in fear-motivated behavior of rats. Pharmacology, biochemistry, and behavior. 1996;54(1):107-11.
410. Loos M, Koopmans B, Aarts E, Maroteaux G, van der Sluis S, Neuro BMPC, et al. Within-strain variation in behavior differs consistently between common inbred strains of mice. Mamm Genome. 2015;26(7-8):348-54.
411. Can A, Dao DT, Arad M, Terrillion CE, Piantadosi SC, Gould TD. The mouse forced swim test. J Vis Exp. 2012(59):e3638.
412. Bogdanova OV, Kanekar S, D'Anci KE, Renshaw PF. Factors influencing behavior in the forced swim test. Physiology & behavior. 2013;118:227-39.
413. Can A, Dao DT, Terrillion CE, Piantadosi SC, Bhat S, Gould TD. The tail suspension test. J Vis Exp. 2012(59):e3769.
414. Luczynski P, McVey Neufeld KA, Oriach CS, Clarke G, Dinan TG, Cryan JF. Growing up in a Bubble: Using Germ-Free Animals to Assess the Influence of the Gut Microbiota on Brain and Behavior. Int J Neuropsychopharmacol. 2016;19(8).
415. Guida F, Turco F, Iannotta M, De Gregorio D, Palumbo I, Sarnelli G, et al. Antibiotic-induced microbiota perturbation causes gut endocannabinoidome changes, hippocampal neuroglial reorganization and depression in mice. Brain, Behavior, and Immunity. 2017.
416. Freitag TL, Hartikainen A, Jouhten H, Sahl C, Meri S, Anttila VJ, et al. Minor Effect of Antibiotic Pre-treatment on the Engraftment of Donor Microbiota in Fecal Transplantation in Mice. Front Microbiol. 2019;10:2685.
417. Kim HJ, Rowe M, Ren M, Hong JS, Chen PS, Chuang DM. Histone deacetylase inhibitors exhibit anti-inflammatory and neuroprotective effects in a rat permanent ischemic model of stroke: multiple mechanisms of action. J Pharmacol Exp Ther. 2007;321(3):892-901.
418. Ayensu WK, Pucilowski O, Mason GA, Overstreet DH, Rezvani AH, Janowsky DS. Effects of chronic mild stress on serum complement activity, saccharin

preference, and corticosterone levels in Flinders lines of rats. Physiology & behavior. 1995;57(1):165-9.
419. Kazemian N, Ramezankhani M, Sehgal A, Khalid FM, Kalkhoran AHZ, Narayan A, et al. The trans-kingdom battle between donor and recipient gut microbiome influences fecal microbiota transplantation outcome. Sci Rep. 2020;10(1):18349.
420. Knudsen JK, Bundgaard-Nielsen C, Hjerrild S, Nielsen RE, Leutscher P, Sorensen S. Gut microbiota variations in patients diagnosed with major depressive disorder-A systematic review. Brain Behav. 2021:e02177.
421. Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, et al. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell. 2015;161(2):264-76.
422. LeBlanc JG, Chain F, Martin R, Bermudez-Humaran LG, Courau S, Langella P. Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. Microb Cell Fact. 2017;16(1):79.
423. Silva YP, Bernardi A, Frozza RL. The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication. Front Endocrinol (Lausanne). 2020;11:25.
424. Louis P, Flint HJ. Formation of propionate and butyrate by the human colonic microbiota. Environmental microbiology. 2017;19(1):29-41.
425. House PK, Vyas A, Sapolsky R. Predator cat odors activate sexual arousal pathways in brains of Toxoplasma gondii infected rats. PLoS One. 2011;6(8):e23277.
426. Ursell LK, Clemente JC, Rideout JR, Gevers D, Caporaso JG, Knight R. The interpersonal and intrapersonal diversity of human-associated microbiota in key body sites. The Journal of allergy and clinical immunology. 2012;129(5):1204-8.
427. Madan A, Thompson D, Fowler JC, Ajami NJ, Salas R, Frueh BC, et al. The gut microbiota is associated with psychiatric symptom severity and treatment outcome among individuals with serious mental illness. Journal of Affective Disorders. 2020;264:98-106.
428. Liskiewicz P, Pelka-Wysiecka J, Kaczmarczyk M, Loniewski I, Wronski M, Baba-Kubis A, et al. Fecal Microbiota Analysis in Patients Going through a Depressive Episode during Treatment in a Psychiatric Hospital Setting. J Clin Med. 2019;8(2).
429. Ye X, Wang D, Zhu H, Wang D, Li J, Tang Y, et al. Gut Microbiota Changes in Patients With Major Depressive Disorder Treated With Vortioxetine. Frontiers in psychiatry. 2021;12:641491.
430. Simmons WK, Burrows K, Avery JA, Kerr KL, Taylor A, Bodurka J, et al. Appetite changes reveal depression subgroups with distinct endocrine, metabolic, and immune states. Mol Psychiatry. 2020;25(7):1457-68.
431. Gafoor R, Booth HP, Gulliford MC. Antidepressant utilisation and incidence of weight gain during 10 years' follow-up: population based cohort study. BMJ. 2018;361:k1951.

432. Althubaiti A. Information bias in health research: definition, pitfalls, and adjustment methods. J Multidiscip Healthc. 2016;9:211-7.
433. Jacka FN, Cherbuin N, Anstey KJ, Butterworth P. Does reverse causality explain the relationship between diet and depression? J Affect Disord. 2015;175:248-50.
434. Hebert JR, Clemow L, Pbert L, Ockene IS, Ockene JK. Social desirability bias in dietary self-report may compromise the validity of dietary intake measures. Int J Epidemiol. 1995;24(2):389-98.
435. Firth J, Marx W, Dash S, Carney R, Teasdale SB, Solmi M, et al. The Effects of Dietary Improvement on Symptoms of Depression and Anxiety: A Meta-Analysis of Randomized Controlled Trials. Psychosomatic medicine. 2019;81(3):265-80.
436. Odamaki T, Kato K, Sugahara H, Hashikura N, Takahashi S, Xiao JZ, et al. Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. BMC Microbiol. 2016;16:90.
437. Kessler RC, Birnbaum HG, Shahly V, Bromet E, Hwang I, McLaughlin KA, et al. Age differences in the prevalence and co-morbidity of DSM-IV major depressive episodes: results from the WHO World Mental Health Survey Initiative. Depress Anxiety. 2010;27(4):351-64.
438. Lish JD, Dime-Meenan S, Whybrow PC, Price RA, Hirschfeld RM. The National Depressive and Manic-depressive Association (DMDA) survey of bipolar members. J Affect Disord. 1994;31(4):281-94.
439. Beck A, Crain AL, Solberg LI, Unutzer J, Glasgow RE, Maciosek MV, et al. Severity of depression and magnitude of productivity loss. Ann Fam Med. 2011;9(4):305-11.
440. Pizzagalli DA. Depression, stress, and anhedonia: toward a synthesis and integrated model. Annual review of clinical psychology. 2014;10:393-423.
441. Liu RT, Walsh RFL, Sheehan AE. Prebiotics and probiotics for depression and anxiety: A systematic review and meta-analysis of controlled clinical trials. Neurosci Biobehav Rev. 2019;102:13-23.
442. Chao L, Liu C, Sutthawongwadee S, Li Y, Lv W, Chen W, et al. Effects of Probiotics on Depressive or Anxiety Variables in Healthy Participants Under Stress Conditions or With a Depressive or Anxiety Diagnosis: A Meta-Analysis of Randomized Controlled Trials. Front Neurol. 2020;11:421.
443. Huang R, Wang K, Hu J. Effect of Probiotics on Depression: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. Nutrients. 2016;8(8).
444. Nikolova VL, Cleare AJ, Young AH, Stone JM. Updated Review and Meta-Analysis of Probiotics for the Treatment of Clinical Depression: Adjunctive vs. Stand-Alone Treatment. J Clin Med. 2021;10(4).
445. Cai T, Shi X, Yuan LZ, Tang D, Wang F. Fecal microbiota transplantation in an elderly patient with mental depression. International psychogeriatrics. 2019;31(10):1525-6.

CHAPTER 9. APPENDICES

Appendix A. – Artist attributions

Figure 2 - Bacteria (artsholic), GI tract (freepik), pharmacy logo (vectorstock), fast food (21kompot), medicine (vectorstock). All vectors acquired from vectorstock.com
Figure 3 - Multiple bacteria (artsholic), GI tract (freepik), immune cells (anastasia8), single bacteria (soponyono), foods (vectorstoc). All vectors acquired from vectorstock.com
Figure 8 – Tube under "DNA purification" (Krolja), DNA (Awareness)
Figures 1, 4, 5, 6, 7, 8, 9 – Mette Henriksen, designer at North Denmark Regional Hospital

Appendix B. – Paper I, Supplementary Material 1

The search was conducted on November 13th using three databases; PubMed, Embase and PsychINO. The search strategies are described below;

Database: Pubmed search strings
((((("mood disorders"[MeSH Terms] OR mood disorder[Text Word])) OR ("depressive disorder"[MeSH Terms] OR "depression"[MeSH Terms] OR depression[Text Word]))) AND (((((("microbiota"[MeSH Terms] OR microbiota[Text Word])) OR ("fecal microbiota transplantation"[MeSH Terms] OR fecal microbiota transplantation[Text Word])) OR microbiome[Text Word]) OR "brain gut axis"[All Fields]).

Database: Embase <1974 to 2020 Week 46>
Search Strategy:
---
1 exp mood disorder/ (536354)
2 mood disorder*.mp. (59009)
3 depressi*.mp. (730993)
4 1 or 2 or 3 (806592)
5 exp microflora/ (130859)
6 fecal microbiota transplantation/ (4621)
7 brain gut axis.mp. (1200)
8 microbiome.mp. (42028)
9 microbiota.mp. (67148)
10 5 or 6 or 7 or 8 or 9 (151670)
11 4 and 10 (2479)
12 remove duplicates from 11 (2453)

13 limit 12 to dc=18000101-20201113 (2287)

Database: PsycINFO <1806 to November Week 3 2020>
Search Strategy:
---
1 exp affective disorders/ (147813)
2 mood disorder*.mp. (23418)
3 depressi*.mp. (367524)
4 1 or 2 or 3 (381677)
5 microbiota.mp. (1074)
6 microbiome.mp. (709)
7 brain gut axis.mp. (130)
8 5 or 6 or 7 (1479)
9 4 and 8 (396)

Appendix C. Paper I, Supplementary Material 2

Analyses Based on Next Generation Sequencing	***Naseribafrouei et al.* (42)**	***Jiang et al.* (43)**		***Zheng et al.* (2016) (44)**	***Kelly et al.* (46)**	***Lin et al.* (47)**	***Chen et al.* (2018) (48)**	
Relative abundance of phylum in MDD compared to HC		*A-MDD*	*R-MDD*				*Male*	*Female*
Actinobacteria		↓		↑				↑
Bacteroidetes	↓	↑	↑	↓			↓	
Firmicutes	↑	↓						
Fusobacteria		↑						
Proteobacteria		↑						
Analyses Based on Next Generation Sequencing	***Naseribafrouei et al.* (42)**	***Jiang et al.* (43)**		***Zheng et al.* (2016) (44)**	***Kelly et al.* (46)**	***Lin et al.* (47)**	***Chen et al.* (2018) (48)**	
Relative abundance of family in MDD compared to HC		*A-MDD*	*R-MDD*				*Male*	*Female*
Actinobacteria - Actinomycineae				↑				
Actinobacteria - Atobopiaceae								
Actinobacteria - Bifidobacteriaceae								↑
Actinobacteria - Coriobacteriaceae				↑				↑
Actinobacteria - Eggerthellaceae								
Actinobacteria - Micrococcaceae								
Bacteroidetes - Bacteroidaceae		↓	↑	↓				
Bacteroidetes - Barnesiellaceae								
Bacteroidetes - Cytophagaceae								
Bacteroidetes - Flavobacteriaecea								
Bacteroidetes - Porphyromonadaceae		↑	↑					
Bacteroidetes - Prevotellaceae		↓			↓			
Bacteroidetes - Sphingobacteriaceae								
Bacteroidetes - Rikenellaceae		↑	↑	↓				
Firmicutes - Acidaminococcaceae		↑	↑	↓				
Firmicutes - Christensenellaceae								
Firmicutes - Clostridiaceae								
Firmicutes - Clostridiales XI incertae sedis				↑				
Firmicutes - Enterococcaceae								
Firmicutes - Erysipelotrichaceae		↓		↑			↑	
Firmicutes - Eubacteriaceae				↑				↑
Firmicutes - Heliobacteriaceae								
Firmicutes - Lachnospiraceae	↓	↓	↓	↑ and ↓			↑ and ↓	↑ and ↓
Firmicutes - Lactobacillaceae				↑				
Firmicutes - Oscillospiraceae								
Firmicutes - Peptococcaceae								
Firmicutes - Peptostreptococcaceae								
Firmicutes - Ruminococcaceae		↓	↓	↑			↓	↑ and ↓
Firmicutes - Streptococcaceae				↑				
Firmicutes - Thermoanaerobacteriaceae					↑			
Firmicutes - Veillonellaceae		↓	↓	↓				
Fusobacteria - Fusobacteriaceae		↑						
Patescibacteria - Saccharimonadaceae								
Proteobacteria - Alicaligenaceae								
Proteobacteria - Enterobacteriaceae		↑	↑					
Proteobacteria - Pasteurellaceae								↑
Proteobacteria - Sutterellaceae								↓

Rong et al. (49)	*Chung et al.* (50)	*Huang et al.* (51)	*Vinberg et al.* (52)	*Chen et al.* (2020) (53)	*Lai et al.* (54)	*Liu et al.* (55)	*Mason et al.* (56)	*Stevens et al.* (57)	*Zheng et al.* (2020) (58)	*Total observations*	
				Young MA						↑	↓
↑	↑									4	1
↓	↓								↑	3	5
↑	↑	↓	↓							3	3
										1	
	↓									1	1

Rong et al. (49)	*Chung et al.* (50)	*Huang et al.* (51)	*Vinberg et al.* (52)	*Chen et al.* (2020) (53)	*Lai et al.* (54)	*Liu et al.* (55)	*Mason et al.* (56)	*Stevens et al.* (57)	*Zheng et al.* (2020) (58)	*Total observations*	
				Young MA						↑	↓
				↑						2	
					↑					1	
↑	↑				↑				↑	5	
↑				↑						4	
					↑					1	
					↑					1	
				↓	↓				↑ and ↓	2	5
						↓					1
					↓						1
					↓						1
↑										3	
	↓			↓							4
					↓						1
										2	1
				↓	↑					3	2
			↓			↑		↓		1	2
		↓		↓							2
										1	
					↑	↑				2	
										2	1
				↑						2	
					↑					1	
	↑	↓		↑				↑ and ↓	↑	7	7
					↑					2	
					↑					1	
					↑					1	
	↑			↓						1	1
↑		↓		↓		↓		↓		3	8
	↑				↑					3	
										1	
↑					↑					2	3
										1	
								↑		1	
	↓										1
									↓	2	1
↓										1	1
				↓							2

Analyses Based on Next Generation Sequencing	*Naseribafrouei et al.* (42)	*Jiang et al.* (43)		*Zheng et al.* (2016) (44)	*Kelly et al.* (46)	*Lin et al.* (47)	*Chen et al.* (2018) (48)	
Relative abundance of genus in MDD compared to HC?		*A-MDD*	*R-MDD*				*Male*	*Female*
Actinobacteria - Actinomycineae - Actinomyces								↑
Actinobacteria - Bifidobacteriaceae - Bifidobacterium								↑
Actinobacteria - Coriobacteriaceae - Atopobium							↑	↑
Actinobacteria - Coriobacteriaceae - Adlercreutzia								
Actinobacteria - Coriobacteriaceae - Coriobacterium								
Actinobacteria - Coriobacteriaceae - Eggerthella					↑			↑
Actinobacteria - Coriobacteriaceae - Gordonibacter							↓	↑
Actinobacteria - Coriobacteriaceae - Olsenella								↑
Actinobacteria - Coriobacteriaceae - Slackia								
Actinobacteria - Micrococcaceae - Rothia								
Bacteroidetes - Bacteroidaceae - Bacteroides		↓	↑				↑	
Bacteroidetes - Porphyromonadaceae - Barnesiella								
Bacteroidetes - Porphyromonadaceae - Parabacteroides		↑	↑					
Bacteroidetes - Prevotellaceae - Paraprevotella					↑			
Bacteroidetes - Prevotellaceae - Prevotella		↓	↓		↓	↑		
Bacteroidetes - Rikenellaceae - Alistipes	↑	↑	↑	↓				
Clostridium sensu stricto								
Clostridium XI						↑		
Clostridium XVIII								
Clostridium XIX		↑						
Clostridium (unspecified)								
Firmicutes - Acidaminococcaceae - Acidaminococca								
Firmicutes - Acidaminococcaceae - Phascolarctobacterium		↑	↑	↓				
Firmicutes - "Bacillales" - Gemella								
Firmicutes - Clostridiaceae - Faecalibacterium		↓	↓	↓				↑
Firmicutes - Clostridiaceae - Fusicantenibacter								
Firmicutes - Clostridiaceae - Lachnoclostridium								
Firmicutes - Enterococcaceae - Enterococcus								
Firmicutes - Erypsipelotrichaceae - Bulleidia								
Firmicutes - Erypsipelotrichaceae - Holdemania					↑			
Firmicutes - Erypsipelotrichaceae - Turicibacteria					↑			
Firmicutes - Erypsipelotrichaceae incertae sedis							↑	
Firmicutes - Eubacteriaceae - Anaerovorax							↓	
Firmicutes - Eubacteriaceae - Eubacterium								↑
Firmicutes - Heliobacteriaceae - Heliobacterium								
Firmicutes - Lachnospiracea incertae sedis		↑		↑ and ↓				
Firmicutes - Lachnospiracea - Agathobacter								
Firmicutes - Lachnospiraceae - Anaerostipes				↑				↑
Firmicutes - Lachnospiraceae - Blautia		↑		↑				↑
Firmicutes - Lachnospiraceae - Clostridium XIVa				↓				
Firmicutes - Lachnospiraceae - Coprococcus				↓				
Firmicutes - Lachnospiraceae - Dorea				↑				
Firmicutes -Lachnospiraceae - Howardella								↓
Firmicutes -Lachnospiraceae - Lachnoclostridium								
Firmicutes -Lachnospiraceae - Lachnospira								
Firmicutes - Lachnospiraceae - Roseburia		↑	↑	↓				↑
Firmicutes - Lachnospiraceae - Sellimonas								
Firmicutes - Lachnospiraceae - Tyzerella								
Firmicutes - Lactobacillaceae - Lactobacillus								
Firmicutes - Oscillospiraceae - Oscillibacter	↑	↑	↓					
Firmicutes - Peptococcaceae - Desulfitobacterium								
Firmicutes - Peptostreptococcaceae - Peptostreptococcus								
Firmicutes - Peptoniphilaceae - Parvimonas				↑				
Firmicutes - Ruminococcaceae - Anaerofilum					↑			
Firmicutes - Ruminococcaceae - Clostridium IV				↑				
Firmicutes - Ruminococcaceae - Flavonifracter								
Firmicutes - Ruminococcaceae - Phocea								
Firmicutes - Ruminococcaceae - Ruminiclostridium								
Firmicutes - Ruminococcaceae - Ruminococcus		↓	↓					
Firmicutes - Ruminococcaceae - Subdoligranulum								
Firmicutes - Streptococcaceae - Streptococcus						↑		
Firmicutes - Thermoanaerobacteraceae - Gelria					↑			
Firmicutes - Veillonellaceae - Dialister		↓	↓		↓			
Firmicutes - Veillonellaceae - Megasphera								
Firmicutes - Veillonellaceae - Megamonas		↑		↓				
Firmicutes - Veillonellaceae - Veilonella							↑	
Fusobacteria - Fusobacteriaceae - Fusobacterium								
Proteobacteria - Alcaligenaceae - Asccharobacter								↑
Proteobacteria - Desulfovibrionaceae - Desulfovibrio								↑
Proteobacteria - Enterobacteriaceae - Citrobacter								
Proteobacteria - Enterobacteriaceae - Escherichia/Shigella			↓					
Proteobacteria - Enterobacteriaceae - Klebsiella						↑		
Proteobacteria - Pseudomonadaceae - Pseudomonas								
Proteobacteria - Oxalobacteriaceae - Oxalobacter								
Proteobacteria - Sutterellaceae - Parasutterella		↑						
Proteobacteria - Sutterellaceae - Sutterella								↓
Spirochaetota - Sphaerochetaceae - Sphaerocheta								
Synergistetes - Synergistaceae - Pyramidobacter							↓	↓

Rong et al. (49)	*Chung et al.* (50)	*Huang et al.* (51)	*Vinberg et al.* (52)	*Chen et al.* (2020) (53)	*Lai et al.* (54)	*Liu et al.* (55)	*Mason et al.* (56)	*Stevens et al.* (57)	*Zheng et al.* (2020) (58)	*Total observations*	
				Young MA							
										1	
↑	↑				↑					4	
↑					↑					4	
	↑									1	
					↑					1	
↑	↑			↑	↑					6	
										1	1
↑					↑					3	
					↑					1	
					↑					1	
↑				↓	↓			↓	↑	4	4
						↓					1
	↑							↑		4	
										1	
↑ and ↓	↓									2	4
										3	1
				↓							1
	↑			↓						2	1
				↓	↑					1	1
										1	
↑										1	
					↑					1	
				↓				↑		3	1
		↑								1	
		↓		↓		↓		↓		1	7
						↓					1
					↑			↓		1	1
					↑	↑				2	
		↑								1	
	↑							↑		3	
										1	
										1	
				↓							2
↑				↑		↓				3	1
					↑					1	
										2	1
								↓			1
	↑	↓								4	1
											1
		↓		↓		↓					3
		↓								1	1
											1
								↑		1	
								↓			1
								↓		4	2
						↑				1	
						↓					1
↑ and ↓					↑					2	1
↑				↓	↑			↑ and ↓		5	3
					↑					1	
		↑								1	
		↑								2	
										1	
										1	
						↑		↑		2	
								↑		1	
								↑ and ↓		1	1
	↑					↓			↓	2	4
						↓					1
↑ and ↓	↑			↑	↑					5	1
										1	
											3
					↑					1	
	↓			↑						1	2
									↓		1
										1	
↑						↓				2	1
									↑	1	
↑								↑		2	1
↑										2	
		↑								1	
		↑								1	
										1	
	↓			↓							3
					↑					1	
											2

ISSN (online): 2246-1302
ISBN (online): 978-87-7210-956-5

AALBORG UNIVERSITY PRESS