DOES SEX MATTER? AN *IN VITRO* STUDY ON SEX-SIMULATED MICROBIOME IN RESPONSE TO A PSYCHOTROPIC DRUG

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ABSTRACT

Sex hormone and gut microbiota interactions are known to play a significant role in mental health and psychotropic medication responses. This study aimed to explore the sexually differentiated impact of psychotropic medication on the gut microbiome composition and diversity, with a focus on sex steroid hormones. Our findings demonstrate that psychotropic medications influence gut microbial diversity differently in male and female models. Notably, females exhibited a higher abundance of Bacteroidota and lower Firmicutes at 24h, while males displayed an opposite trend. Additionally, the α-diversity was significantly lower in females, indicating reduced microbial evenness, although no significant differences in β-diversity were observed. Psychotropic treatment resulted in increased microbial diversity in both sexes, with shifts in key taxa, such as *Eubacterium coprostanoligenes* and *Sutterella* in females, along with alterations in short-chain fatty acid production, particularly in acetate, propionate, and butyrate. These results suggest that sex hormones play an important role in modulating gut microbiome composition and their interactions with psychotropic drugs. These findings emphasize the need to consider sex-specific microbiome profiles and hormonal status when optimizing psychotropic treatments for mental health problems.

Keywords: sex hormones, gut microbiota, psychotropic medication, short-chain fatty acids, α-diversity, β-diversity, sex-specific effects, *in vitro* fermentation.

RÉSUMÉ

Les interactions entre les hormones sexuelles et le microbiote intestinal jouent un rôle significatif dans la santé mentale et la réponse aux médicaments psychotropes. Cette étude visait à explorer l'impact différencié selon le sexe des médicaments psychotropes sur la composition et la diversité du microbiote intestinal, avec un accent particulier sur les hormones stéroïdes sexuelles. Nos résultats ont démontré que les médicaments psychotropes influencent la diversité microbienne intestinale différemment chez les modèles masculins et féminins. Notamment, les femmes ont présenté une abondance plus élevée de Bacteroidota et une diminution des Firmicutes à 24h, tandis que les hommes ont affiché une tendance opposée. De plus, l'α-diversité était significativement plus faible chez les femmes, indiquant une diminution de l'homogénéité microbienne, bien qu'aucune différence significative en β-diversité n'ait été observée. Le traitement psychotrope a entraîné une augmentation de la diversité microbienne dans les deux sexes, avec des changements au niveau de certains taxons clés tels que *Eubacterium coprostanoligenes* et *Sutterella* chez les femmes, ainsi que des altérations dans la production d'acides gras à chaîne courte, notamment l'acétate, le propionate et le butyrate. Les résultats suggèrent que les hormones sexuelles jouent un rôle important dans la modulation de la composition du microbiote intestinal et de ses interactions avec les médicaments psychotropes. Ces conclusions soulignent la nécessité de prendre en compte les profils microbiens spécifiques à chaque sexe ainsi que l'état hormonal lors de l'optimisation des traitements psychotropes pour la santé mentale.

Mots-clés: hormones sexuelles, microbiote intestinal, médicaments psychotropes, acides gras à chaîne courte, diversité α, diversité β, effets spécifiques au sexe, fermentation *in vitro*.

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Abbreviations

16S rRNA - 16S Ribosomal RNA

α-Diversity - Alpha Diversity

ANOVA - Analysis of Variance

BMI - Body Mass Index

β-Diversity - Beta Diversity

β-glucuronidase - Beta-Glucuronidase

CNS - Central Nervous System

CO_2 - Carbon Dioxide

CR - Control Reactor

CPP - Central Precocious Puberty

DA - Differential Abundance

E+P- - Elevated Estrogen and Low Progesterone

E+P+ - Elevated Estrogen and Progesterone

E-P- - Low Estrogen and Progesterone

E1 - Estrone

E2 - Estradiol

E3 - Estriol

EVs - Extracellular Vesicles

GABA - Gamma-Aminobutyric Acid

GAD - Generalized Anxiety Disorder

GC-FID - Gas Chromatography with Flame Ionization Detector

GnRH - Gonadotropin-Releasing Hormone

IR - Inoculum Reactor

KW - Kruskal-Wallis test

LinDA - Linear Discriminant Analysis

MDD - Major Depressive Disorder

MGBA - Microbiota-Gut-Brain Axis

NaOH - Sodium Hydroxide

N_2 - Nitrogen Gas

nmol/L - Nanomoles per Liter

PC - Principal Coordinate Analysis

PCR - Polymerase Chain Reaction

PCOS - Polycystic Ovary Syndrome

pmol/L - Picomoles per Liter

SCFA - Short-Chain Fatty Acids

SD - Standard Deviations

sIgA - Secretory Immunoglobulin A

TR - Test Reactor

TR1-3 - Test Reactors 1-3

V3-V4 - Variable regions 3 and 4 of the 16S rRNA gene

v/v - Volume/Volume

w/v - Weight/Volume

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GENERAL INTRODUCTION

Understanding the intricate connections between mental health and physiological processes has emerged as an important focus of contemporary research (World Health Organization, 2017). Psychiatric disorders, recognized as leading causes of disability globally, have garnered considerable attention owing to their profound impact on individuals and societies alike (Bains & Abdijadid, 2023). These disorders encompass a spectrum of highly prevalent and debilitating illnesses that significantly contribute to the burden of the disease. While the microbiota-gut-brain axis has been robustly linked to various neuropsychiatric disorders, including depression, anxiety, and psychosis, establishing a definitive cause-effect relationship remains a challenge (Chahwan et al., 2019; Kazemi et al., 2019; Ramírez-Carrillo et al., 2020; Uemura et al., 2019).

Research suggests that microbial dysbiosis, characterized by alterations in microbial diversity and function, is often accompanied by severe mental disorders, with depression being a prominent example (X. Hu et al., 2023). Notably, the use of antibiotics, implicated in reducing microbial diversity, has been associated with depression, suggesting a central role for gut microbial diversity in its pathophysiology (Pouranayatihosseinabad et al., 2023). Additionally, factors such as diet and environmental exposure have been linked to changes in gut microbiota composition, further underscoring the influence of the gut microbiome on mental health (Herselman et al., 2022).

Moreover, emerging evidence highlights sex-specific disturbances along the gut microbiota-brain axis as potential contributors to divergent mental health phenotypes in men and women (Audet, 2019; Jašarević et al., 2016). Recent findings by Bashir et al. (2024) showed that women with high depressive symptoms had increased levels of *Escherichia, Shigella,* and *Parabacteroides*, whereas women experiencing high perceived stress showed an increased abundance of *Escherichia, Shigella*, and *Blautia*. This dysbiosis is particularly significant in *Escherichia* and *Shigella*, which are potentially pathogenic bacteria, and correlates with alterations in neuroactive pathways, such as the glutamatergic, GABAeric, dopaminergic, and kynurenine pathways (Bashir et al., 2024). Notably, women exhibit a higher susceptibility to psychiatric disorders and demonstrate differential responses to psychotropic treatments, likely influenced by sex-based physiological and molecular differences that affect antidepressant drug dynamics (LeGates et al., 2019).

The role of the gut microbiome extends beyond its direct interaction with the brain to influence systemic inflammation and metabolic processes, which are increasingly recognized as important factors in mental health (D. Zheng et al., 2020). Chronic inflammation, often linked to dysbiosis, has been implicated in the pathophysiology of depression and other psychiatric disorders (Clapp et al., 2017). Similarly, metabolic disturbances associated with altered gut microbiota may contribute to psychiatric symptoms, suggesting that a comprehensive understanding of these pathways could inform novel therapeutic strategies (Grau-Del Valle et al., 2023; Liu et al., 2023).

The interplay between sex hormones, gut microbiome diversity, and metabolism of psychotropic drugs presents a complex yet intriguing area for further exploration (Manosso et al., 2021; Shobeiri et al., 2022). Hormonal fluctuations, particularly during puberty, menstruation, and menopause, can significantly alter the gut microbiota composition and function (Jašarević et al., 2016; Valeri & Endres, 2021), potentially influencing mental health outcomes (Cussotto et al., 2018). Preliminary studies suggest that specific probiotic strains and prebiotic fibers may have beneficial effects on mood and cognitive function, opening new avenues for clinical applications (Ķimse et al., 2024). Understanding these multifaceted microbiome-host interactions holds promise for elucidating novel therapeutic strategies and personalized nutritional interventions for psychiatric disorders (Zhao et al., 2024).

The connection between sex hormones, gut microbiome diversity, and metabolism of psychotropic medications presents a complex yet intriguing area for further exploration. Understanding these multifaceted interactions holds promise for the elucidation of novel therapeutic strategies and personalized interventions for psychiatric disorders. Thus, this study aimed to delve into the nuanced dynamics of the sexually divergent gut microbiome and its interplay with psychotropic medication, shedding light on potential avenues for targeted interventions in psychiatric care.

CHAPTER 1:

Gut Feeling: Exploring The Intertwined Trilateral Nexus of Gut Microbiota, Sex Hormones, and Mental Health

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Abstract

The complex interplay between the gut microbiota, sex hormones, and mental health is emerging as a pivotal factor in understanding and managing psychiatric disorders. Beyond their traditional roles, sex hormones exert profound effects on various physiological systems including the gut microbiota. Fluctuations in sex hormone levels, notably during the menstrual cycle, influence gut physiology and barrier function, shaping gut microbiota composition and immune responses. Conversely, the gut microbiota actively modulates sex hormone levels via enzymatic processes. This bidirectional relationship underscores the significance of the gut-brain axis in maintaining mental well-being. This review explores the multifaceted interactions between sex hormones, the gut microbiota, and mental health outcomes. We highlight the potential of personalized interventions in treating psychiatric disorders, particularly in vulnerable populations such as premenopausal women and individuals with depressive disorders. By elucidating these complex interactions, we aim to provide insights for future research into targeted interventions, enhancing mental health outcomes.

Keywords: Gut Microbiota; Sex Hormones; Mental Health Disorders; Microbiome-Targeted Interventions; Sex-Specific Responses

1. Introduction

The complex interplay between sex hormones, gut microbiota, and mental health represents a developing area of research with significant implications for health management (Cryan et al., 2019; Ganci et al., 2022). Recent advancements in microbiome research have introduced the concept of the "microsexome", which describes the phenomenon of sexual dimorphism of the microbiome and entails multidirectional interactions between sex hormones, various parameters of the microbiome, and the immune system (Mulak et al., 2022). The microbiota-gut-brain axis (MGBA), a bidirectional communication network linking the central nervous system (CNS) and gastrointestinal tract, plays a pivotal role in regulating physiological processes and mental well-being (Carabotti et al., 2015). This axis encompasses a diverse array of signaling mechanisms, including endocrine, humoral, metabolic, and immune pathways, through which the gut microbiota and sex hormones exert reciprocal influences on CNS function and mental health (Appleton, 2018; Santos-Marcos et al., 2023).

Emerging evidence highlights the significant impact of the gut microbiota on mental health outcomes (Basiji et al., 2023). Disruptions in the composition and function of gut microbiota have been implicated in stress, anxiety, and depression (Grau-Del Valle et al., 2023). Communication between the gut microbiota and CNS occurs through the production of microbiota-derived metabolites, neuroactive substances, and modulation of immune responses, emphasizing the crucial role of MGBA in maintaining mental well-being (Brydges et al., 2021; Huo et al., 2017; Mosaferi et al., 2021). More specifically, a meta-analysis showed that depleted levels of *Faecalibacterium* and *Coprococcus* and enriched levels of *Eggerthella* were consistently observed across major depressive disorder (MDD), bipolar disorder, psychosis, schizophrenia, and anxiety (Nikolova et al., 2021). Furthermore, recent studies have shed light on the multifaceted interactions between sex hormones, gut microbiota, and mental health outcomes (Barth et al., 2015; Chen et al., 2018; S.-Y. Kim et al., 2023). Sex hormones, such as

androgens and estrogens, not only play critical roles in sexual differentiation and reproductive processes, but also exert profound effects on various physiological systems, including cardiovascular, immune, and neuroendocrine functions (Campbell & Jialal, 2024). Additionally, sex hormones contribute to CNS function by influencing neurogenesis, neuroprotection, learning, memory, and mood regulation (McEwen & Milner, 2017). Furthermore, sex-specific differences in gut microbiota composition, hormone regulation, and mental health outcomes have been increasingly recognized (Fang et al., 2021; Jamilian et al., 2018; Ostadmohammadi et al., 2019; Shobeiri et al., 2022).

In this paper, we comprehensively review the current understanding of the complex interplay between the gut microbiota and sex hormones, particularly focusing on their intertwined interactions and their potential impact on mental health. By synthesizing current knowledge in this field, we aimed to provide insights for future research directions and facilitate the development of personalized interventions for individuals with mental health disorders.

2. Overview of The Gut Microbiota in the Early Stages of Life

Microbial colonization of the intestine occurs even before birth as a result of the microbiota present in the amniotic fluid and placenta (Collado et al., 2016; Jiménez et al., 2008). Previous research has indicated the presence of bacteria and bacterial components, including DNA, in the meconium, amniotic fluid, and placenta itself, without causing noticeable effects on either the course of pregnancy or the well-being of the newborn (Bushman, 2019; Chu et al., 2016; Jiménez et al., 2008; L. Stinson et al., 2020). Stinson et al. (2019) collected meconium and amniotic fluid samples and subjected them to full-length 16S rRNA gene sequencing using PacBio SMRT cell technology for high-resolution profiling of fetal gut and amniotic fluid bacterial microbiomes. Bacterial DNA was found in all meconium samples and in the majority of amniotic fluid samples, with *Pelomonas puraquae* dominating the meconium microbiome,

suggesting that the fetus is exposed to bacterial DNA and metabolites prior to birth (L. F. Stinson et al., 2019). While the aforementioned studies have shown the presence of microorganisms in prenatal intrauterine locations, a recent study contradicts this hypothesis (Kennedy et al., 2023). The currently available data do not substantiate the presence of robust and stable microbial populations under typical non-pathogenic conditions (Kennedy et al., 2023). Therefore, more research is needed to better understand microbial interactions at the maternal-fetal interface (Bolte et al., 2022). Recent advancements have highlighted the role of microbiota-derived extracellular vesicles (EVs) in maternal-fetal communication (Kaisanlahti et al., 2023; Menon et al., 2023). These EVs, found in the amniotic fluid of healthy pregnant women, resemble those found in the maternal gut microbiota and carry a diverse protein cargo, including bacterial proteins originating from predominant phyla, such as Bacteroidetes and Firmicutes (Kaisanlahti et al., 2023). Importantly, the presence of bacterial EVs in amniotic fluid suggests a potential mechanism by which the maternal microbiota communicates with the fetus (Kaisanlahti et al., 2023).

The method of delivery significantly influences the early development of gut microbiota (C. Zhang, Li, et al., 2021). In newborns delivered vaginally, *lactobacilli* and *Prevotella* are most commonly present in the gut microbiota, which originate from the mother's vaginal microbiota (Biasucci et al., 2010). In contrast, infants born via cesarean section acquire gut microbiota from the skin, resulting in an abundance of *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* (Dominguez-Bello et al., 2010). Reyman et al. (2019) assessed the effect of delivery mode on gut microbiota in 74 vaginally delivered and 46 cesarean section-born infants (Reyman et al., 2019). Significant differences have been observed in the composition and stability of the gut microbiota in infants, particularly during the initial months of life (Reyman et al., 2019). Notably, *Bifidobacterium* was found to be more abundant in vaginally delivered children, consistent with the existing literature on the subject (Wampach et al., 2017).

Following birth, the intestinal microbes in infants are primarily sourced from the mother's skin, breastfeeding, and/or formula feeding. One of the earliest influences on gut microbiota is diet (Bezirtzoglou et al., 2011). The process of intestinal colonization is significantly influenced by feeding, with breastfed and formula-fed infants exhibiting differences in their microbial composition (Coker et al., 2021). Breast milk composition plays a pivotal role in shaping the gut microbial community (Van Den Elsen et al., 2019). In breastfed infants, the prevailing species in the gut microbiota are *lactobacilli* and *Bifidobacterium* (B. Yang et al., 2019). Breast milk, owing to its abundant supply of unique oligosaccharides, is the most effective stimulant for the development of a balanced infant microbiome. The oligosaccharides present in breast milk are readily broken down by certain species, leading to an increase in short-chain fatty acids (SCFA) (Williams et al., 2019). SCFAs play various roles in intestinal physiology by contributing to homeostasis, promoting epithelial barrier function, and regulating the turnover of intestinal epithelial cells (Koh et al., 2016). Human milk contains all types of immunoglobulins (Igs), with the most predominant being secretory IgA (sIgA) followed by sIgG (Demers-Mathieu et al., 2018). In contrast, infants fed formula tended to show a predominance of *Enterococcus*, *Enterobacteria*, *Bacteroides*, *Clostridium*, and *Streptococcus* in their gut microbiota (Wang et al., 2020).

As infants grow, primary microbial communities established during infancy may have a significant impact on their immunity. The primary microbiota of infants during this crucial period plays an important role in protecting them from illnesses associated with compromised immunity (Y. Yao et al., 2021). These primary microbial communities undergo a gradual transformation over time, increasing and becoming more diverse and achieving relative stability by the age of three years, when these microbial communities closely resemble those found in the adult gut microbiota (Koenig et al., 2011).

Gut microbiota continues to develop after infancy, and diet is key to defining its structure, diversity, and shape (MetaHIT Consortium et al., 2014; J. Yang et al., 2021). Long-term and short-term alterations in dietary composition can affect the structure and activity of gut microbiota (Ecklu-Mensah et al., 2022; D. So et al., 2018; Tomova et al., 2019). The predominant microbial communities in the human gut are closely linked to the ratio of microbiota-accessible carbohydrates, dietary fats, and proteins in the overall dietary makeup (Senghor et al., 2018). Restricting microbiota-accessible carbohydrates not only results in a loss of bacterial diversity but also leads to a reduction in SCFAs, affecting various physiological processes, including energy homeostasis, lipid metabolism, and inflammation (Nogal et al., 2021). Although total dietary proteins and amino acid composition also influence SCFA production, the molecular mechanisms underlying these effects are not fully understood (Oliphant & Allen-Vercoe, 2019).

Beyond environmental influences on the gut microbiota, another layer of complexity emerges with sex-related differences in the gut microbial composition (Y. S. Kim et al., 2020). Interestingly, although sex differences in gut microbes are present in infancy, these disparities undergo significant increases during puberty, concomitant with changes in endogenous sex hormone levels (Jašarević et al., 2016; Valeri & Endres, 2021).

3. A Trilateral Intertwined Relationship

The complex communication between sex hormones, gut microbiota, and mental health underscores a dynamic trilateral relationship with important implications for human well-being (S.-Y. Kim et al., 2023; Niemela et al., 2024). At the core of this intertwined relationship lies the MGBA, a complex network that facilitates bidirectional communication between the CNS and gastrointestinal tract (Rutsch et al., 2020). Through sophisticated signaling mechanisms encompassing endocrine, humoral, metabolic, and immune pathways, MGBA maintains a

delicate balance crucial for physiological processes and mental well-being (Mitrea et al., 2022) (Figure 1.2).

3.1. Sex Hormones Influence the Gut Microbiome and Mental Health

3.1.1. Role of Sex Hormones in Shaping the Gut Microbiome

Although it is traditionally believed that the gut microbiome remains unaffected by biological sex during childhood (Yoon & Kim, 2021), a recent study challenged this assumption (Chen et al., 2021). Investigating the developmental trajectory of the gut microbiota and sex-specific microbial characteristics in preterm twins and triplets during their initial 28 days in the neonatal intensive care unit revealed significant differences between the female twins/triplets and male twin groups in gut microbiome diversity, composition, and predicted metabolic profiles. Furthermore, it highlights the significant dissimilarities between females and males within the co-twin/triplet pairs of the mixed-sex twin/triplet group (Chen et al., 2021).

During puberty, gonadal sex hormones significantly influence the gut microbiome (Valeri & Endres, 2021). Accumulating evidence indicates that gut microbiota may play an important role in influencing the onset of puberty through metabolic and hormonal mechanisms (Korpela et al., 2021). Furthermore, there is a possibility that the composition of gut microbiota is intricately linked to hormonal signals (Dong et al., 2020). This hormonal transition becomes particularly evident as girls progress through puberty, with their fecal microbiota adopting a more adult-like profile (Korpela et al., 2021). Unlike boys, girls exhibited a statistically significant increase in the relative abundance of estrogen-metabolizing *Clostridia* and a concurrent decrease in *Bacteroidia* during pubertal development. Hormonal fluctuations during puberty can also affect immune reactions to gut microbes, resulting in shifts in the microbial composition (Yurkovetskiy et al., 2013). Moreover, changes in bile acid metabolism,

influenced by sex hormones, may further alter the gut microbiota composition, thus affecting pubertal timing via metabolic signaling pathways involving bile acids (Org et al., 2016). Given the complexity of puberty as a biological process, there may be additional uncharacterised mechanisms at play. These findings underscore the evolving role of the gut microbiota in shaping the timing of puberty, potentially through metabolic and hormonal interactions, supporting the notion that gut microbiota composition may be responsive to hormonal signals (Kheloui et al., 2023; Korpela et al., 2021).

Huang et al. (2023) analyzed the gut microbiome profile and metabolic alterations in patients with central precocious puberty (CPP), a disease in which the development of secondary sexual characteristics begins earlier than expected owing to the early release of gonadotropin-releasing hormone (GnRH) (Huang et al., 2023). The genera *Bifidobacterium* and *Streptococcus* were highly enriched in CPP and both were associated with GnRH signal transduction. The fecal metabolites estrone sulfate, 3-dehydroepiandrosterone sulfate, androsterone glucuronide, 11β-hydroxyprogesterone, and corticosterone were significantly different between CPP patients and healthy controls. Notably, estrone sulfate and androsterone glucuronide were highly enriched in CPP, which was correlated with GnRH activation. The enrichment of specific genera and the presence of distinct metabolites suggest a potential interplay between sex hormones and the gut microbiome, highlighting the complex relationship between endocrine function and microbial composition during puberty (Carson et al., 2023). A survey of the literature investigating the relationship between sex and the gut microbiome strongly supports the hypothesis that human and rodent gut microbiomes differentiate during puberty, and that this differentiation results in sex-specific communities by adulthood (Korpela et al., 2021; Markle et al., 2013; Yuan et al., 2020).

Studies have consistently identified distinct gut microbial communities between the sexes (De La Cuesta-Zuluaga et al., 2019; Org et al., 2016; X. Zhang et al., 2021). In a study involving

341 female and 348 male mice from 89 matched strains, significant differences in microbiota composition and diversity were observed between the sexes within individual strains (Org et al., 2016). Permutational multivariate analysis indicated that both sex and strain exerted significant effects on microbiota. Further investigation revealed that the phyla *Actinobacteria* and *Tenericutes* and several taxa, including *Allobaculum*, *Anaeroplasma*, and *Erwinia*, were more abundant in males, whereas *SMB53, Dorea, Coprococcus*, and *Ruminococcus* from the family *Lachnospiraceae* were more abundant in females. Subsequent analyses with specific strain subsets and multiple cages confirmed the consistency of the observed sex-related differences, highlighting the impact of both host sex and strain on gut microbiota composition (Org et al., 2016).

In the same study, distinct sex differences in gut microbiota composition were identified in both regular chow and high-fat diets in three mouse strains (Org et al., 2016). Combining the mouse strains in different treatment groups revealed significant sex effects on the microbiota. Hormonal status had a more pronounced effect on microbiota composition in male mice fed a chow diet, whereas in female mice, this effect was more evident in response to a high-fat diet. Post-gonadectomy testosterone administration prevented significant changes in the gut microbiota composition of both the strains. Notably, *Ruminococcacea* abundance of Ruminococcaceae varied significantly between gonadectomized and sham control male mice fed a high-fat/high-sucrose diet. In females, a distinct separation was observed in response to the high-fat/high-sucrose diet, with strain-specific differences in *Akkermansia* abundance between the sham control and gonadectomized mice (Org et al., 2016).

Mueller et al. (2006) found sex-specific differences in *Bacteroides-Prevotella* with higher levels in men than in fertile women in a sample of 230 healthy individuals (Mueller et al., 2006). Recent findings further suggest that hormonal changes during the postpartum phase may influence the composition and diversity of gut microbiota, potentially contributing to variations

in mental well-being among mothers (Matsunaga et al., 2024). Furthermore, in a recent meta-analysis, Yang et al. (2022) analyzed the differences in gut microbiota in women before and after menopause (M. Yang et al., 2022). Primary findings revealed no differences in *Firmicutes*, *Bacteroidetes*, or *Proteobacteria* during menopause at the phylum level. However, at the genus level, postmenopausal women exhibited a higher relative abundance of *Odoribacter* and *Bilophila* than did premenopausal women. Furthermore, *Sutterella*, *Roseburia*, and *Blautia* showed a declining pattern after menopause, whereas *Prevotella*, *Parabacteroides*, and *Bacteroidetes* showed an increasing trend. Additionally, estradiol levels are positively correlated with the presence of *Gammaproteobacteria*, particularly *Shewanella putrefaciens* and *Erwinia amylovora* (M. Yang et al., 2022).

Sex hormones directly modulate bacterial metabolism through steroid receptors, a regulatory mechanism that significantly affects the intestinal microbial diversity (Hussain et al., 2021). This influence extends to the regulation of gene expression, protein production, and various processes within the gut microbiota (Gancz et al., 2023). Sex hormones have bidirectional effects on the gut microbiome. In reciprocal dynamics, the microbial population actively shapes sex hormone levels, thereby conditioning the physiological responses (García-Gómez et al., 2013). Pregnancy introduces an additional layer into this complex interaction with progesterone, assuming a critical role in influencing crucial physiological changes for fetal development and maintaining an optimal gestational environment (Soma-Pillay et al., 2016). Recognized as the principal gestation hormone, progesterone influences the gut microbial community, as evidenced by the increased relative abundance of *Bifidobacterium in vitro* (Nuriel-Ohayon et al., 2019). However, research on the impact of sex hormones on the gut microbiome has largely focused on estrogen and androgen rather than progesterone.

Recent studies have explored the complex relationship between serum testosterone levels and the composition of the gut microbiota, shedding light on bidirectional interactions (d'Afflitto

et al., 2022; Del Castillo-Izquierdo et al., 2022). Matsushita et al. (2022) identified a positive correlation between serum testosterone levels and relative abundance of Firmicutes in elderly men. Remarkably, they observed that the abundance of Firmicutes significantly influences serum testosterone levels (Matsushita et al., 2022). Furthermore, Markle et al. (2013) demonstrated that in female mice, elimination of commensal microbiota increased circulating testosterone levels (Markle et al., 2013). Shin et al. (2019) stratified participants into low, medium, and high groups based on serum testosterone and estradiol levels in both men and women. Notably, the high-testosterone group exhibited a decrease in *Atopobium* and an increase in *Acinetobacter*, *Dorea*, *Ruminococcus*, and *Megammonas*. Among these, *Ruminococcus* is the most responsive to testosterone levels (J.-H. Shin et al., 2019).

It has been established that the microbiota can affect estrogen levels, and reciprocally, the composition and diversity of the microbiota may be influenced by estrogen levels (d'Afflitto et al., 2022). The estrobolome is defined as a collection of specific genes within the microbiome, whose products are capable of metabolizing estrogens through the expression of β-glucuronidase (Figure 1.1), an enzyme that facilitates the deconjugation of both dietary and non-dietary estrogens, implying that gut microbial communities have a significant influence on hormonal profiles (Baker et al., 2017). For example, in mice, gavage with *Klebsiella aerogenes*, isolated from the fecal samples of women, led to an estradiol decline. Notably, a previous study identified the estradiol-degrading enzyme responsible for this effect as 3β-hydroxysteroid dehydrogenase (D. Li et al., 2023).

Experiments involving the injection of radiolabeled estrogen into women showed that approximately 65% of estradiol (E2), 48% of estrone (E1), and 23% of estriol (E3) were retrieved from the bile (Adlercreutz & Järvenpää, 1982). Additionally, approximately 10–15% of E1, E2, and E3 were found in a conjugated state in feces, indicating that the majority of deconjugated estrogens undergo reabsorption through deconjugation activity (Adlercreutz &

Järvenpää, 1982). Disruption of the balance between reabsorption and excretion ratios can result in significant fluctuations in estrogen levels within the body, potentially leading to estrogen-related diseases (S. Hu et al., 2023).

Another piece of evidence for the metabolism of sex hormones by the gut microbiome is the presence of metabolites in the wastewater (Almazrouei et al., 2023; González et al., 2020). Estrogens, progestins, and androgens are produced and released in large quantities by both human and animal fecal matter, ultimately making their way into wastewater systems (Combalbert & Hernandez-Raquet, 2010; Jauković et al., 2017). This poses a significant environmental concern as aquatic organisms are highly susceptible to contamination by steroid hormones, leading to morphological changes, behavioral alterations, developmental abnormalities, fertility issues, and reproductive disorders (Adeel et al., 2017).

3.1.2. Sex Hormones modulate the Gut-Brain Axis

3.1.2.1. The Gut-Brain Axis

The intricate communication between the microbiota, gut, and brain, known as the microbiota-gut-brain axis (MGBA), has immense therapeutic potential (Cryan et al., 2019). Signaling mechanisms within MGBA are diverse and involve bidirectional pathways that connect the central nervous and digestive systems (Carabotti et al., 2015). Through the hypothalamic–pituitary–adrenal (HPA) axis and autonomic nervous system, the brain influences intestinal functions, including functional immune effector cells, mood, cognition, and mental health (Appleton, 2018). Conversely, the gut modulates central nervous system (CNS) functions by employing microbiota-derived metabolites, neuroactive substances, gut hormones, and neurotransmitters. These include a range of compounds such as serotonin, kynurenine, indoles, dopamine, glutamate, γ-aminobutyric acid (GABA), acetylcholine, short- and medium-chain

fatty acids, secondary bile acids, and branch-chain amino acids (Brydges et al., 2021; Huo et al., 2017).

This bidirectional network extends beyond mere anatomical connections and incorporates endocrine, humoral, metabolic, and immune pathways (Appleton, 2018). The autonomic nervous system, HPA axis, and gastrointestinal tract nerves collectively form the gut-brain axis, enabling reciprocal influence (Cryan et al., 2019). Huo et al. (2017) investigated the effect of intestinal microbes on the HPA axis by analyzing the behavior, hormone levels, and receptor expression within the HPA axis, employing a chronic restraint stress model in mice under either germ-free or specific pathogen-free conditions (Huo et al., 2017). The heightened levels of corticotropin-releasing hormone, adrenocorticotropic hormone, and cortisol in germ-free restraint-stressed mice signify exaggerated activation of the HPA axis. The enhanced release of stress hormones may be a physiological response to perceived stressors in the absence of certain gut pathogens. This study implies that the composition of the gut microbiota has a substantial impact on behavioral and hormonal responses to stress (Huo et al., 2017). Kelly et al. (2016) demonstrated that transferring gut microbiota from depressed patients to rats with depleted gut microbiota induces the development of depressive features such as anhedonia and anxiety-like behaviors, along with a physiological profile resembling that of depressed individuals (Kelly et al., 2016). Similarly, Zheng et al. (2016) showed that fecal microbiota transplantation in germ-free mice with depressive microbiota derived from MDD patients resulted in depression-like behaviors compared with colonization with 'healthy microbiota' from healthy individuals (P. Zheng et al., 2016). Multiple authors have further supported these observations by showcasing animal models with fecal microbiota transplantation from donors with mental disorders such as depression, anxiety, and stress (Jang et al., 2021; Knudsen et al., 2021; N. Li et al., 2019).

3.1.2.2. Sex Hormones modulate the Gut-Brain Axis

In addition to the complex dynamics of MGBA, sex hormones exert significant effects on both the gut and the brain (He et al., 2021). These hormones, known for their roles in sexual differentiation and reproductive processes, also affect various physiological systems, including cardiovascular, skeletal, immune, and muscular (Campbell & Jialal, 2024). Moreover, sex hormones contribute to CNS function by promoting neurogenesis, neuroprotection, learning, and memory, and by influencing brain structure and cognitive function (McEwen & Milner, 2017; Sun et al., 2019). The effects of sex hormones are mediated through various receptors, such as intracellular or membrane-associated receptors, including the androgen and estrogen receptors (ERα and ERβ), as well as transmembrane receptors, such as zinc transporter protein 9 and G protein-coupled estrogen receptor 1 (Diotel et al., 2018). The interaction between gut microbiota and sex hormones in MGBA involves several mechanisms. For example, the gut microbiota regulates bile acid metabolism into secondary bile acids, which in turn influence the microbiota structure (Guzior & Quinn, 2021). Furthermore, enzymatic processes in the gut microbiota, particularly β-glucuronidases and hydroxysteroid dehydrogenase, can affect sex hormone levels and activity (Ervin et al., 2019). Additionally, phytoestrogens, which are plant compounds activated by gut microbiota, exhibit structural and functional similarities to estrogens, inducing physiological effects through cell signaling and affecting estrogen receptors, thereby triggering epigenetic effects (Domínguez-López et al., 2020; Rietjens et al., 2017).

The menstrual cycle affects not only the gut microbiota composition but also the immune response (Hussain et al., 2021; Roomruangwong et al., 2019). Roomruangwong et al. (2019) showed that peak IgA responses to lipopolysaccharides (LPS) in gram-negative bacteria occur around day 28, coinciding with increased bacterial proliferation during the secretory phase. Conversely, lower IgA levels were observed around days 7–14, suggesting variations in LPS

load and potential bacterial translocation during menstruation. These fluctuations may contribute to the pathophysiology of premenstrual and peri-menstrual syndrome symptoms, as evidenced by the association between IgA responses to LPS and symptom domains, such as physio-somatic symptoms, breast-craving, and anxiety (Roomruangwong et al., 2019). Furthermore, the impact of the menstrual cycle extends beyond immune responses, including hormonal modulation of the gut physiology and barrier function. Fluctuations in sex hormone levels, particularly progesterone, influence colonic transit time, smooth muscle contraction, and barrier integrity by modulating tight and adherens junctions (Coquoz et al., 2022; Matos et al., 2016; Nie et al., 2018). Increased gut permeability during the luteal phase, coupled with relative corpus luteum insufficiency, may contribute to increased bacterial translocation and subsequent IgA production, potentially exacerbating premenstrual symptoms (Roomruangwong et al., 2019).

Sex hormones, specifically androgens and estrogens, play a role in shaping mental health outcomes (Barth et al., 2015). Androgens exhibit anxiolytic properties, potentially causing lower rates of anxiety in males. In males, decreased testosterone levels are associated with sleep disruptions such as nocturnal awakenings, reduced sleep efficiency, and symptoms of depression (Cherrier et al., 2015; Shigehara et al., 2018). As men age and experience declining testosterone levels, they become more susceptible to depressive symptoms (Zarrouf et al., 2009). Administering testosterone to individuals with depressive disorders may alleviate these symptoms (Pope et al., 2003). Conversely, disrupted sleep patterns can lead to decreased testosterone levels owing to altered nighttime secretion. Furthermore, a meta-analysis showed that testosterone treatment significantly reduced depressive symptoms in men compared with placebo (Walther et al., 2019).

Estrogens, on the other hand, enhance the HPA axis activity. Several studies have consistently indicated that elimination of most endogenous estrogens through ovariectomy leads to a

reduction in stress-induced adrenocorticotropin and corticosterone levels (Figueiredo et al., 2007; Garcia et al., 2018). Conversely, estradiol treatment produces opposing effects (El-Khatib et al., 2020; Walf & Frye, 2010). In the paraventricular nucleus, estradiol has been demonstrated to enhances stress-induced neuronal activation and elevates the expression of *Crh* and *Avp* genes (Ogura et al., 2008). This heightened HPA axis activity in response to estradiol may explain the higher rates of anxiety and depression observed in women (Oyola & Handa, 2017; Patchev, 1996). Furthermore, a strong body of evidence suggests that fluctuations in sex hormones among women are the primary biological factors contributing to sex differences in depression risk (Albert, 2015; Kundakovic & Rocks, 2022; Thériault & Perreault, 2019). Moreover, adult female mice exhibit a higher expression of inflammation-related genes in microglial cells than male mice, potentially contributing to neuroinflammation (Lynch, 2022). These microglia, as resident immune cells of the CNS, respond to changes in gut microbiome composition (D'Alessandro et al., 2022). Microbial effectors, including SCFAs and tryptophan-derived metabolites, influence the intestinal barrier integrity, immune system activity, and vagal nerve stimulation (Silva et al., 2020). Thus, there is a strong bidirectional relationship between endogenous sex hormones and the gut microbiome, which may impact neurobiological and neuroendocrine functions in the host through the gut-brain axis (Cussotto et al., 2018).

Investigating sex-specific comparisons, which are relatively underexplored or unreported in many studies, may play a crucial role in understanding the complex interactions in mental health (Jaggar et al., 2020). Table 1.1 provides a summary of studies investigating the interactions between sex hormones, the gut microbiota, and mental health.

3.2. Gut Microbiota Influence Host Sex Hormone Levels and Mental Health

Microbial communities within the gut produce enzymes that can metabolize sex hormones, thereby influencing their levels and activities in the body (Ervin et al., 2019; D. Li et al., 2022,

2023). These enzymes enable gut bacteria, such as *Bacteroides* and *Clostridium,* to reactivate estrogens by cleaving glucuronide bonds with deconjugating enzymes such as β-glucuronidase (Ervin et al., 2019). This process, particularly via deglucuronidation, regulates active estrogen levels within the body. Estrogens are rapidly deactivated in the liver via conjugation reactions (Cotton et al., 2023), thereby preventing their interaction with receptors and facilitating their elimination from the body (Baker et al., 2017). Gut bacteria also convert and modulate estrogen-like compounds (Nakatsu et al., 2014), with certain bacterial strains, such as those in the Coriobacteriaceae family, metabolizing compounds structurally similar to estrogens, affecting estrogen receptor activity and gene expression (Seyed Hameed et al., 2020).

Similarly, androgens are modulated by gut microbiota, affecting male physiological characteristics and disease susceptibility. Bacterial enzymes can synthesize and degrade testosterone, affecting systemic hormone levels and disease outcomes (D. Li et al., 2022; Sambyal & Singh, 2020). Clinical studies have linked alterations in microbiota composition to changes in androgen levels, implicating the microbiota in conditions such as polycystic ovary syndrome and prostate cancer (Guo et al., 2016; Wheeler & Liss, 2019). In depression, the degradation of testosterone by the microbiota has been associated with low hormone levels and depressive symptoms, highlighting the potential of microbiota-targeted interventions to modulate hormone levels and alleviate symptoms (D. Li et al., 2022).

Dysbiosis can disrupt the metabolism of estrogens and androgens by altering the abundance and activity of bacteria involved in hormone synthesis and degradation (Qi et al., 2021). This dysregulation can lead to fluctuations in systemic hormone levels, which have been linked to various health conditions including depression (K. M. Albert & Newhouse, 2019). SCFAs influence reproductive health, particularly in women (Mirzaei et al., 2023), by affecting sex steroid hormone levels through signaling pathways involving leptin, GnRH, and gonadotropin production (X. Yao et al., 2023). Butyric acid controls progesterone and estradiol synthesis in

granulosa cells via the cAMP signaling pathway, which is possibly mediated by GPR41/43 receptors (N. Lu et al., 2017). In a retrospective cross-sectional study involving patients undergoing *in vitro* fertilization or intracytoplasmic sperm injection and embryo transfer, fecal propionate levels were significantly higher in the group with no pregnancies than in the group with clinical pregnancies (X. Yao et al., 2023). High fecal propionate concentration is negatively associated with clinical pregnancy outcomes and positively correlated with fasting serum insulin, triglycerides, and insulin resistance (X. Yao et al., 2023). Additionally, peptide YY and glucagon-like peptide-1, which are influenced by SCFAs, regulate gonadotropin secretion and serve as important signals in the female reproductive system (Taylor et al., 2009). Studies have shown that disruptions in gonadotropin levels may contribute to depression-like behaviors (Padda et al., 2021) observed in stressed animals, highlighting a potential pathway through which alterations in reproductive hormones intersect with mental health (Sahin et al., 2022).

Previous research has suggested that gut microbiota has a potential influence on mental health and hormone regulation (Mosaferi et al., 2021). Evidence shows that the microbiota significantly influences the hippocampal serotonergic system, with observed sex-specific effects, particularly in male germ-free animals exhibiting serotonergic alterations, decreased BDNF expression in the hippocampus, and fewer anxiety-like behaviors (Clarke et al., 2013). In individuals with MDD, the relative abundance of *Actinobacteria* and *Bacteroidetes* differed significantly between women and men compared to their respective healthy counterparts. Specifically, *Actinobacteria* increased in women with MDD, whereas *Bacteroidetes* decreased in men with MDD, suggesting notable sex differences in the gut microbiota composition (Chen et al., 2018). Cross-sex transfer of the cecal microbiota between male and female mice affects depressive-like behaviors, with recipients of the female cecum displaying decreased grooming

behavior compared to both controls and recipients of the male cecum, suggesting increased depressive-like behavior (Hinks et al., 2023).

Recently, researchers reported that *Klebsiella aerogenes*, isolated from fecal samples of women experiencing depression, led to a decline in estradiol levels and induced depression-like behavior in mice (D. Li et al., 2023). Similarly, *Mycobacterium neoaurum*, isolated from fecal samples of men with testosterone deficiency and depression, degrades testosterone *in vitro*, reduces serum and brain testosterone levels, and causes depression-like behaviors in rodents (D. Li et al., 2022). Notably, *Akkermansia* spp., a gut microbe associated with metabolic health and stress responses, showed contrasting associations with anxiety in male and female mice. Male mice exposed to chronic social defeat stress exhibited a lower abundance of *Akkermansia*, which is potentially associated with increased anxiety (McGaughey et al., 2019). Conversely, *Akkermansia* is positively associated with increased anxiety in female mice, highlighting sex-specific differences in microbial responses to stress (Acharya et al., 2023).

3.3. Mental Health Influences Microbiota and Sex Hormone Levels

Recent studies have demonstrated the impact of mental health on sex hormone levels. For example, Pletzer et al. (2021) investigated gonadal hormone responses to social evaluative stress in both males and females (Pletzer et al., 2021). The results revealed that stress significantly affected testosterone and estradiol levels in both the sexes. Specifically, testosterone levels decreased and estradiol levels increased after stress. Surprisingly, some participants exhibited a decrease in estradiol levels in response to stress, showing individual variability in gonadal hormone stress response. Importantly, these gonadal stress responses are largely independent of cortisol levels, suggesting that the endocrinological stress response extends beyond the HPA axis (Pletzer et al., 2021; Zueger et al., 2023). Stress, particularly chronic stress, disrupts gut microbiota and contributes to dysbiosis (F. Gao et al., 2022). Stress

signals conveyed through the autonomic and circulatory systems and immune cells can trigger inflammation and promote the growth of pathogenic bacteria (Rust et al., 2023). Both acute and chronic stressors have been shown to alter the composition of gut bacteria, affecting various regions of the gastrointestinal tract (Leigh et al., 2023). Dysbiosis induced by stress can have wide-ranging effects on immune function, metabolism, stress reactivity, mood, and sex hormone regulation, potentially exacerbating mental health symptoms and increasing the risk of infection and autoimmune diseases (Anand et al., 2022). Stress and depression can also lead to increased gut barrier permeability, commonly referred to as a "leaky gut," allowing bacteria to enter the circulation and trigger inflammation (H.-N. Kim et al., 2018; Ohlsson et al., 2019).

4. Sex-specific Psychotropic Response and Interplay with the Gut Microbiota

Men and women demonstrate distinct responses to pharmacotherapy, owing to variations in drug absorption, metabolism, and distribution within the body (Soldin & Mattison, 2009). These differences stem from variations in pharmacokinetic and pharmacodynamic factors including bioavailability, volume of distribution, and plasma protein binding. Additionally, hormonal fluctuations throughout a woman's life, such as those associated with menstrual cycles, pregnancy, and lactation, further influence the drug response by altering pharmacokinetic parameters and the activity of drug-metabolizing enzymes (Mitchell et al., 2009). Despite these disparities, the contrast in drug kinetics tends to diminish during menopause as estrogen and progesterone production declines, leading to a convergence in drug responses between the sexes (Seeman, 2020). Psychotropic medications, which are commonly used to manage psychiatric disorders, are no exception to sex-specific responses (Bolea-Alamanac et al., 2018).

Women tend to utilize psychotropic medications more frequently than men (Boyd et al., 2015). A study encompassing 1999 individuals aged ≥ 20 years revealed a prevalence of psychotropic drug use of 11.7%, with a notable discrepancy between the sexes (7.3% among men and 15.8% among women). In particular, the utilization of antidepressants was significantly higher among women, accounting for 44.3% of their usage, compared to 25.5% among men (Estancial Fernandes et al., 2018). Women also tend to take multiple psychotropic medications and are more vulnerable to adverse drug reactions, including nonspecific side effects such as nausea, dizziness, and sexual dysfunction (Shetageri et al., 2016). Women have a significantly higher incidence of hypothyroidism, and tend to experience substantial weight gain during lithium treatment (Gitlin, 2016). Endocrinological abnormalities such as hyperandrogenism and menstrual irregularities are also more prevalent in women undergoing mood-stabilizing treatments (Qadri et al., 2018).

Beyond direct pharmacological effects, emerging research suggests that psychotropic medications can affect the gut microbiota, adding another layer to the complexity of sex-specific responses to treatment (Ait Chait et al., 2023a; Ortega et al., 2023). Most psychopharmaceuticals exhibit antibacterial activity, which may influence the microbial diversity over time (Ait Chait et al., 2020a). In a previous study of 40 patients diagnosed with depression and/or anxiety, changes in microbial diversity were observed, particularly in those receiving antipsychotics, where a significant decrease in diversity was noted (Tomizawa et al., 2021). Furthermore, β-diversity analysis revealed notable compositional changes in the gut microbiota structure between patients receiving antipsychotics and those who did not, indicating the potential influence of these medications on the microbial composition (Tomizawa et al., 2021). Antipsychotic dosage showed a negative correlation with gut microbial diversity, suggesting a dose-dependent effect on the microbiome. Selective serotonin reuptake inhibitors are associated with alterations in microbial abundance, such as decreased *Turicibacteraceae*

and increased *Eubacterium ramulus* (Rogers et al., 2013). Additionally, the use of mirtazapine, fluoxetine, and nortriptyline has been linked to changes in gut microbiome composition, including the expansion of antimicrobial resistance genes and increased colonization by pathogens such as *Clostridium difficile* and *Enterococcus faecium* (Nagata et al., 2022; Rogers et al., 2013).

5. Nutritional Interventions and Sex-specific responses

Several studies have investigated the effects of interventions targeting both sex hormones and the gut microbiota on mental health parameters (Acharya et al., 2023; Fang et al., 2021; Ostadmohammadi et al., 2019). For example, the antidepressant-like effects of the aqueous extract of pomegranate (AE-PG) have been investigated in ovariectomized female rats (Valdés-Sustaita et al., 2017). AE-PG demonstrated behavioral profiles similar to those of citalopram and estrogen, with a combination of citalopram and AE-PG producing synergistic effects. The potential mechanisms underlying the antidepressant-like effects of AE-PG include inhibition of monoamine oxidase enzymes, metabolism of ellagitannins by gut microbiota resulting in the production of urolithins, and activation of estrogen receptors, particularly ERβ, which is linked to changes in the sensitivity of the serotonergic system (Valdés-Sustaita et al., 2017). Additionally, co-administration of vitamin D and probiotics in women with polycystic ovary syndrome led to improvements in depressive and anxious behaviors, as indicated by reduced scores on various depression and anxiety scales. This synergistic effect was attributed to the regulation of the gut-brain axis, neuroprotection, and modulation of neurotransmitters such as GABA and serotonin (Ostadmohammadi et al., 2019).

Fang et al. (2021) investigated whether berberine could alleviate anxiety by modulating the intestinal microbiota under estrogen-deficient conditions in specific pathogen-free ovariectomized rats. These findings revealed positive associations, wherein genera enriched by

berberine treatment, such as *Bacteroides*, *Lactobacillus*, and *Akkermansia*, were positively correlated with equol metabolism. Equol production is significant because of its potential implications in treating menopausal symptoms, including anxiety. This study emphasizes the role of the gut microbiota in isoflavone metabolism and its potential in treating menopausal symptoms (Fang et al., 2021). Similarly, Acharya et al. (2023) analyzed the associations between gut microbiota composition, estrogen treatment, a high-fat diet (HFD), and anxiety-like behavior in mice. Microbial taxa enriched by a HFD, including *Ruminococcaceae, Mogibacteraceae, Peptostreptococcaceae, Erysipelotrichaceae, Lactococcus, A. muciniphila, Coprococcus, Coprobacillus, Adlercreutzia*, and *Oscillospira*, were positively correlated with increased anxiety-like behavior. Notably, *Mogibacteraceae*, *Peptostreptococcaceae*, and *Coprococcus* decreased in mice treated with estrogen, indicating that the proliferation of these microbial communities induced by HFD may increase the risk of anxiety and exacerbate it in the absence of estrogens (Acharya et al., 2023). In addition to emerging research, probiotic formulations already available on the market that address women's health needs, such as vaginal, urinary, and digestive health support, as well as those targeting menopausal symptoms and postmenopausal bone health, are gaining popularity. The probiotic supplement market for women's health is experiencing robust growth, driven by heightened consumer awareness and demand for health products (Technavio, 2024). This growth trajectory is expected to continue, with projections indicating a substantial increase in market size from 2023 to 2028, reaching an estimated USD 660.82 million (Technavio, 2024).

6. Current limitations and future perspectives

While the aforementioned studies provide valuable insights into the intertwined and complex interactions between sex-specific responses, the gut microbiome, and mental health outcomes, most of them are constrained by the limitations inherent in using animal models such as murine

models. Researchers often employ techniques such as gonadectomy to examine the influence of sex hormones on different parameters, allowing them to study the effects of sex hormone depletion or supplementation on various biological processes (Sovijit et al., 2021). Other methods, such as germ-free models and fecal microbiota transplantation, offer complementary approaches to understanding the complex relationships between sex hormones, gut microbiome, and mental health (N. Li et al., 2019). For instance, germ-free models allow researchers to study the effects of specific microbial colonization on physiological processes, whereas fecal microbiota transplantation facilitates investigations into how microbial communities influence mental health outcomes (Hinks et al., 2023; Jang et al., 2021). Although these models offer controlled environments and experimental manipulations, they may not capture the full complexity of human physiology and behavior, including the dynamics of the gut microbiome (Nguyen et al., 2015; C. Zhang, Franklin, et al., 2021).

To overcome certain limitations and enhance the translational relevance of preclinical research involving the gut microbiome, there is a compelling opportunity to incorporate *ex vivo* models such as bioreactors into experimental designs (Van Den Abbeele et al., 2023). Unlike animal models, bioreactors offer a unique platform that enables researchers to culture complex microbial communities in environments that closely mimic the human gastrointestinal tract (Davis Birch et al., 2023). Several models have been developed to replicate the complexities of the human gut microbiome (Von Martels et al., 2017). Humanized gut bioreactors recreate diverse microbial communities found in the human gut by inoculating them with fecal samples obtained from human donors (Bayer et al., 2021). Age-specific gut models, for example, have been developed to mimic microbial communities at different life stages from infancy to old age (An et al., 2021; Fehlbaum et al., 2015; Pham et al., 2019). These models allow the study of personalized responses to interventions, capturing individual variations in microbial composition and activity shaped by genetics, diet, lifestyle, and medical history (Von Martels

et al., 2017). They enable researchers to study how the gut microbiota changes throughout life and its impact on health and disease across different age groups in a controlled yet physiologically relevant context (Fehlbaum et al., 2015). Moreover, *ex vivo* models allow for the exclusion of confounding host effects, thereby providing a clearer understanding of the direct impact of interventions on gut microbiota composition and function (Van De Steeg et al., 2018). This approach facilitates the identification of specific microbial pathways and mechanisms underlying observed effects on mental health outcomes (Isenring et al., 2023).

Furthermore, the application of *ex vivo* models holds promise in elucidating sex-specific responses to dietary interventions and their impact on mental health outcomes. By tailoring these models to specific populations, such as females and males, researchers can explore sex differences in the composition and function of gut microbiota in response to various nutritional stimuli. For example, investigations into the effects of hormonal fluctuations throughout the menstrual cycle or hormonal therapies in women, as well as testosterone levels in men, offer avenues for uncovering sex-specific mechanisms underlying the gut-brain axis (Zou et al., 2023). However, fermentation bioreactor models face challenges in fully replicating the intricacies of the gut environment, such as the dynamics of peristalsis, mucus layers, and host-tissue interactions (Costa & Ahluwalia, 2019). Integrating cell cultures or tissues with microbial communities within these systems offers a promising solution (Mittal et al., 2023). By developing co-culture systems that mimic physiological barriers, such as the gut epithelial barrier and mucosal immune system, researchers can delve into complex host-microbe interactions that occur within the gut (Ramadan & Jing, 2016; W. Shin & Kim, 2018). Despite inherent complexities and limitations, the integration of *ex vivo* models into research paradigms not only enhances the robustness of preclinical studies, but also paves the way for the development of personalized interventions tailored to individual physiological and microbial

profiles, thus advancing our understanding and treatment of mental health disorders (Isenring et al., 2023).

Future research should explore the sex-specific mechanisms underlying the interactions between sex hormones, the gut microbiota, and mental health disorders. This includes elucidating how sex hormones modulate immune responses, metabolism, and neurotransmitter production, which in turn influence gut microbiota composition and its impact on mental health. Such investigations hold promise for the discovery of novel therapeutic targets and personalized treatment strategies for individuals affected by these conditions. Furthermore, longitudinal studies that track changes in gut microbiota composition and mental health outcomes over time in response to hormonal fluctuations, psychotropic medications, and interventions are essential for understanding the dynamic nature of these interactions. These studies provide valuable insights into the causal relationships between gut microbiota dysbiosis, hormonal imbalances, and mental health disorders. Further exploration of clinical interventions targeting both sex hormones and gut microbiota is crucial. Investigating the efficacy of interventions such as probiotics, prebiotics, and dietary supplements in modulating gut microbiota composition and improving mental health outcomes, particularly in vulnerable populations such as premenopausal and postpartum women with depressive disorders, could pave the way for novel therapeutic approaches.

7. Conclusion

The complex interplay between sex hormones, the gut microbiota, and mental health highlights the complexity of understanding and treating psychiatric disorders. Studies examining mental health disorders have revealed sex-specific associations between the gut microbiota composition, hormonal fluctuations, and mental health outcomes. These findings suggest that the composition and diversity of the gut microbiota may contribute differently to mental health

symptoms in men and women, and are influenced by sex hormones and related physiological mechanisms. Furthermore, interventions targeting both sex hormones and the gut microbiota show promise in modulating mental health parameters, offering novel therapeutic avenues for psychiatric disorders. Additionally, the effect of psychotropic medications on the gut microbiota adds another layer of complexity to the sex-specific response to treatment. Although these medications are crucial for managing psychiatric disorders, their potential to alter microbial diversity highlights the need for personalized treatment approaches. Understanding the bidirectional relationship between psychotropic medications, sex hormones, and the gut microbiota may lead to more effective and tailored interventions for mental health disorders.

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Table 1.1. Summary of studies reporting sex hormone-influenced interactions between gut microbiota and mental health

Mental Health Condition	Population	Gut microbiota associated with sex hormones	Potential mechanisms	Reference
Anxiety	Men and women categorized into anxious and non-anxious groups	• Lower relative abundances of *Oscillospiraceae*, *Monoglobaceae*, and *Lachnospiraceae*_NK4A136 in anxious men. • Lower relative abundance of *Prevotella* in anxious women. • Sex-specific associations between microbial taxa and anxiety symptoms. • Enrichment of *Shigella* in anxious women.	• The differences in gut microbiota composition and diversity are influenced by sex steroid hormones and related genes, affecting immune system responses and metabolism. • Alterations in immune responses and metabolism may shape microbial diversity and specific taxa colonization in the gut, impacting overall physiology and contributing to anxiety symptoms.	(S.-Y. Kim et al., 2023)
	Ovariectomized C57BL/6J mice	• Progesterone stimulates *Lactobacillus* spp. growth	• Progesterone may regulate local microbiota composition by promoting the growth of specific bacteria and reducing IL6 mRNA expression in cecum tissue, suppressing the inflammatory response in ovariectomized mice. • Decreased progesterone levels may lead to dysbiosis, diminishing *Lactobacillus* spp. abundance and stimulating inflammatory responses through kynurenine production. This inflammation is associated with depression and anxiety, potentially by downregulating BDNF expression.	(Sovijit et al., 2021)
	C57BL/6J female mice	• Estradiol treatment was associated with increased abundances of *Muribaculaceae*, *Sutterella* and	• Estradiol may influence neuronal activity in anxiety-related brain regions like the paraventricular nucleus of the hypothalamus and	(Acharya et al., 2023)

		decreased abundances of *Peptostreptococcaceae*, *Mogibacteriaceae*.	the medial preoptic area, potentially contributing to its anxiolytic effects. • Estradiol treatment attenuates high fat diet-induced alterations in gut microbiota composition, with specific microbial taxa linked to anxiety-like behavior and metabolic health. Some taxa negatively correlate with anxiety, while others associate with weight gain.	
Major Depressive Disorder (MDD)	Premenopausal women with depression and female mice with fecal transplants derived from these depressed women	• Gut microbiota from depressed premenopausal women showed higher efficiency in degrading estradiol compared to microbiota from non-depressed women. • *Klebsiella aerogenes* TS2020, isolated from female patients with depression, demonstrated the ability to degrade estradiol.	• 3β-Hydroxysteroid dehydrogenase enzyme expressed by gut bacteria as a key player in estradiol degradation, potentially leading to depression-like behaviors in mice.	(D. Li et al., 2023)
	First-episode drug-naïve MDD patients and healthy controls	• Increased *Actinobacteria* and decreased *Bacteroidetes* levels in female and male MDD patients, respectively. • *Actinobacteria* and *Bacteroidia* are potential sex-specific biomarkers for MDD diagnosis. • *Bacteroidetes* decreased only in male MDD patients, *Actinobacteria* increased only in female MDD patients.	• Suggested causal role of gut microbiome dysbiosis in depressive behaviors. • Gut microbiota can regulate bile-acid pool size and affect blood-brain barrier and HPA axis. • Metabolites from gut microbiota impact the gut–brain axis and host immunity. • Gut microbiota influences tryptophan metabolism and regulates microglia homeostasis.	(Chen et al., 2018)

Postpartum depressive disorder (PPD)	PPD patients and healthy controls	• Lower relative abundance of Firmicutes phyla in PPD patients. • Reduced levels of *Faecalibacterium*, *Phascolarctobacterium*, *Butyricicoccus*, and *Lachnospiraceae*, increased levels of *Enterobacteriaceae* in PPD patients. • Progesterone level was positively correlated with *Prevotella*, *Eubacterium*, *Ruminococcus*. Testosterone level was positively correlated with *Erysipelotrichaceae*, *Alistipes*, *Burkholderiaceae*, while estradiol was positively correlated with *Burkholderiaceae* and *Turicibacter*.	• The interaction of sex hormones and gut microbiota was suggested to play a substantial role in PPD, with *Faecalibacterium* and *Lachnospiraceae* being correlated with disease severity.	(Y. Zhou et al., 2020)
Neuroticism	Men and women	• Neuroticism was correlated with *Haemophilus* spp. and Conscientiousness with *Lachnospiraceae* in men.	• Negative emotions can trigger chronic inflammation, impacting gut permeability and allowing harmful bacterial components to enter the bloodstream. • Decreased abundance of butyrate-producing bacteria may fuel inflammation and influence personality traits. • Microbial activities involved in amino acid degradation might influence neurotransmitter synthesis, potentially affecting personality traits. • Gut microbiota composition and function could affect physiological pathways like the HPA axis, thereby influencing personality traits.	(H.-N. Kim et al., 2018)

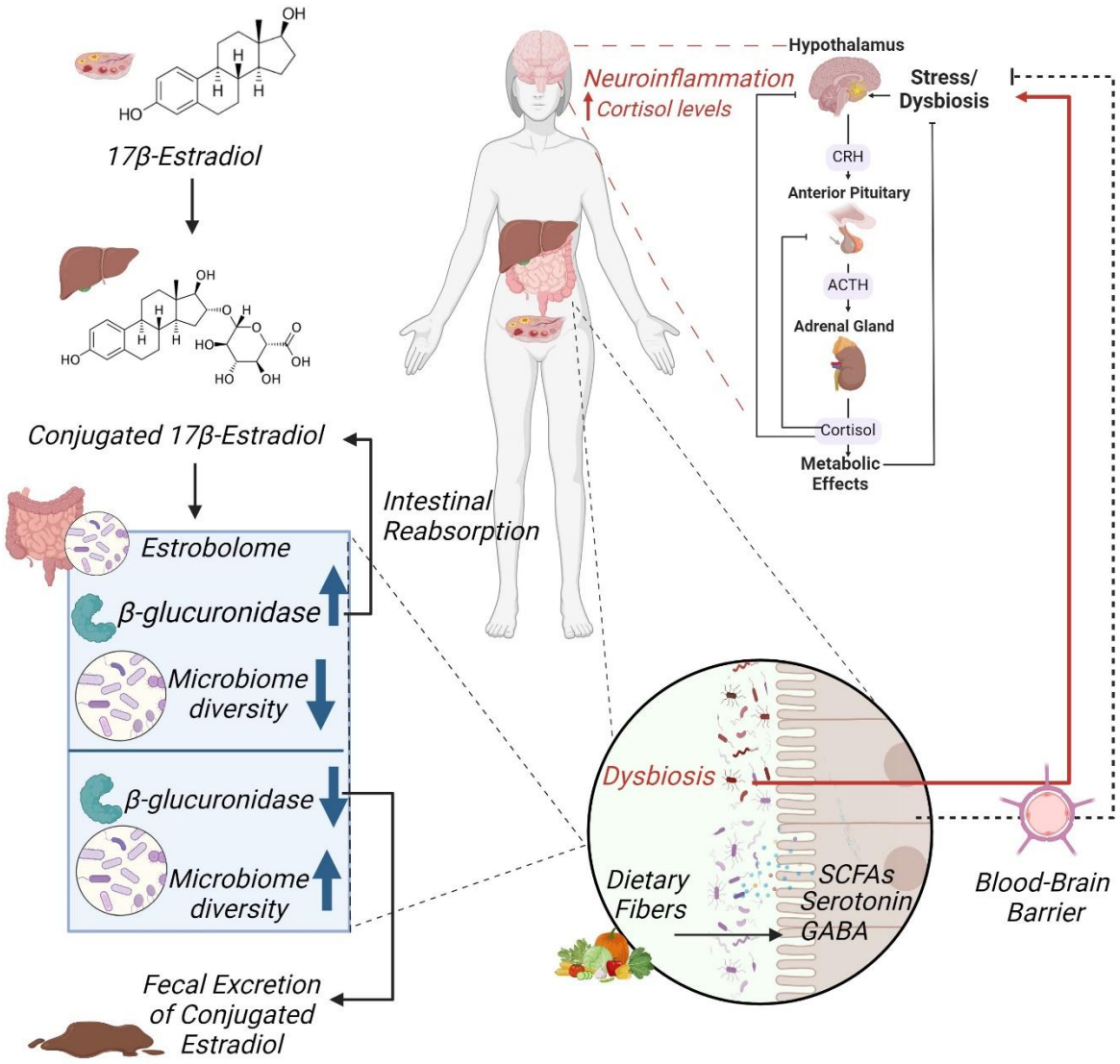

Figure 1.1. Molecular mechanisms linking microbiota, sex hormones, and mental health. There are several proposed molecular mechanisms underlying the connection between the microbiota, sex hormones, and mental health, involving complex interactions within the gut-brain axis. Neurotransmitter Production: Gut microbiota plays a role in synthesizing neurotransmitters such as serotonin, dopamine, and GABA, crucial for mood regulation and stress response. Neuroinflammation: Dysbiosis can increase gut permeability, allowing bacterial products to enter systemic circulation. This triggers immune responses and neuroinflammation, contributing to mental health disorders. Hormonal Regulation: Sex hormones influence the gut microbiota's composition and function. Conversely, gut microbiota can metabolize and modulate sex hormone levels through the β-glucuronidase enzyme, affecting hormone signaling and homeostasis. Steroid hormone such as 17beta (β)-estradiol are metabolized and recycled in the body through a process involving gut microbial beta (β)-glucuronidase enzymes. These enzymes, as important players in the estrobolome, influence both the excretion and the levels of circulating conjugated 17beta (β)-estradiol in the body. SCFA Production: Gut bacteria ferment dietary fibers to produce SCFAs such as acetate, propionate, and butyrate. SCFAs possess anti-inflammatory properties and regulate

neurotransmitter synthesis, gut barrier function, and immune responses, thereby impacting mental health. Stress Axis Regulation: The gut microbiota communicates with the HPA axis, regulating the body's stress response. Dysbiosis can dysregulate the HPA axis, leading to abnormal cortisol levels and heightened stress responses associated with anxiety and depression.

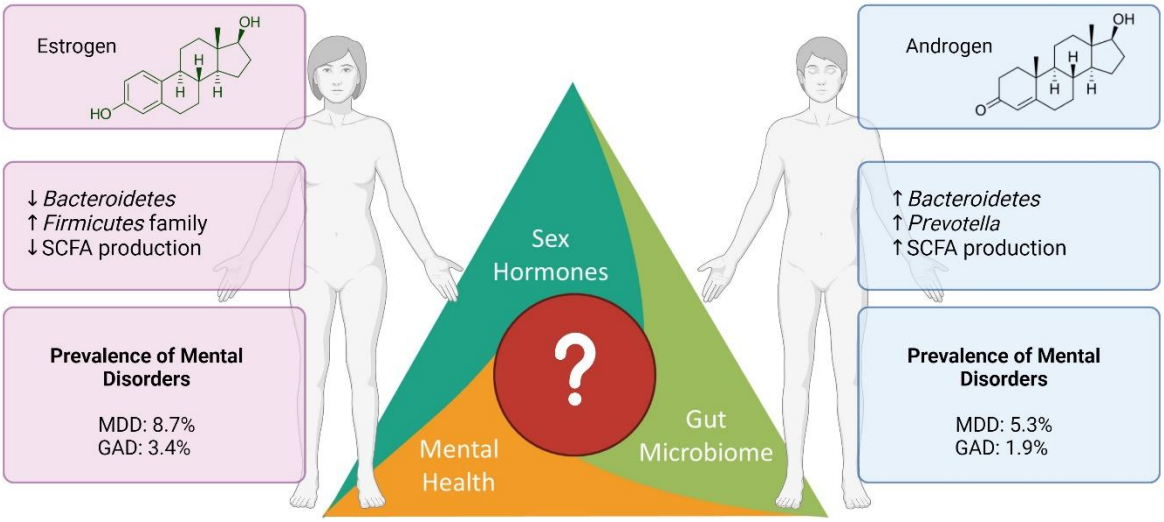

Figure 1.2. A trilateral intertwined relationship between sex hormones, gut microbiota, and mental health.
Sex hormones impact the gut-brain axis, influencing neurogenesis, cognitive function, and various physiological systems through androgen and estrogen receptors. They interact with gut microbiota, regulating bile acid metabolism and activating enzymes that affect hormone levels. Sex hormones also influence gut microbiota composition and immune response, modulating gut physiology and barrier function, and affecting colonic transit time and permeability. Gut microbiota metabolizes sex hormones, impacting their levels and activities. Mental disorders like Major Depressive Disorder (MDD) and Generalized Anxiety Disorder (GAD) show sex-specific prevalence. Mental disorders impact sex hormone levels and gut microbiota composition, leading to dysbiosis and increased gut permeability, exacerbating mental health symptoms and affecting immune function.

Authorship Contributions:

Riadh Hammami: Conceptualization, drafting, and critical revision of the manuscript. Saba Miri: Drafting and critical revision of the manuscript. Luana Leao: Literature acquisition and review, drafting, and critical revision of the manuscript.

Hypothesis and Objectives

Sex steroid hormone levels, such as estrogen and testosterone, could contribute to shaping the gut microbiome and modulating its interactions with drugs, including psychotropic chemicals. Therefore, this study aimed to investigate the interplay between sexually divergent gut microbiome and psychotropic medication.

Specific objectives:

- Develop a sexually divergent microbiome simulated model of the human gut;
- Investigate the impact of sex hormones on gut microbiome composition and metabolism;
- Study the impact of psychotropic medication on the gut microbiome in a developed feminine colonic model.

CHAPTER 2:

Impact of Sex Hormones and Psychotropic Medication on Gut Microbiome in a Simulated Colonic Model

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1. INTRODUCTION

The gut microbiome is increasingly recognized as a key player in mental health, influencing brain function and behavior through the gut-brain axis (Loh et al., 2024). This complex communication between the gut and the brain involves various mechanisms, including the production of neuroactive compounds, modulation of the immune system, and synthesis of essential metabolites such as short-chain fatty acids (SCFA) (Marano et al., 2023). As understanding of this connection deepens, the gut microbiome has emerged as a potential target for therapeutic interventions aimed at treating psychiatric disorders (Mhanna et al., 2024).

Sex hormones not only control reproductive functions but also play a significant role in both brain health and composition of the gut microbiome, making them particularly relevant in the context of mental health (He et al., 2021). Women are more likely to experience psychiatric conditions, including depression and anxiety, owing in part to the fluctuating levels of these hormones throughout their lives, particularly during menstruation, pregnancy, and menopause (Wieczorek et al., 2023). Given the cyclical nature of hormone levels in women, these interactions may contribute to the observed differences in mental health between sexes and across lifespan (Toffol et al., 2015). This hormonal variability not only affects mood and cognitive function but also influences how women metabolize medications, including psychotropic drugs (González-Rodríguez & Seeman, 2019). For instance, postmenopausal decreases in estrogen can reduce the levels and effectiveness of certain antipsychotics, such as olanzapine and clozapine, while increasing the levels of others, potentially altering the therapeutic outcomes (Spina & De Leon, 2014).

The effect of sex hormones on the gut microbiome is especially pertinent when considering the use of psychotropic medications, which are commonly prescribed to manage various psychiatric conditions (Ait Chait et al., 2023a; Ortega et al., 2023). Women tend to use these medications more often than men, with studies showing a prevalence of 15.8% in women compared to 7.3% in men, particularly for antidepressants (Estancial Fernandes et al., 2018). Aripiprazole, for example, used to treat schizophrenia, bipolar disorder, and major depressive disorder as adjunctive therapy (Gettu & Saadabadi, 2023), was found to have higher concentration–dose ratios in women than in men (Jönsson et al., 2019). This suggests that sex-

specific differences in drug metabolism may affect the therapeutic efficacy and side effect profiles of these medications (Bolea-Alamanac et al., 2018). Moreover, psychotropic medications can alter the gut microbiome, potentially affecting their effectiveness and the severity of side effects through mechanisms involving the gut-brain axis (Ait Chait et al., 2020; Ait Chait et al., 2023; Ortega et al., 2023).

Understanding how psychotropic drugs interact with the gut microbiome is crucial for optimizing mental health treatments and minimizing adverse effects, especially considering sex-specific responses to these medications. Women are more likely to take multiple psychotropic drugs and are more vulnerable to adverse reactions, including nonspecific side effects (Mazza et al., 2024). These sex-specific differences in drug utilization and responses further highlight the importance of considering both hormonal and microbial factors in the treatment of psychiatric disorders.

This study investigated the impact of sex hormones (estrogen, progesterone, and testosterone) on the gut microbiome composition and metabolic activity using an *ex-vivo* colonic fermentation model. Additionally, we examined how the psychotropic drug aripiprazole affects the gut microbiota in a newly developed female model of the simulated human colon.

2. METHODS

2.1. Development of sexually divergent human colon model

An in vitro simulated colon was used to develop a sexually divergent model to replicate the representative gut microbiota in both men and women. This model consisted of a two-stage design, including an inoculation reactor with immobilized fecal microbiota used to inoculate four second-stage reactors that were continuously operated in parallel (Fig. 2.1). An *ex-vivo* fermentation model simulates the physiological and microbiological conditions of the human gut (Ait Chait et al., 2023b; Mottawea et al., 2020). The impact of testosterone, estrogen, and progesterone on the structure and functionality of fecal microbiota was evaluated *ex-vivo* in a colon model mimicking the physiological and microbiological conditions of the large intestine (Mottawea et al., 2020). Fecal samples were provided by four donors: two men (ages 40 and 36) and two women of reproductive age (ages 30 and 25) with no previous history of mental disorders. The study was conducted under the certificate of ethics approval number H-02-18-347.

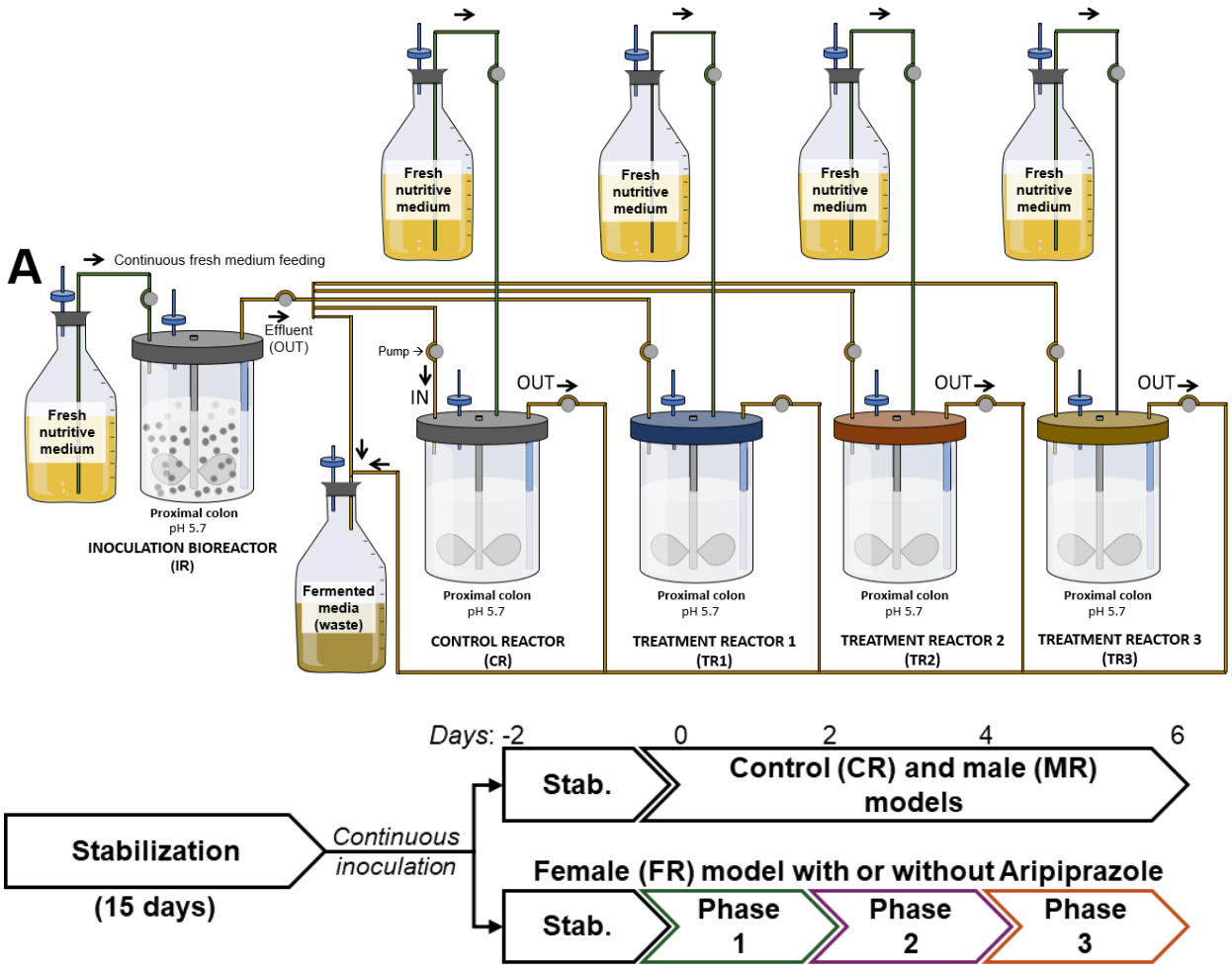

Figure 2.1. Experimental reactor setup of the ex-vivo PolyFermS fermentation model. **IR**: inoculum reactor containing immobilized donor feces (30% v/v); **CR:** control reactor; **TR1-TR3**: test reactors 1-4.

Immobilization of microbiota was performed using fresh fecal samples within 1 h to inoculate gellan (2.5%, w/v) and xanthan (0.25%, w/v) beads under anaerobic conditions, as previously described. (Le Blay et al., 2012). Bacteria immobilized within the beads were protected from harsh external conditions, such as pH fluctuations, temperature changes, and shear forces. This enhanced stability allows the bacteria to maintain their activity and viability over longer periods (Dieni & Ustadi, 2020). The immobilization procedure was based on a dispersion process in a two-phase system, as described by (Lamboley et al., 1997) for kappa-carrageenan/locust bean gum gel bead preparation. The autoclaved polymer solution was cooled to 43°C and inoculated aseptically with fecal inoculum (2%). After inoculation, the polymer solution was stirred into a

freshly autoclaved (15 min at 121°C) hydrophobic phase (commercial canola oil) at 43°C to obtain a suspension of aqueous droplets in the oil. The gel beads were formed by cooling the suspensions. After separation and washing, the beads were hardened by soaking for 30 minutes in CaCl2 (0.1 M). Beads with diameters in the 1.0–2.0 mm range were selected by wet sieving and used for fermentation. The process was completed under aseptic conditions within 1 h (Cinquin et al., 2004). Gel beads (30%) were then transferred to a stirred glass reactor containing fresh MacFarlane culture medium (Macfarlane et al., 1998).

Each fermentation lasted for 23 days. During the first 48 h, the colonic model was run as a batch culture to allow bead colonization, with the nutritive medium being replaced by fresh medium every 12 h. This period was essential for establishing a stable and functional microbial community on the beads, as the microorganisms required time to adjust to the new conditions and nutrients provided by the fresh medium. This ensured that the microbial community was established and became active before any subsequent experimental manipulation was performed (Scannell et al., 2000).

Following the initial 48 h, the medium flow was switched to the continuous mode for the rest of the experiment. After a stabilization period of 15 d, the microbial community in the inoculum reactor (IR) was used to inoculate four second-stage DASGIP® bioreactors (Eppendorf, Mississauga, ON): one Control Reactor (CR: non-treatment control) and three Treatment Reactors (TR1-3) that were challenged with different parallel treatment periods (as described below). A working volume of 100 mL was maintained in each sub-bioreactor by inoculating 5% (v/v) (0.62 mL/h) IR effluent and adding 95% (v/v) (11.88 mL/h) of fresh fermentation medium. The entire second-stage bioreactor ran for another 2 days to stabilize the microbial community. Each reactor reproduced the physiological and microbiological conditions of the adult proximal colon (pH 5.7, stirring at 120 rpm, 37°C, and mean retention time of 8 h) (Ait Chait et al., 2023b; Mottawea et al., 2020). Anaerobiosis was ensured through continuous headspace flushing of N_2 and CO_2 at a 0.9:0.1 ratio, and a constant pH of 5.7 was maintained by adding NaOH (2.5 M). (Mousavi et al., 2022). Once stabilization was reached in all reactors, the bioreactors were subjected to treatment every 24 h, as shown in Table 2.1.

The concentrations of female hormones were determined based on research by Stricker et al. (2006), who identified three distinct phases of the menstrual cycle (E-P-, E+P-, and E+P+) according to estradiol and progesterone serum levels (Fig. 2.2) (Stricker et al., 2006). The hormone

concentrations used in each treatment are shown in Table 2.2. Hormonal treatments and combined treatments with hormones and the psychotropic aripiprazole were added to the media that fed the bioreactors TR2 and TR3, respectively, following the 2-day stabilization period. To simulate the menstrual cycle, the hormonal concentrations were adjusted every two days. The psychotropic aripiprazole was selected following a previous study from our lab, demonstrating its antimicrobial effects on intestinal bacterial pure cultures and the gut microbiome *ex-vivo* (Ait Chait et al., 2020b, 2023b). Stock solutions of aripiprazole at a concentration of 20 mg/mL were prepared according to the manufacturer's recommendations, filter-sterilized (0.22 μm), and stored at -20°C until use. The collected samples were separated (centrifugation at 14,000 × g for 5 min at 4°C), the pellet was used for metagenomic DNA extraction, and the supernatant was used for short chain fatty acid (SCFA) analysis.

Table 2.1. Sampling details according to the treatments

Treatments	Sampling time	Cycle phase	Number of samples
Control (CR)	0, 12, 24, 48h	-	6 (d0, d1, d2, d3, d4, d5, d6)
Male (TR1)	0, 12, 24, 48h	-	6 (d0, d1, d2, d3, d4, d5, d6)
Female (TR2)	0, 12, 24, 48h	E-P-, E+P-, and E+P+	9 (d0, d1, d2b, d2a, d3, d4b, d4a, d5, d6)
Female + Aripiprazole (TR3)	0, 12, 24, 48h	E-P-, E+P-, and E+P+	9 (d0, d1, d2b, d2a, d3, d4b, d4a, d5, d6)

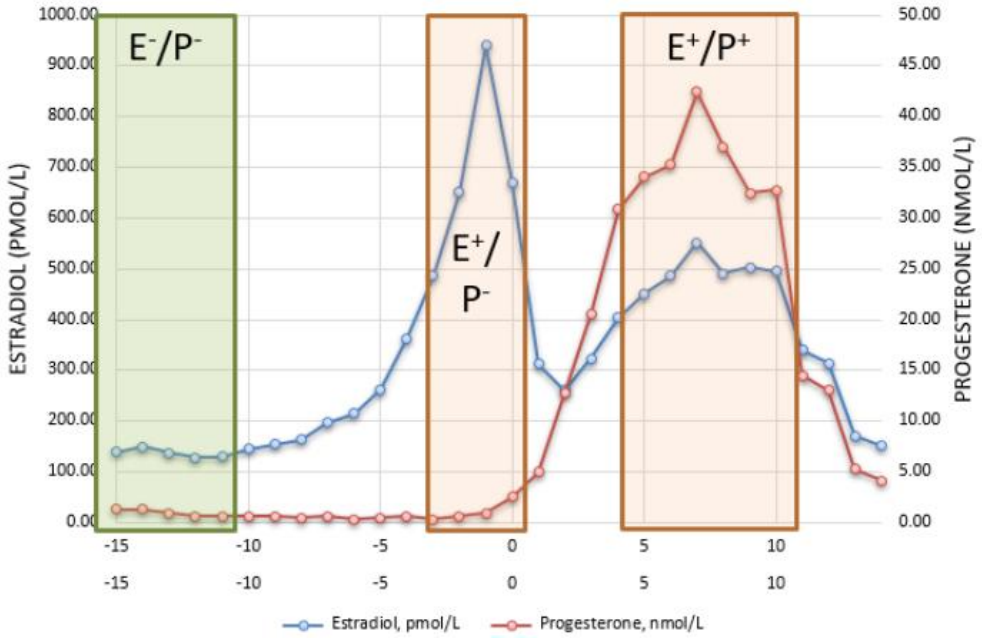

Figure 2.2. Estradiol and progesterone concentrations during the menstrual cycle.

Table 2.2. Hormone concentrations for each treatment

Female gut	**E-P- (Phase 1)**	**E+P- (Phase 2)**	**E+P+ (Phase 3)**	**Reference**
Estradiol (pmol/L)	130	650	464	Stricker et al. (2006)
Progesterone (nmol/L)	1	2	34	
Testosterone (nmol/L)	0.32	0.32	0.32	Skiba et al. (2019)
Male gut				
Testosterone (nmol/L)	16.2	16.2	16.2	Zhu et al. (2022)

2.2. Microbiome genomic analysis

DNA extraction

Genomic DNA was extracted from the pellet of fecal slurry and fermentation samples using a Fast DNA Spin Kit following the manufacturer's instructions. Briefly, mechanical lysis was performed in two cycles of 40 s each at a speed of 6.0 m/s in a Bead Mill-24 Homogenizer, with a 5-minute cooling period on ice between the two cycles (Mottawea et al., 2016). The amount of extracted DNA was quantified by using a Qubit fluorometer.

16S rRNA gene sequencing

The microbial profiles of the fecal slurry and fermentation samples were assessed using tag-encoded 16S rRNA gene MiSeq-based high-throughput sequencing. The V3-V4 regions of the 16S rRNA gene were amplified using dual-barcoded primers, and an amplicon library for sequencing was prepared according to the Illumina standard protocol. The amplicon libraries were pooled in equimolar amounts and paired-end sequenced using the Illumina MiSeq platform with the 600 bp MiSeq Reagent Kit v3 following the standard protocol.

We processed the V3-V4 region sequences were processed using the Qiime2 pipeline (version 2023.2) (Bolyen et al., 2019). DADA2 was employed for quality control, truncation, and denoising of reads, resulting in an amplicon sequence variant (ASV) feature table. Taxonomic annotation was performed using a classifier trained on the targeted 16S rRNA regions from the Silva database (release 13.8) (Yilmaz-Ersan & Kurdal, 2014). Downstream analyses were performed using '*R*' (v.4.3.1) (https://www.R-project.org/). Alpha diversity was estimated using the observed features and Shannon and Simpson's metrics. Beta diversity among samples was calculated using the Bray-Curtis distance and visualized using Principal Coordinate Analysis (PCoA). The contribution of different treatments to the diversity of the gut microbiota community will be assessed from the Bray-Curtis distance matrix using pairwise permutational multivariate analysis of variance with 999 permutations (McMurdie & Holmes, 2013). To identify differential taxa among different treatments, a linear regression model was conducted on the relative abundance of taxa at various levels using linear models for differential abundance analysis (LinDA) (Zhou et al., 2022). The results were deemed significant if the *p*-value was < 0.05.

2.3. Quantification of short-chain fatty acids using GC-FID

SCFA (butyrate, acetate, and propionate) production in fermentation samples from all sub-reactors was assessed using Gas Chromatography combined with a Flame Ionization Detector (GC-FID) following the methods described in previous studies (Ait Chait et al., 2023b; Monk et al., 2016). To summarize the procedure briefly, supernatants obtained from the fermentation samples underwent centrifugation (at 14,000 × g for 30 min at 4°C) followed by filtration using a 0.22 µm filter for sterilization. For quantification, a mixture of formic acid and 2-ethyl butyric acid will serve as an internal standard, with each sample receiving a concentration of 0.5 mM. Approximately 100 µL of each sample was injected into a capillary column, Stabilwax-DA, and

the analysis was performed for 20 min. The resulting peaks were identified and quantified using standards from Millipore Sigma (Oakville, ON, Canada). The results are expressed as the concentration of SCFAs in millimolar (mM) units. All samples underwent duplicate analyses (two technical measurements).

2.4. Statistical Analysis

Kruskal-Wallis test with Dunn post-hoc test (FDR-corrected) was applied when the statistical analysis was required to compare differences between the control, female, male, and psychotropic groups using the rstatix package (v. 0.7.2). Data from Gas Chromatography analyses were analyzed using GraphPad Prism v8.3 (GraphPad Software, USA) to assess the significance of results among treatments simultaneously and among different time points within each treatment. Data are expressed as the mean ± standard deviations (SD), and an ANOVA test with Bonferroni post hoc test for multiple comparisons was applied (P-values < 0.05).

3. RESULTS

3.1. Impact of Sex Hormones on Microbiota Composition

3.1.1. Microbiota Composition

The shift in microbiota composition over time was compared between the study groups during the first two days of fermentation. At the phylum level, the dominant phylum Bacteroidota showed an increase over time in all groups except at time 48h in the female group (Fig. 2.3). In contrast, the relative abundance of Firmicutes and Actinobacteriota dropped sharply from time 12h to 48h, particularly Firmicutes, in all study groups. The relative abundance of Proteobacteria was unstable at an early fermentation time of 6-12h followed by an increase at 24h and 48h (Fig. 2.3). A statistical analysis using Kruskal-Walis (KW) test was conducted to investigate the differences between the groups. Over time, no significant difference was found between the groups, as indicated by *p*-value (≥ 0.05). However, the pairwise comparison analysis showed a statistical significance at 24h between the male and female groups for Bacteroidota and Firmicutes. Bacteroidota was significantly increased in the female group, contrary to Firmicutes, which increased in the male group (*p*-value < 0.05 (Fig. 2.4 A&B). Actinobacteriota abundance showed a large increase in the male group compared to the control at 24h as indicated by the *p*-value of <

0.05, whereas Proteobacteria relative abundance showed no differences over all time points (Fig. 2.4 C&D).

Among the groups, no significant difference was found between the compared phyla in the control group at 6h, 12h, and 24h (data not shown). At 48h, significance was observed by KW analysis with a *p*-value of 0.037, and the relative abundance of Bacteroidota was significantly larger than that of Firmicutes and Actinobacteriota by $p \leq 0.01$ and $p < 0.05$, respectively. For the female group, the KW test revealed large variations between phyla at 6h, 12h, and 24h with *p*-value of 0.03, 0.012, and 0.03, respectively (Fig. 2.5 A-C). Notably, the abundance of Bacteroidota was significantly higher than that of Firmicutes at all time points (Fig. 2.5 A-D). The male group showed no significant difference between the compared phyla for 6h-24h. However, pairwise comparisons revealed large differences between Bacteroidota and Firmicutes at 12h, 24h, and 48h. Bacteroidota were more abundant than Firmicutes at 12 and 48h, contrary to 24h where Firmicutes abundance was the highest.

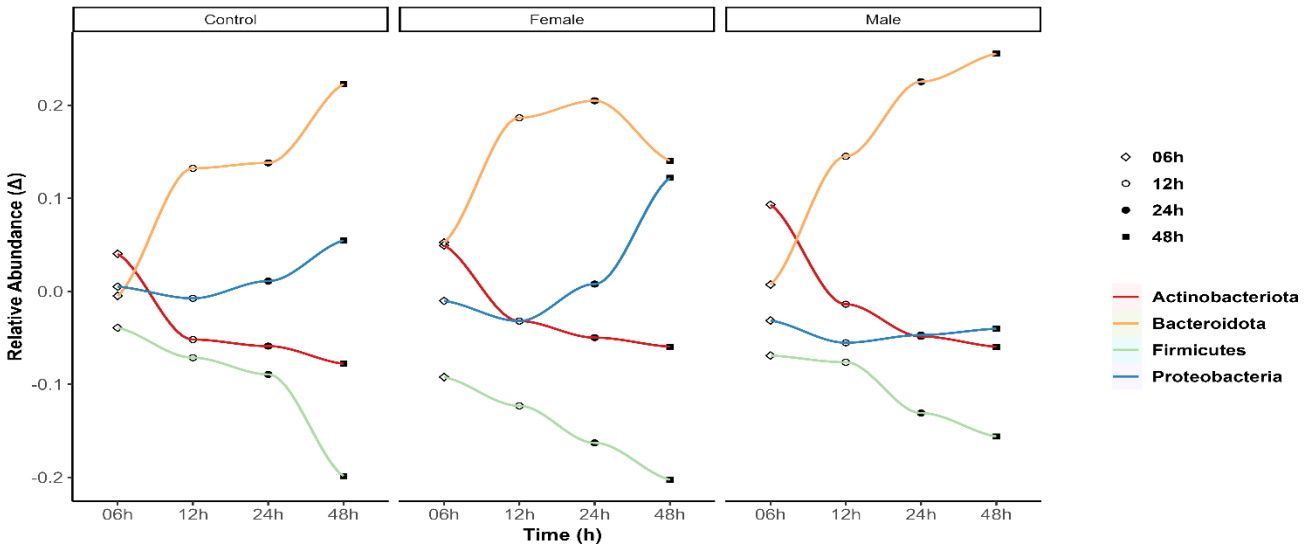

Figure 2.3. Line plots illustrate the changes in the relative abundance of the most abundant phyla enriched in each treatment over time for the first 2 fermentation days.
The relative abundance represents the shift (Δ) from time 0.

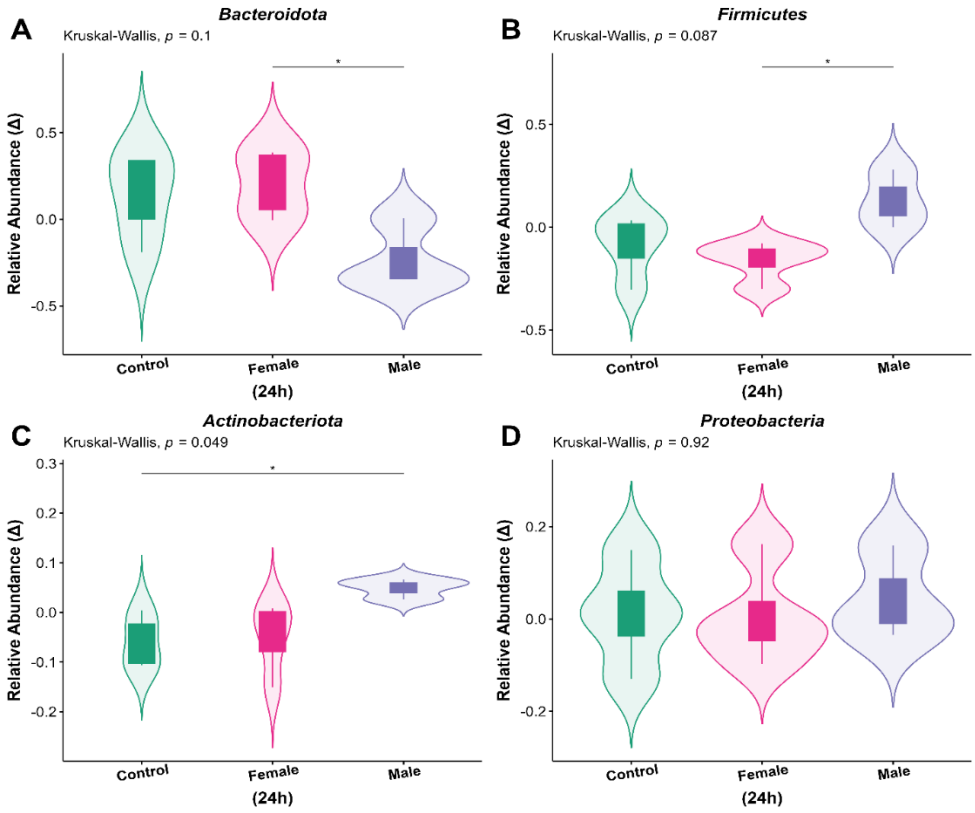

Figure 2.4. Changes in relative abundance between study groups based on time 24h.
Figures indicate the variations for the first 2 days of fermentation using the Kruskal-Wills test. Relative abundance represents the shift (Δ) of the baseline abundance at time 0. *p < 0.05.

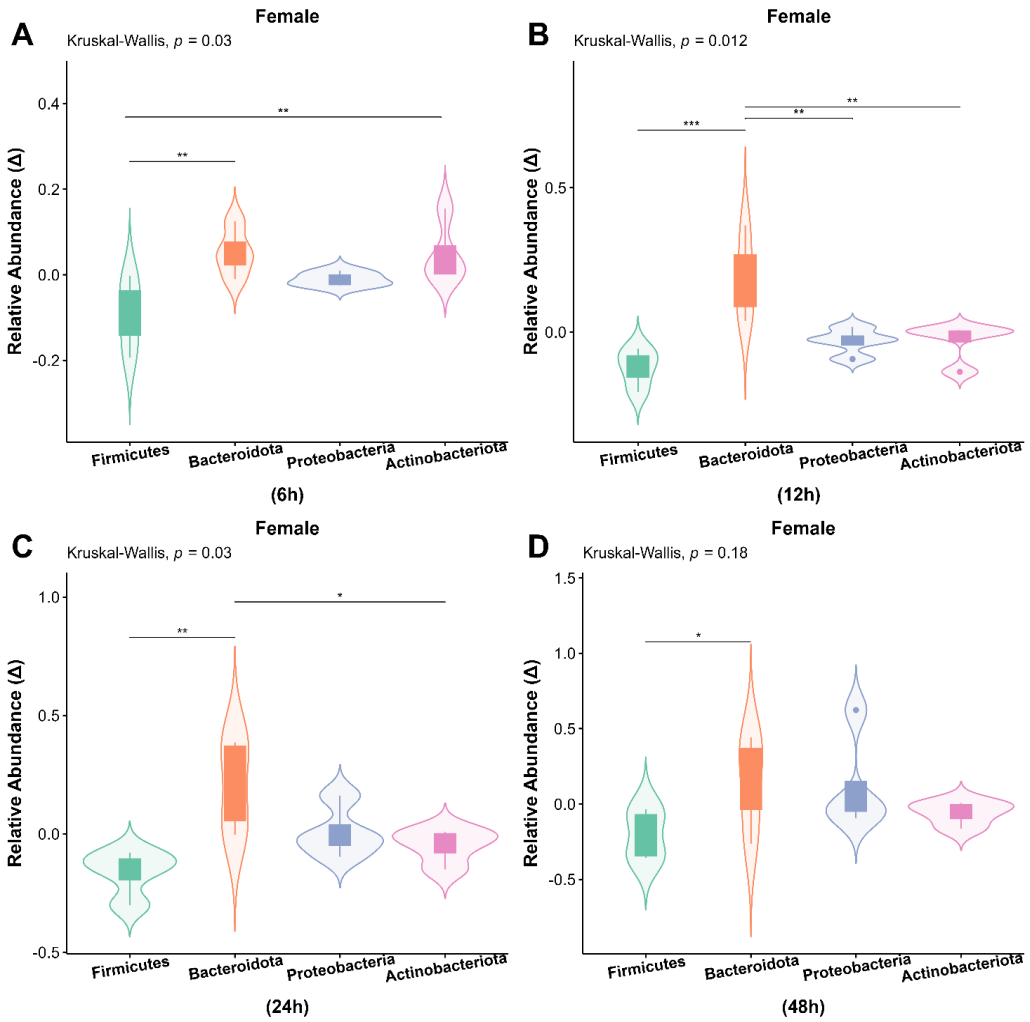

Figure 2.5. Changes in the relative abundance of top abundant phyla among Female groups at different times post-fermentation.
Kruskal-Wallis tests were conducted to assess significant differences in relative abundance variations over the initial two fermentation days. Relative abundance values represent the shift (Δ) from the baseline abundance at time 0. (*p < 0.05, **p ≤ 0.01).

At the family level, the most abundant families were Acidaminococcaceae, Bacteroidaceae, Lachnospiraceae, Prevotellaceae and Veillonellaceae. Almost all the compared groups exhibited an increase in the relative abundance of Bacteroidaceae and Prevotellaceae over time. However, at 48h the female group showed a dramatic decrease in the abundance of Bacteroidaceae after stability between 12-24h (Fig. 2.6). Lachnospiraceae abundance exhibited a similar trend in both the control and psychotropic groups, but remained stable in the male group and decreased in the female group. In contrast, Veillonellaceae consistently declined in relative abundance over time across all groups (control, female, and male) (Fig. 2.6).

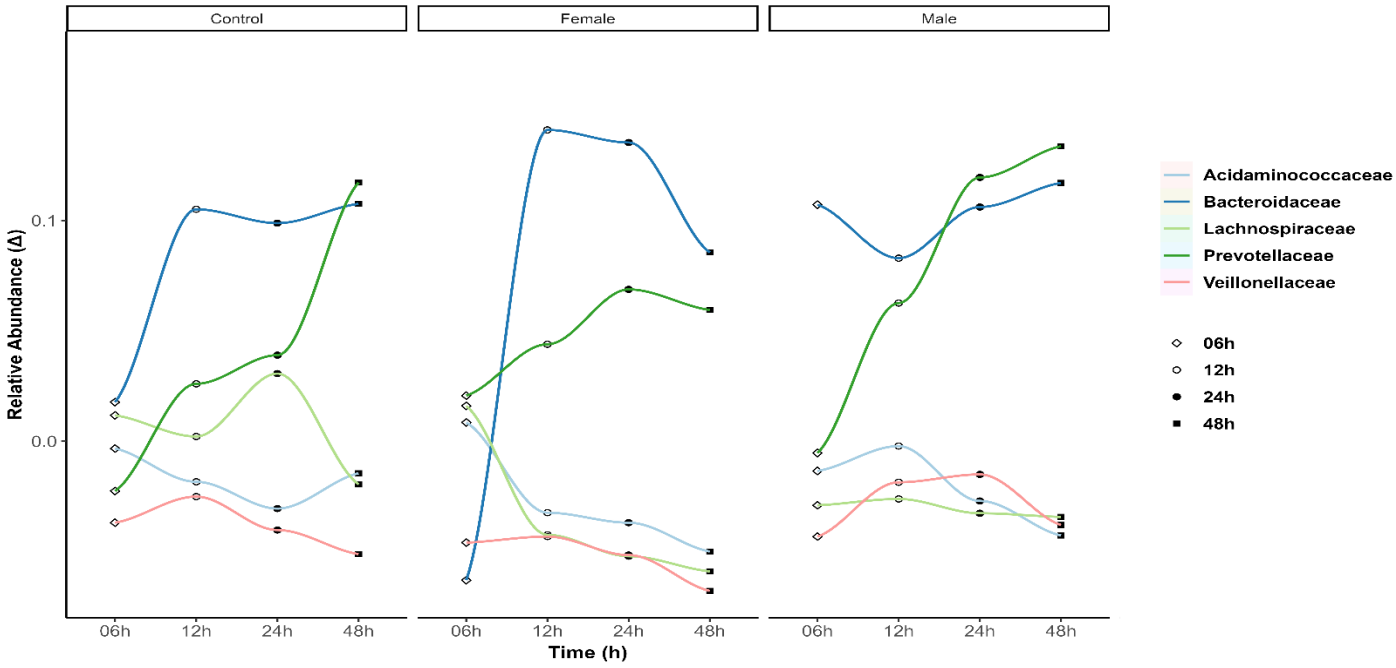

Figure 2.6. Line plots show the shifts in the relative abundance of the most prevalent families over the first two fermentation days, expressed as shift (Δ) from the baseline abundance at time 0.

3.1.2. Exploring the Influence of Donor Sex on Microbiota Composition in a Female Model

The impact of female hormones on microbiota composition was investigated based on donor sex. The microbiota from male donors, donor1 (D1) and donor4 (D4), showed similarity in the relative abundance of Bacteroidota, Proteobacteria, and Actinobacteria over time in the female model. However, a notable difference in the abundance of Firmicutes was observed over time. In D1, Firmicutes abundance increased from 12 to 48 h, whereas in D4, it declined over time (Fig. 2.7 A&D). For female donors, donor2 (D2) and donor3 (D3), while the Bacteroidota abundance dropped particularly after 24h, Actinobacteria showed steady stability in both donors. This stability was observed with Proteobacteria in D2, in contrast to D3, which sharply elevated particularly after 24h. Interestingly, a steady increase and decrease were found in Firmicutes abundance over time in D2 and D3, respectively ((Fig. 2.7 B and C).

Kruskal-Wallis analysis revealed significant differences in the abundance of Bacteroidota, Actinobacteriota, and Proteobacteria among donors ($p < 0.05$), while Firmicutes abundance remained relatively comparable across all individuals ($p > 0.05$) (Fig. 2.8 A-D). However, Firmicutes abundance was higher in D1 and D2 than in D4, as indicated by the p-value (< 0.05)

(Fig. 2.8 A). Notably, Bacteroidota was significantly higher in male than in female donors ($p \leq 0.001$) (Fig. 2.8 B). Conversely, Proteobacteria levels were elevated in female donors, particularly at D3 ($p < 0.05$) (Fig. 2.8 C). Actinobacteriota abundance was also relatively high in female donors, albeit with a large variation between male donors (Fig. 2.8 D).

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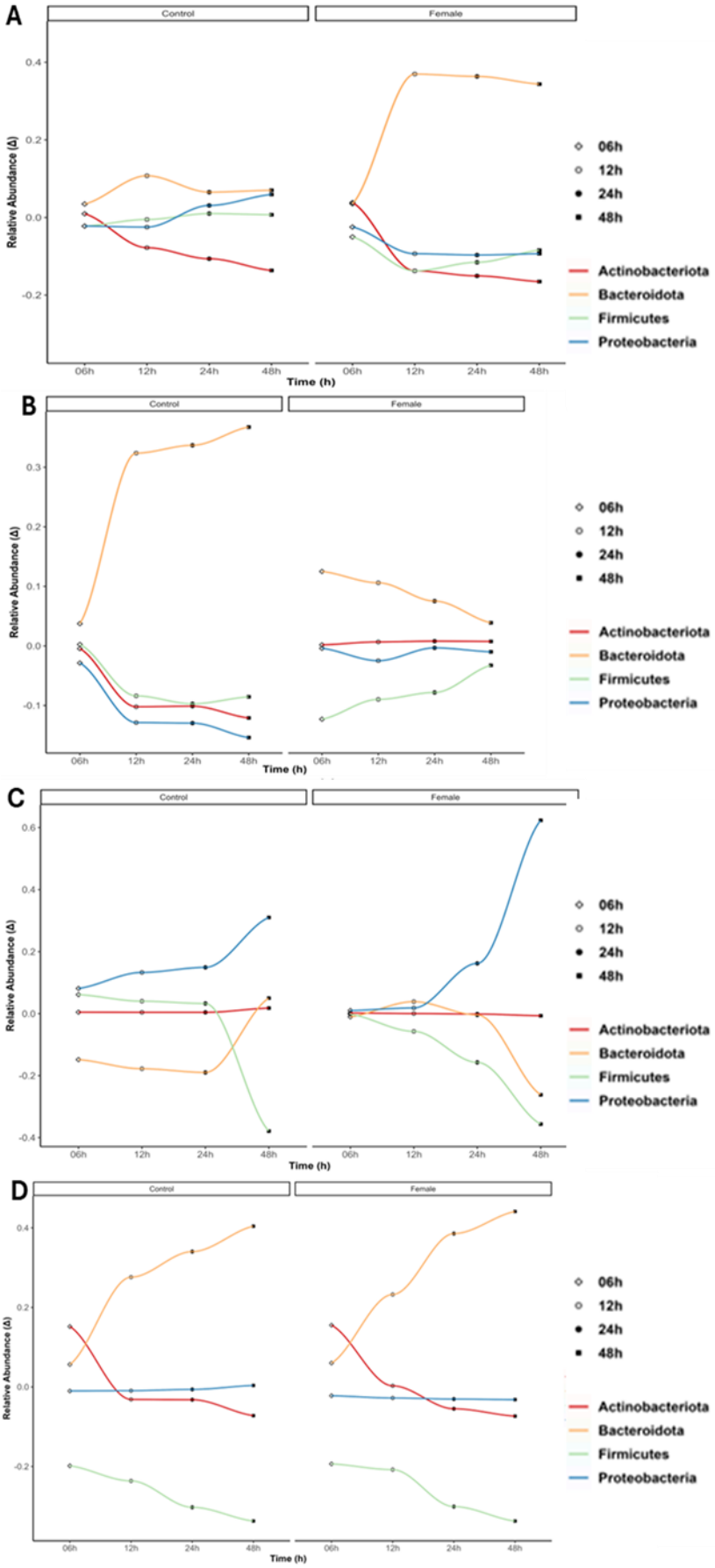

Figure 2.7. Changes in microbiota composition over 2 fermentation days based on sex type with female hormones.

A & D represent the male donors (D1 & D4) and B & C are for female donors (D2 & D3). The relative abundance showed a shift (Δ) from the baseline abundance at time 0.

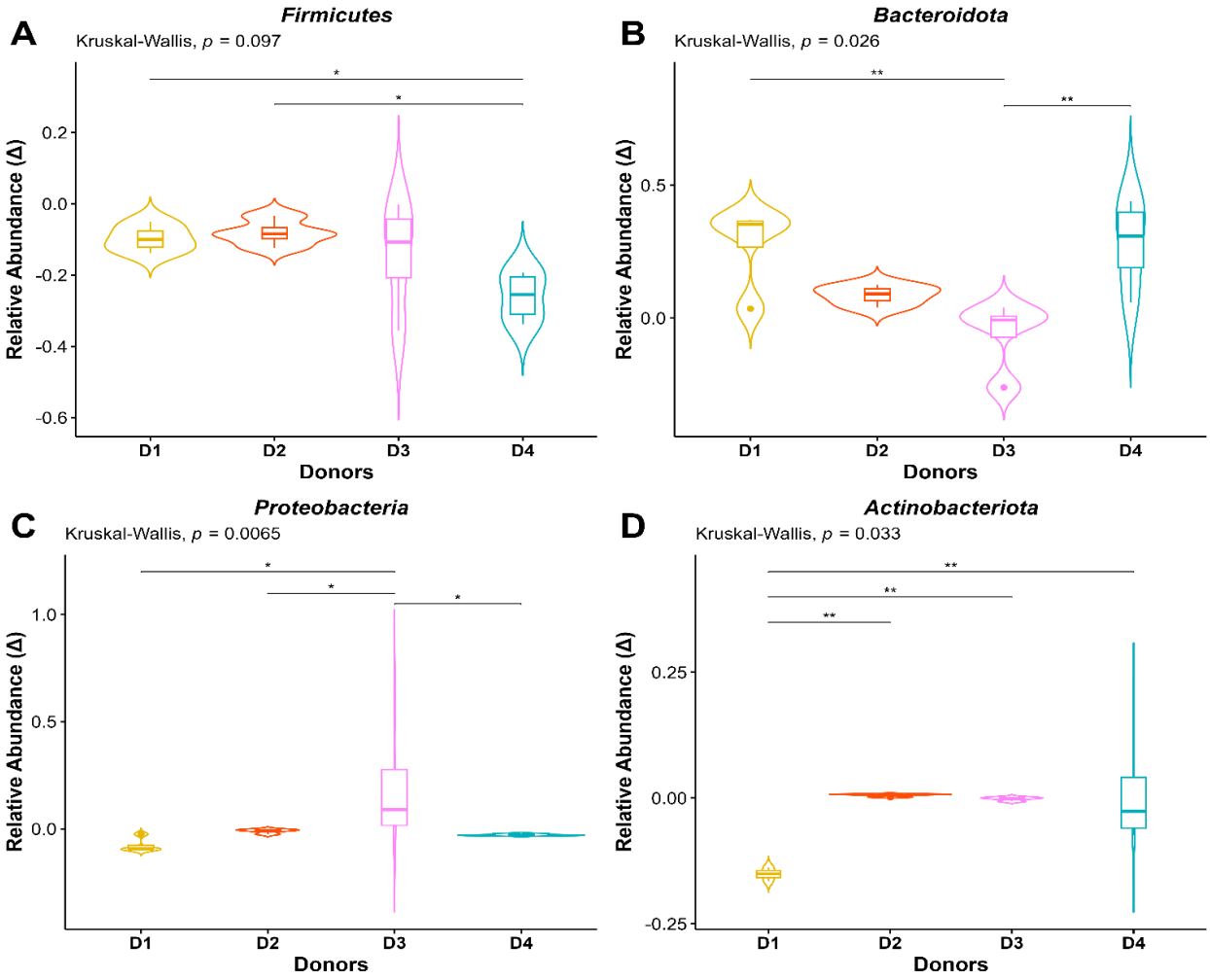

Figure 2.8. Variations in the microbiome composition depending on the donor sex for the top abundant phyla, Firmicutes (A), Bacteroidota (B), Proteobacteria (C), and Actinobacteriota (D).

D1 & D4 are the male donors and D2 & D3 are the female donors. (*p < 0.05, **p ≤ 0.01).

3.1.3. Microbiota Diversity is Affected Over Time by Sex Hormones

The α-diversity was measured using three metrics: Observed, Shannon, and Simpson, and the shift from baseline (Time 0) was calculated and statistically investigated. After two days of fermentation, we found that the female group had significantly lower α-diversity than the control group, as indicated by the KW p-value for Shannon and Simpson's by 0.016 and 0.0037, respectively. Moreover, the female group showed lower observed features, Shannon entropy, and Simpson's diversity compared to the control ($p < 0.05$, observed index; $p \leq 0.01$, Shannon entropy and Simpson's index) (Fig. 2.9 A-C). Additionally, the female group had lower Simpson's index values than the male group ($p < 0.05$ (Fig. 2.9 C). Despite significant differences in the α-diversity

metrics, β-diversity analysis revealed no discernible differences between the study groups (Fig. 2.9 D).

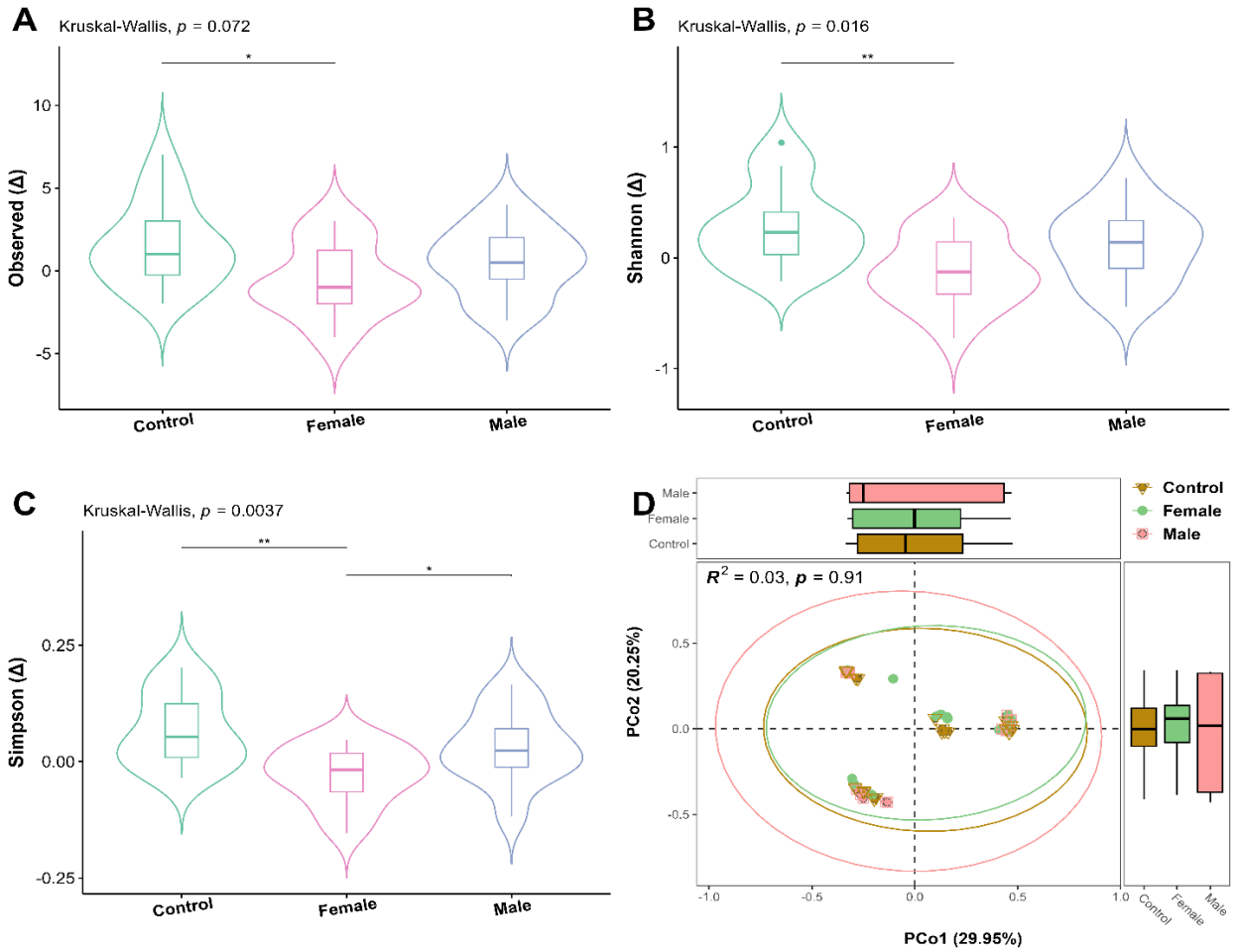

Figure 2.9. Analysis of gut microbiota diversity across 4 study groups.
Panels (A), (B), and (C) present α-diversity analyses, showing the change in Observed Features, Shannon, and Simpson's Index, respectively. (D) shows Beta Diversity analysis through Principal Coordinate Analysis (PCoA). Asterisks indicate statistical significance: *p < 0.05, and **p ≤ 0.01.

3.1.4. Sex hormones significantly shape the microbiota structure

Differential abundance (DA) analysis was performed at the genus level after filtering sequences to remove low-abundance reads (frequency <10) and those present in < 10% of the samples. The findings revealed significant shifts in the gut microbiome composition of females and males compared to that of the control group (Fig. 2.10 A-C). DA findings revealed significant enrichment of *Eubacterium coprostanoligenes*, *Agathobacter*, *Sutterella*, and *Acidaminococcus* in the female group compared to the control group (p = 0.002, p = 0.02, and p = 0.03, respectively). In contrast,

Parasutterella was significantly enriched in the control group (p = 0.03) (Fig. 2.10 A, Table 2.3). While no variations were found in the male group compared to the control, the *Butyricicoccus* taxon showed a declining trend, although this difference was not statistically significant (p=0.05) (Fig. 2.10 B, Table 2.3).

Table 2.3. Results of significant taxa that are differentially abundant between groups using the LinDA linear model.

Phase	Comparison	Genus	Group	p-value
Phase 1	Female vs Control	*Eubacterium coprostanoligenes group*	Female	0.002025
		Agathobacter	Female	0.027187
		Sutterella	Female	0.030921
		Parasutterella	Control	0.031297
		Acidaminococcus	Female	0.037676
	Male vs Control	*Butyricicoccus*	Control	0.055778
	Psychot. vs Control	*Bacillus*	Psychot.	0.016187
		Enterococcus	Psychot.	0.044344
	Female vs Psychot.*	*Enterococcus*	Female	0.001481
	Female vs Psychot.	*Eubacterium coprostanoligenes group*	Female	0.012642
	Female vs Psychot.	*Acidaminococcus*	Female	0.020871
Phase 2	Female vs Control	*Acidaminococcaceae*	Female	0.0456666
Phase 3	Psychot. vs Control	*Blautia*	Psychot.	0.0030525

Psychot.*, the psychotropic group

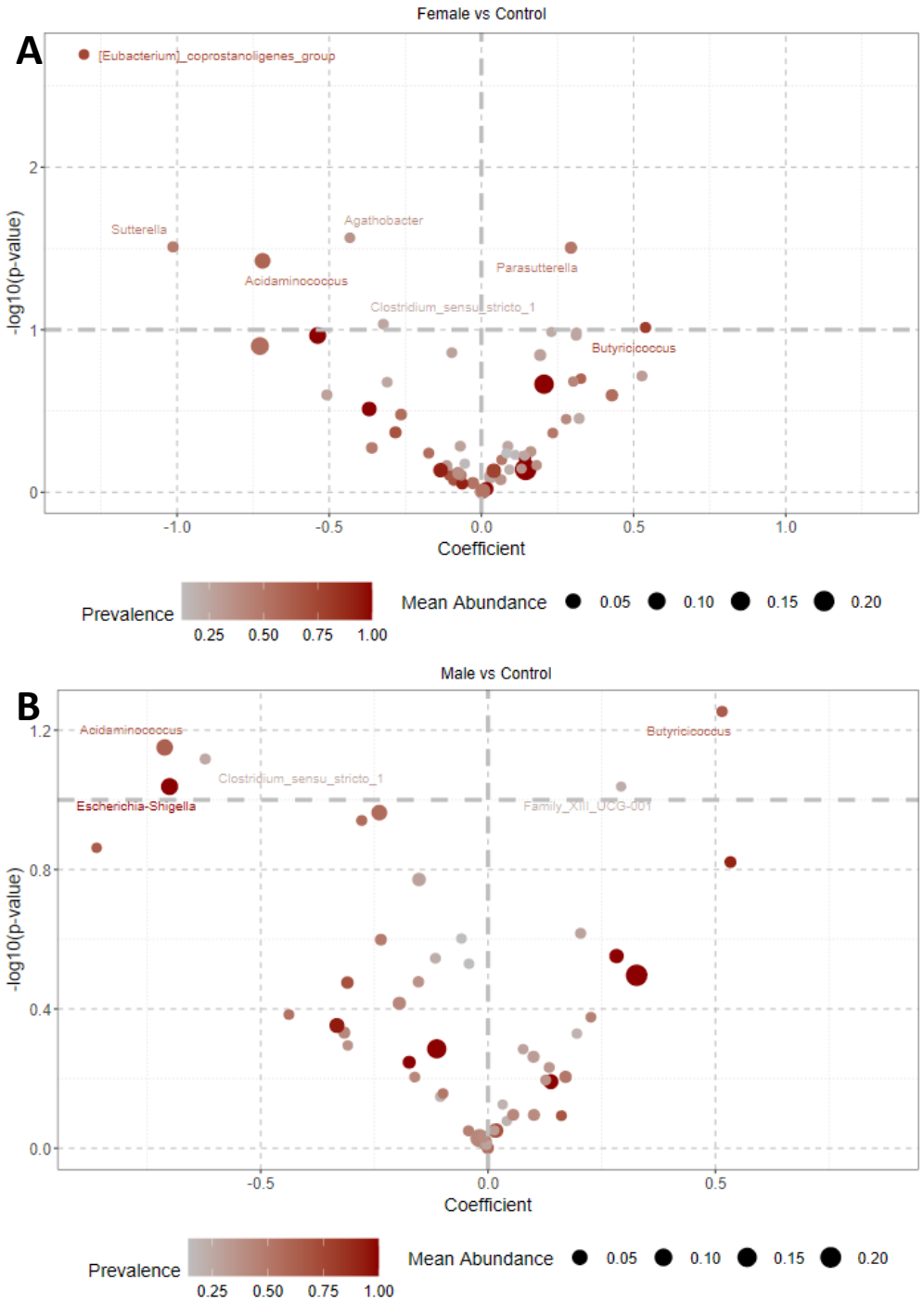

Figure 2.10. Volcano plots illustrate significant differences in specific taxa at the genus level between study groups compared to the control, as identified by the LinDA linear model.

3.1.5. Correlation between SCFAs and the most abundant families

In both the control (Fig. 2.11 A) and female (Fig. 2.11 B) groups, various bacterial families were associated with different SCFAs at specific time points. In the control group, Bacteroidaceae and Veillonaceae were negatively associated with butyrate at 6h, while Lachnospiraceae and Ruminococcaceae were positively associated (Fig. 2.11 A-1). By 12h, Acidaminococcaceae exhibited a positive association with both propionate and butyrate, whereas Bacteroidaceae continued to have a negative association with acetate (Fig. 2.11 A-2). At 24h, Bacteroidaceae remained negatively associated with acetate and Lachnospiraceae displayed positive associations with both propionate and butyrate (Fig. 2.11 A-3). Acidaminococcaceae was positively associated with acetate and propionate, Enterobacteriaceae with butyrate, and Veillonaceae was negatively associated with both acetate and propionate at 48h (Fig. 2.11 A-4).

In the female group, Bacteroidaceae was positively associated with acetate at 6h (Fig. 2.11 B-1), and Enterobacteriaceae was positively associated with propionate. At 12h, Bifidobacteriaceae and Veillonaceae were positively associated with both propionate and butyrate, whereas Oscillospiraceae and Ruminococcaceae were negatively associated with propionate and butyrate (Fig. 2.11 B-2). Finally, at 48h, Bifidobacteriaceae and Coriobacteriaceae were positively associated with both propionate and butyrate (Fig. 2.11 B-4).

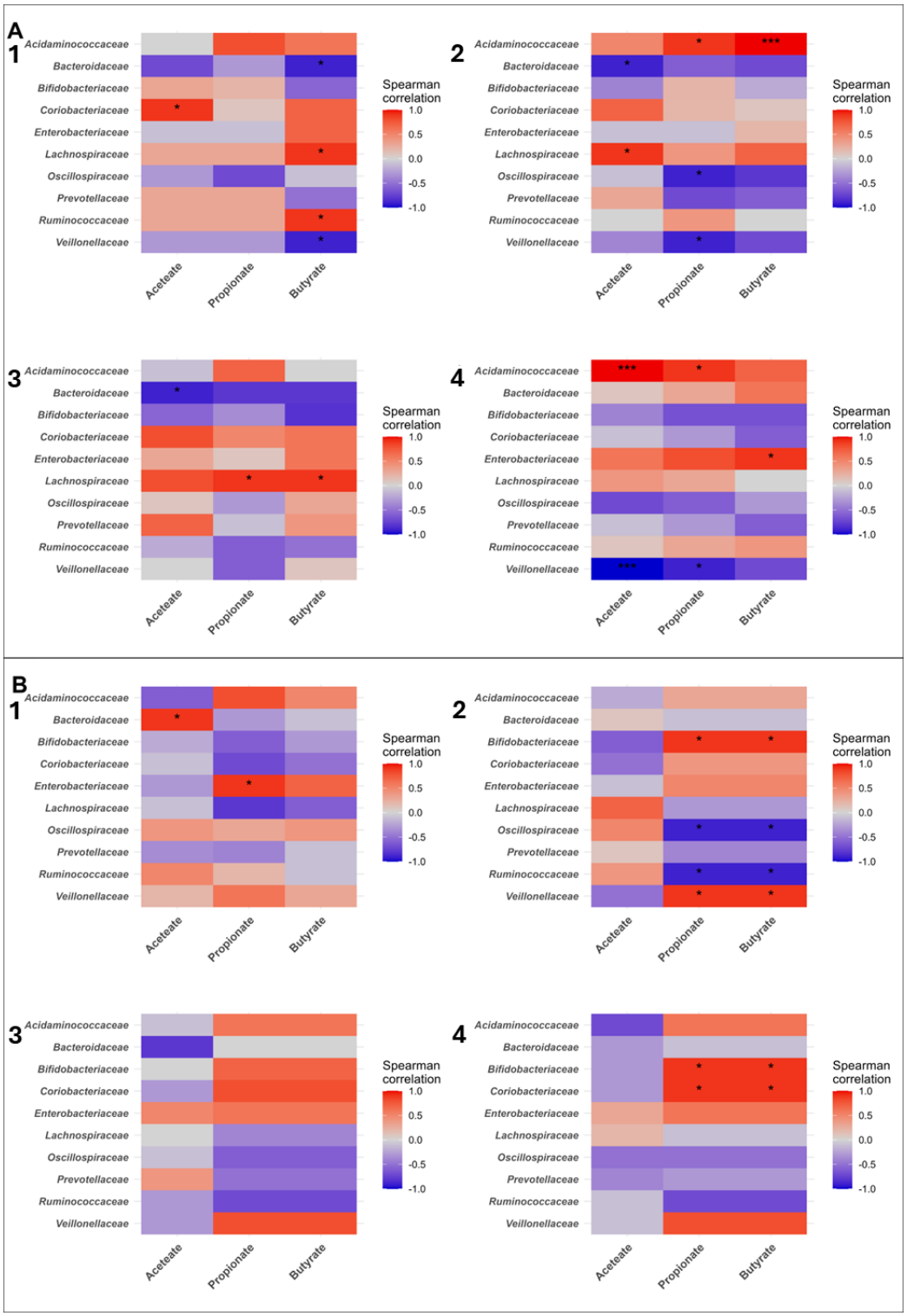

Figure 2.31. Correlation heatmap between the most abundant families and SCFAs Produced by the Control (A) and Female (B) simulators at various time points.

Panels 1-4 display Spearman's correlation coefficients for the time points of 6h, 12h, 24h, and 48h, respectively. In the heatmaps, dark red represents a strong positive correlation, while dark blue indicates a strong negative correlation between the families and SCFAs. *$p < 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$.

3.2. Impact of psychotropic medication on gut microbiome in the developed feminine colonic model

3.2.1. Microbiome composition

In the control group, the profile of phyla was similar across all phases; Bacteroidota and Proteobacteria exhibited a consistent increase in relative abundance. Conversely, Firmicutes and Actinobacteriota showed a steady decline, with more significant reductions at the end of the phase. In contrast, instability in the abundance of phyla for all phases was observed in the female and psychotropic groups, Bacteroidota consistently increased, similar to control, while Firmicutes showed a sharp decrease in phase 1 and was relatively stable in phases 2 and 3. Actinobacteriota maintained a relatively stable relative abundance, although only a minor increase was observed. Proteobacteria demonstrated the greatest variability, especially in the female and psychotropic bioreactors, where they initially decreased but subsequently recovered (Fig. 2.12 A-C).

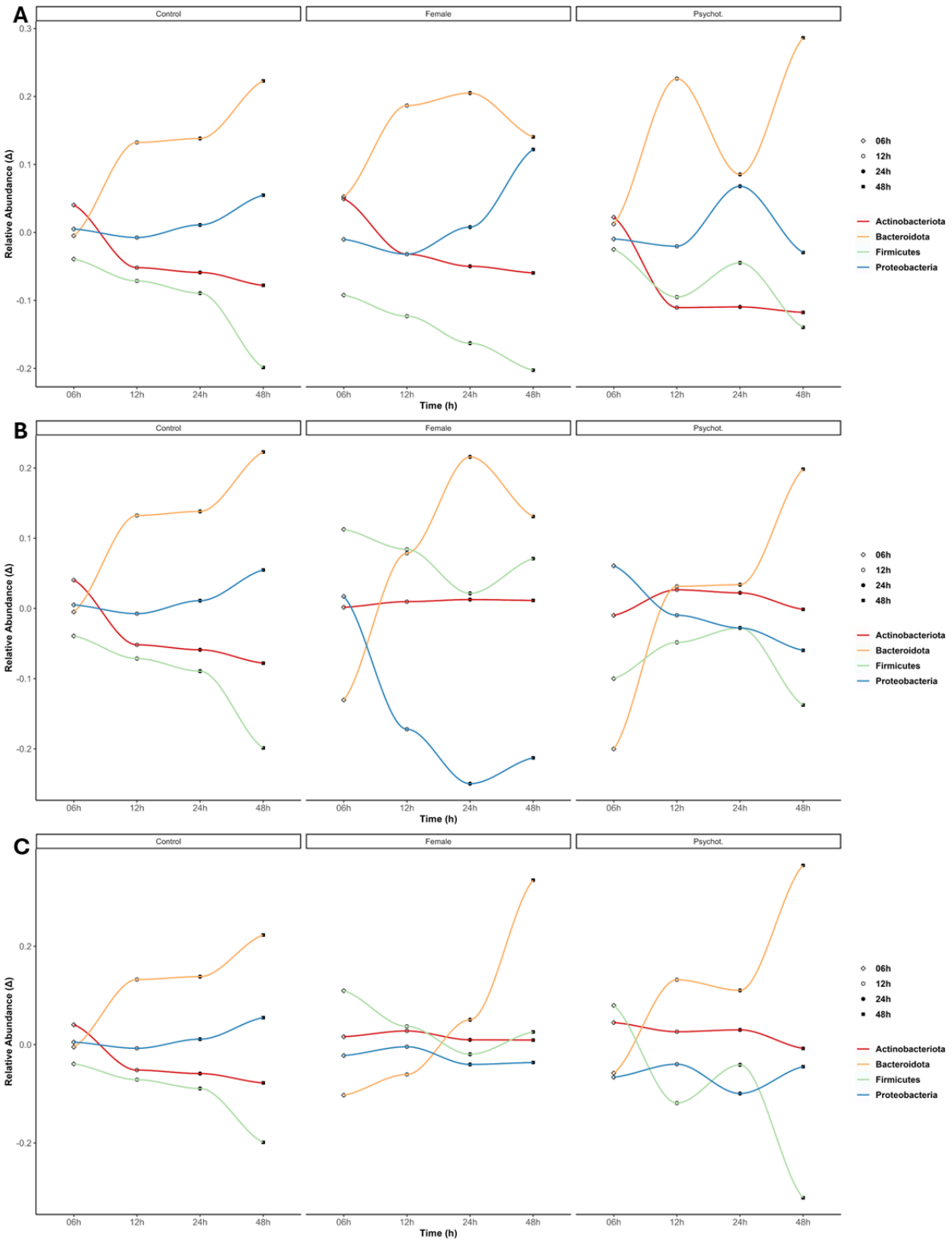

Figure 2.42. Relative abundance at the phyla level over time.
A-C: Relative abundance across three cycles during Phases 1, 2, and 3, showing changes in relative abundance (Δ from time 0) for each treatment group.

The relative abundances of Acidaminococcaceae and Bacteroidaceae were consistent across the Control and Female groups in phase 1 (Fig. 2.13 A). The Psychotropic group, however, showed a marked difference, particularly a higher abundance of Lachnospiraceae at the earlier time point (6h), but this seemed to decrease over time. Over time, bacterial diversity stabilized in both the Control and Female groups, with fewer drastic shifts after 48h. Phase 2 (Fig. 2.13 B) A greater variability was observed between the groups, especially at the 6-hour mark, where the psychotropic group showed a higher abundance of Enterobacteriaceae and Oscillospiraceae compared to the Female group. Enterobacteriaceae became more prominent in the female group at 24h and 48h, while the psychotropic group continued to exhibit more dynamic changes in bacterial family composition. In Phase 3 (Fig. 2.13 C), the differences between the Female and Psychotropic groups became more pronounced. The Female group showed a steady presence of Bacteroidaceae, whereas the psychotropic group exhibited a large increase in Ruminococcaceae and Prevotellaceae after 48h. The Control group remained relatively stable across all time points, suggesting less disruption compared with the other two conditions.

After normalizing the data with a rarefication approach, alpha diversity was investigated using Observed, Shannon, and Simpson's metrics. The Observed and Simpson's metrics were not significant at any time point in any of the three phases. However, a large difference in Shannon diversity (Δ) between phase 1 and phase 2 at 48h ($p < 0.05$) was found, indicating a notable shift in the microbial community composition and diversity over time (Fig. 2.14 A). In the Beta Diversity Analysis through Principal Coordinate Analysis (PCoA) presented in Fig. 2.14 B, there were no significant differences observed between the groups across the cycle phases.

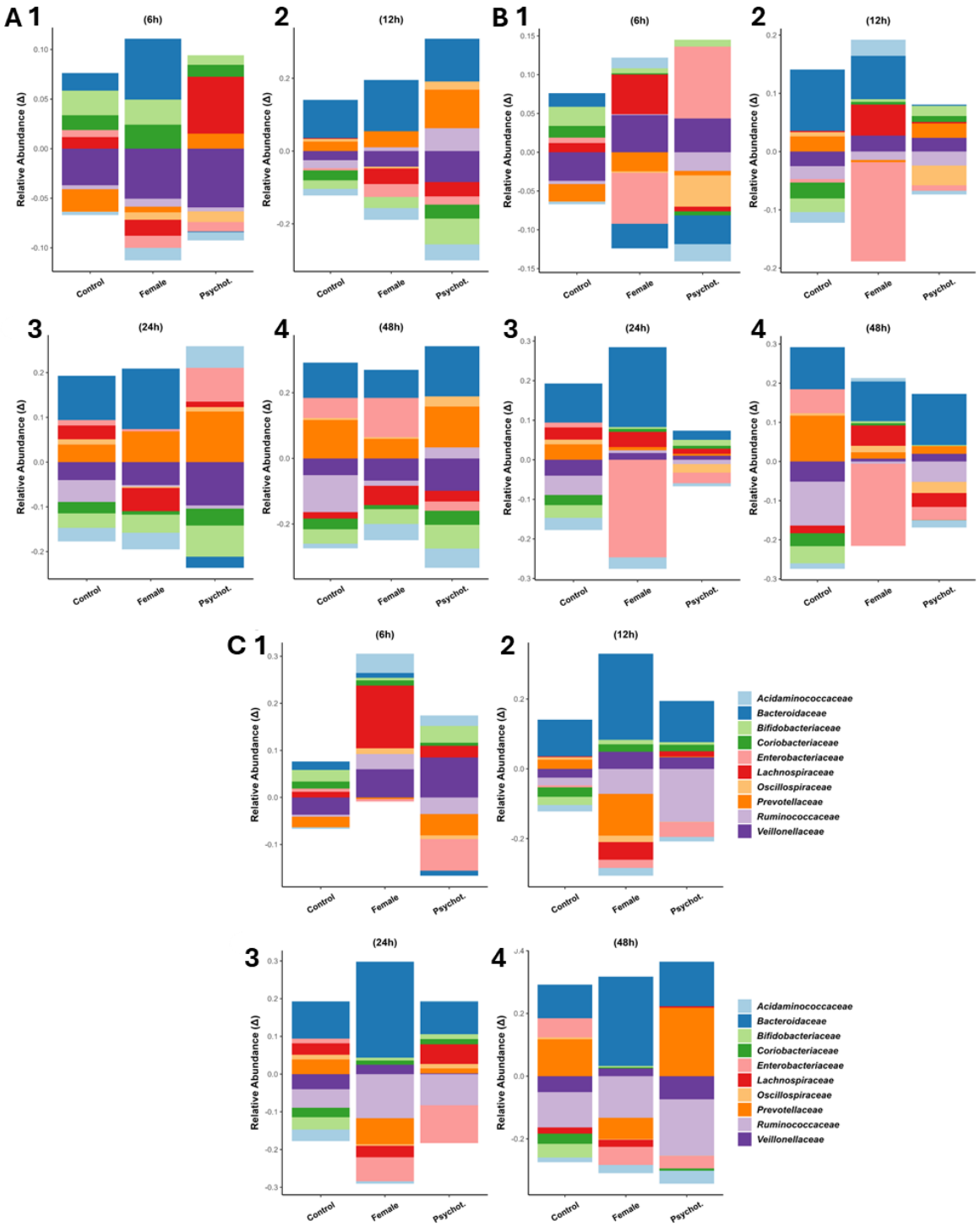

Figure 2.53. Changes in the abundance of the most abundant families over time.
A-C: Top Families' relative abundance across three cycles during Phases 1, 2, and 3 in females, respectively. Time points 1, 2, 3, and 4 correspond to 6 hours, 12 hours, 24 hours, and 48 hours, respectively.

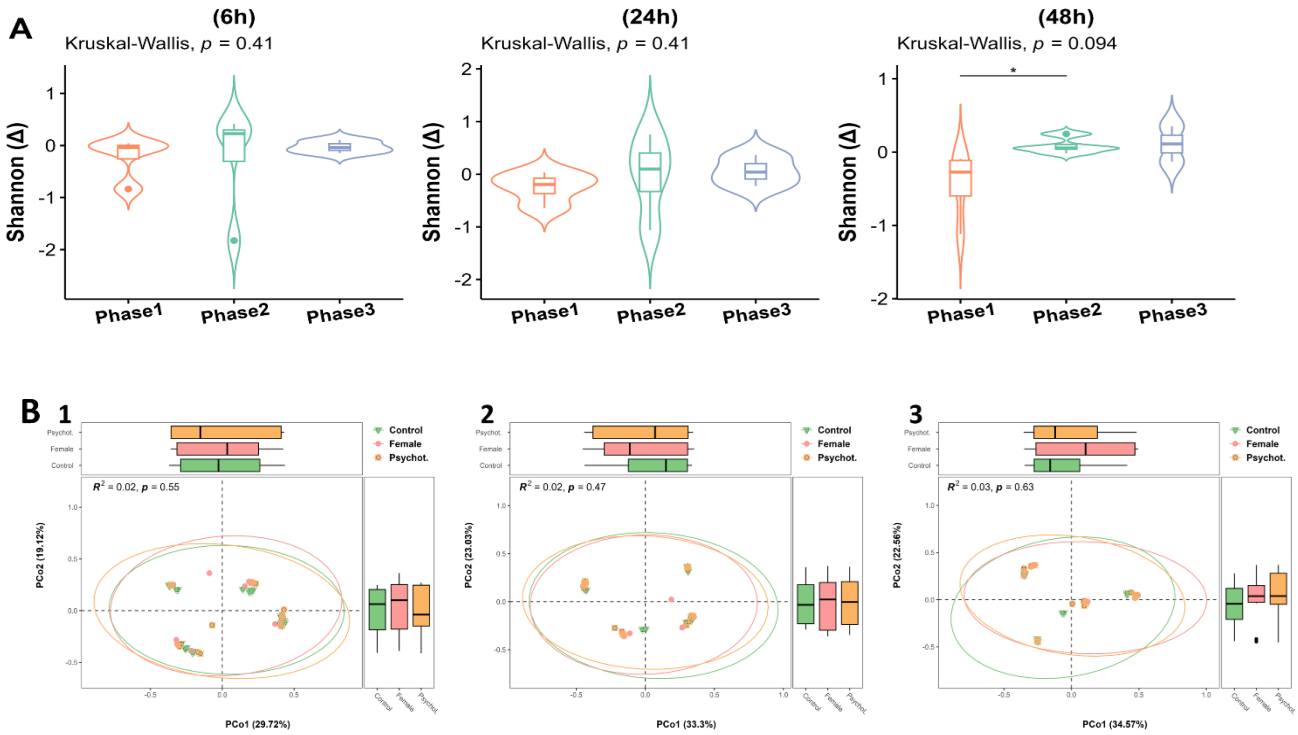

Figure 2.64. (A) Shannon Diversity by Cycle Phase. Panels display changes in Shannon diversity by cycle phase (Δ shift from diversity at time 0). (B) Beta Diversity Analysis Through Principal Coordinate Analysis (PCoA).
Panels A, B, and C present PCoA plots for cycles during Phases 1, 2, and 3, respectively. Asterisks indicate statistical significance: *p < 0.05.

Over time (6h, 12h, 24h, and 48h), alpha diversity analysis showed temporal changes in the microbial community composition in phase 1. The findings revealed significant differences between the study groups at 6 and 24h post-fermentation but not at 12 or 48h. All three α-diversity indices indicated lower diversity in the female group than in the psychotropic group at 6h ($p < 0.05$) (Fig. 2.15 A). Although the Observed index showed no significant differences at 24h, both Shannon and Simpson's metrics indicated a notable decrease in alpha diversity in the psychotropic group compared to the control and male groups ($p < 0.05$) (Fig. 2.17 A). In phase 2, significant variations were observed at 24 and 48h (Fig. 2.15 B). We observed a significant difference in diversity between the psychotropic and control group at 24h using the three metrics. The psychotropic group displayed a lower diversity. Interestingly, this trend was reversed at 48 h, with the female group showing a higher diversity than the psychotropic group ($p < 0.05$). In Phase 3, the psychotropic group showed lower diversity than the control group, as indicated by the observed index (Fig. 2.15 C).

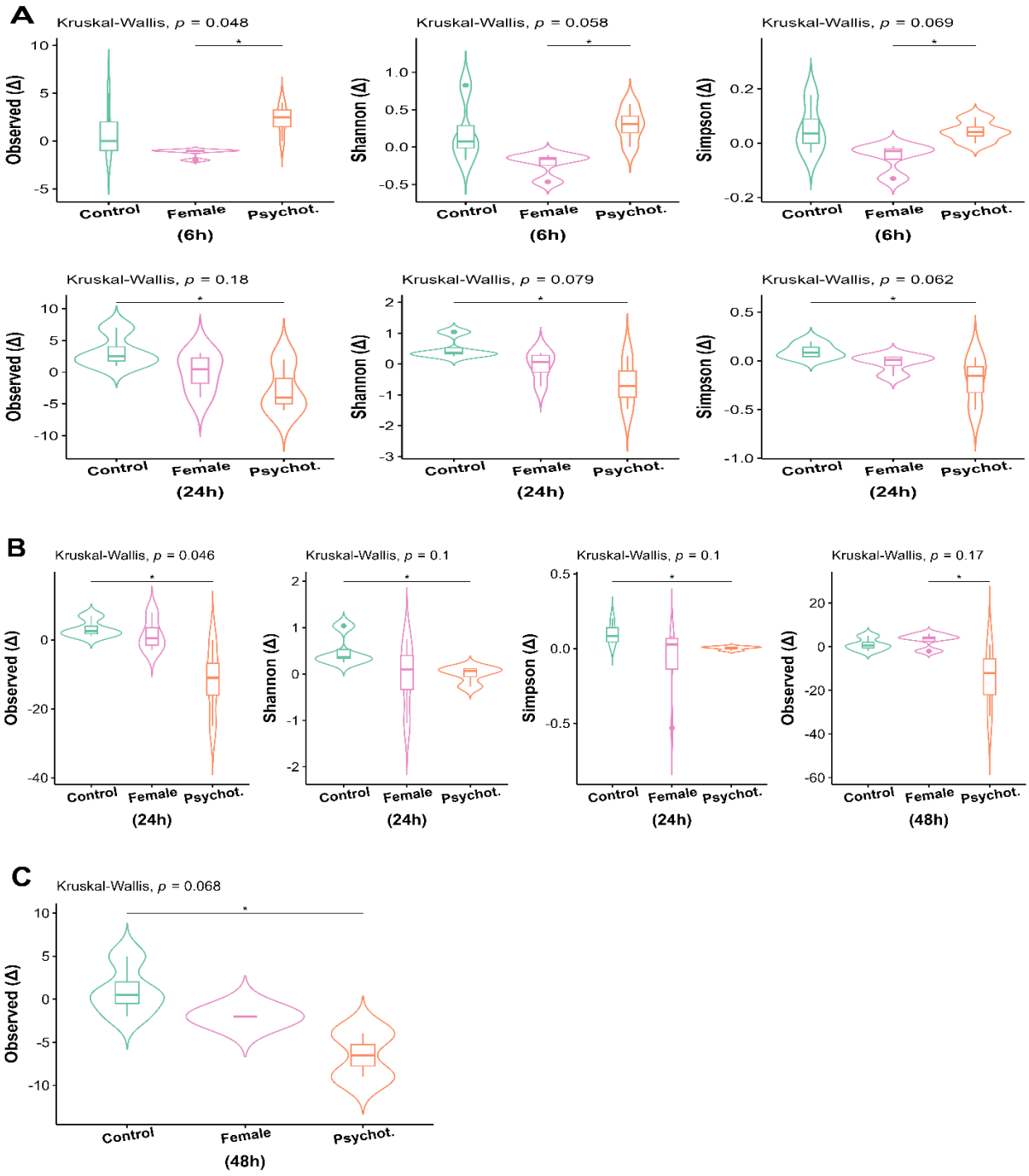

Figure 2.7. Alpha diversity analysis over time (6h, and 24h) for the first 2 days of fermentation.

Figure A-C illustrates the differences between the four study groups at 6h and D-F for 24h of Observed Features, Shannon, and Simpson's metrics. Asterisks indicate statistical significance: *$p < 0.05$.

Differential abundance analysis revealed variations between the groups across the three phases. When comparing the female group to the psychotropic, *Enterococcus* showed a negative shift (p = 0.001), indicating a decrease in its abundance under the psychotropic influence in phase 1. Similarly, *Eubacterium coprostanoligenes* group and *Acidaminococcus* exhibited reductions (p= 0.01, 0.02, respectively), suggesting a negative impact of psychotropic drugs on these bacteria (Fig. 2.16 A, Table 2.3). Acidaminococcaceae thrived significantly in the female group (p= 0.04) compared to the control at phase 2 (Fig. 2.16 B). In phase1, psychotropic medication was found to have a significant impact on microbiota composition, promoting the growth of *Bacillus* while suppressing *Enterococcus* (p = 0.01, p = 0.04, respectively) (Fi. 2.16 C, Table 2.3). However, in Phase 3, *Blautia* was largely elevated in the psychotropic (p= 0.003) compared to the control (Fig. 18 C).

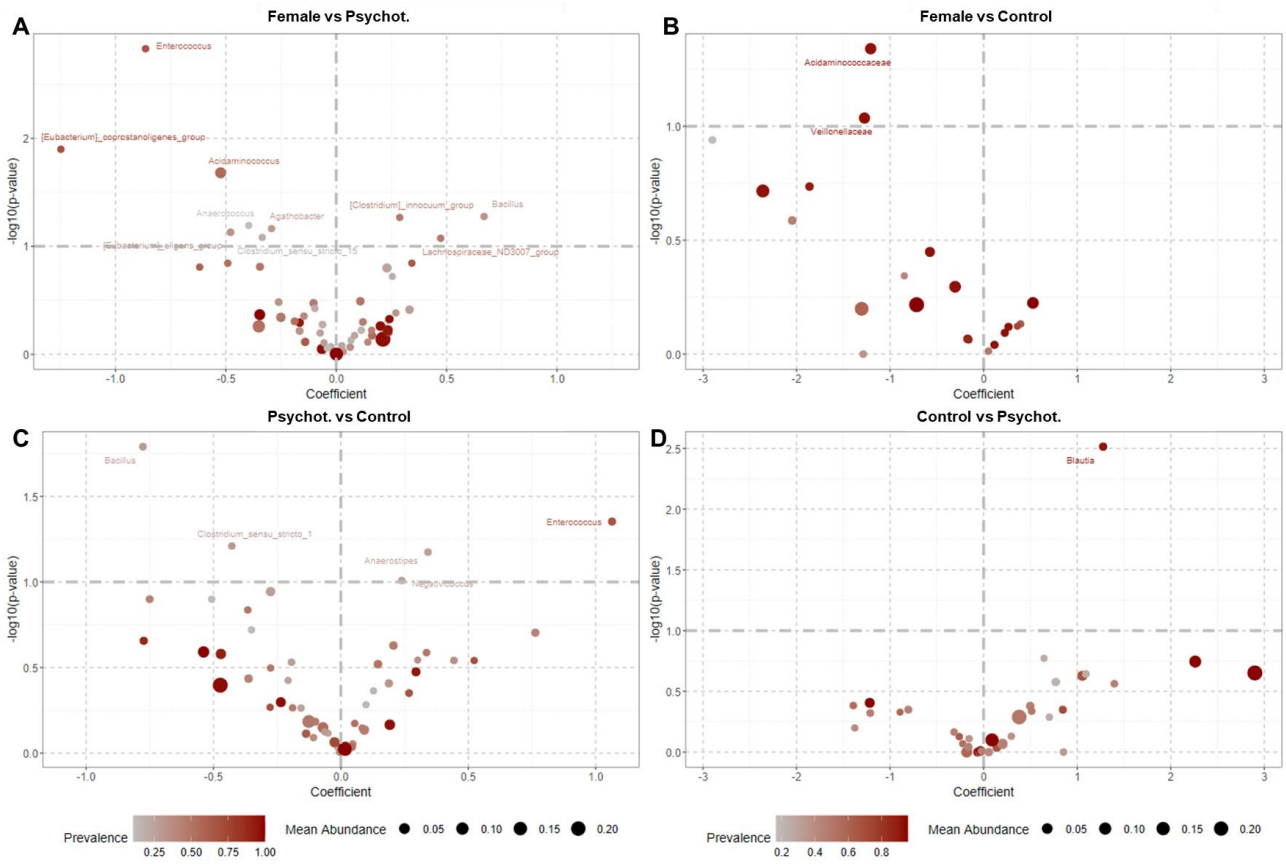

Figure 2.86. Volcano plots highlight significant differences in specific taxa between the study groups and the control.
(A) DA between female and psychotropic in phase 1, (B) between female and control in phase 2, and (C) and (D) between control and psychotropic in phase 1&3 respectively.

3.2.2. SCFA production levels by phases

A comparative analysis of SCFA concentrations across the study groups revealed distinct temporal profiles. In the first 48 h of treatment, SCFA concentrations in the control group were higher for all three analyzed SCFAs, following a similar trend of gradual decline over time. The other treatment groups also displayed a decrease in SCFA levels over time (Fig. 2.17 A). In the female group, SCFA concentrations decreased sharply during phase 3 compared with the other phases, showing a more pronounced reduction over time for all three SCFAs (Fig. 2.17 B). In the psychotropic group, acetate levels increased after 12 h in Phase 3 and then decreased after 24 h. In contrast, propionate and butyrate showed steady increases throughout the time points (Fig. 2.17 C).

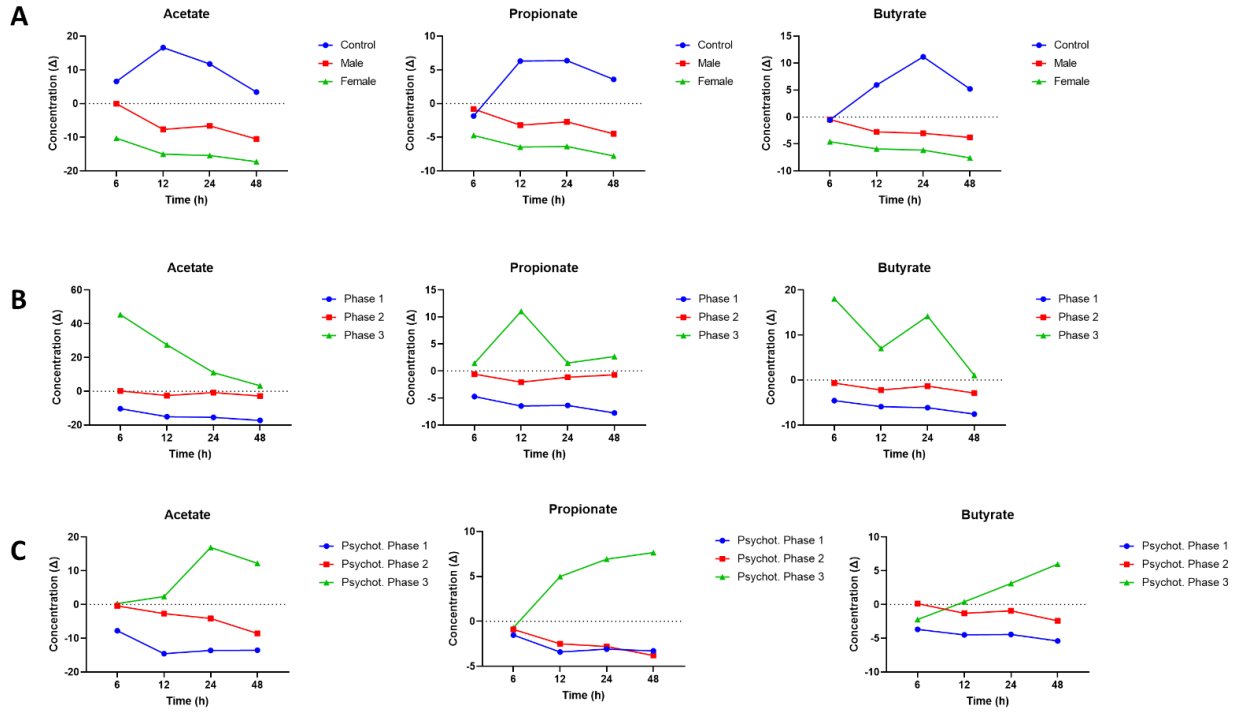

Figure 2.97. SCFA Concentration by Time Point and Cycle Phase.
Panels show changes in SCFA concentration relative to baseline (Δ SCFA = time point - baseline). A- SCFA concentration over time at the first 48h. B- SCFA concentration by cycle phase in the female bioreactor over time. C- SCFA concentration by cycle phase in the psychotropic bioreactor over time.

4. DISCUSSION

The aim of this study was to investigate the impact of the sex steroid hormones on the gut microbiota. This study aimed to develop a female simulated model of the colon and assess the microbiome response to antidepressant drugs. The findings of this study support the hypothesis that sex hormones play an important role in modulating gut microbiome composition and metabolism.

4.1. Sex-Specific Differences in Gut Microbiome

Our results demonstrated that the gut microbiome of male and female models exhibited differences in bacterial abundance. At the phylum level, Bacteroidota was increased in all study groups, with the exception of a decline at 48h in the female group. Pairwise comparisons revealed differences at 24h between male and female groups, where Bacteroidota was notably higher in females, while Firmicutes increased in males.

The observed differences in bacterial abundance between male and female colonic models, particularly the higher levels of Bacteroidota in females and Firmicutes in males at 24h, align with findings from the literature, such as d'Afflitto et al. (2022). Their meta-analysis revealed that in healthy women, higher estrogen levels were associated with a greater abundance of Bacteroidetes and a reduction in Firmicutes, particularly within the Ruminococcaceae family (d'Afflitto et al., 2022). These results contrast with those of several previous studies that reported a lower abundance of Bacteroidota and a higher proportion of Firmicutes in females than in males. For instance, Koliada et al. (2021) found that the relative abundance of Firmicutes and Actinobacteria was significantly higher in females, whereas that of Bacteroidetes was significantly lower (Koliada et al., 2021). Similarly, Kaliannan et al. (2018) reported that the Firmicutes/Bacteroidota ratio was elevated in female mice (Kaliannan et al., 2018). Further complicating the picture, a study in Spain found that Bacteroidota abundance was lower in men than in women with Body Mass Index (BMI) >33 (Haro et al., 2016). This variability suggests that factors such as BMI, diet, and overall health could also influence the relationship between sex and microbial composition (X. Gao et al., 2018). The discrepancies between our findings and those of previous studies may be attributed to variations in hormonal treatments or the conditions under which the studies were conducted. For

instance, the hormonal environment in our study might have been different from those reported in studies showing lower Bacteroidota and higher Firmicutes in females.

Microbial diversity, as measured by α-diversity, further supports the influence of sex hormones on gut microbiota. The lower Simpson's index values in the female group suggest that females exhibit less evenness in their microbiota, indicating that certain bacterial taxa dominate the ecosystem more than in males. Despite the differences observed in α-diversity, no significant differences in β-diversity were found between the study groups, indicating that while the evenness of the microbial communities may vary, the overall composition of microbial species remains similar across the sexes. Notably, these results were obtained in the first two days of treatment when bioreactors mimicked the follicular phase with low estrogen and progesterone (E-P-) levels, potentially influencing the observed microbial patterns. These findings contradict some existing literature on the effects of sex hormones on the gut microbiota. Shin et al. investigated the association between serum levels of sex steroid hormones and gut microbiome diversity. They found that although no significant changes in microbiota richness were observed among groups with varying levels of testosterone or estradiol, significant differences in diversity and evenness were noted. Specifically, men with high testosterone levels and women with high estradiol levels showed significantly higher diversity and evenness scores than their medium-level counterparts (J.-H. Shin et al., 2019).

In our study, low hormone levels may have contributed to the observed reduction in microbial evenness in females, consistent with previous studies showing an inverse relationship between estradiol and α-diversity (Insenser et al., 2018). Additionally, the lack of an association between estrogen and diversity in premenopausal women, as observed by Flores et al. (2012), may help explain the variability in findings across studies (Flores et al., 2012). The hormonal context, phase of the menstrual cycle, and individual hormonal levels are likely key factors in determining the impact of estrogen on gut microbiota diversity (Krog et al., 2022).

When examining the influence of the sex of the microbiota donor, the data revealed that female sex hormones impacted bacterial communities across all donors, indicating a notable effect of hormonal treatment on microbiota composition. Although the donor's original sex still affected the microbial response, hormonal treatment induced consistent directional changes, such as a decreasing trend in Actinobacteriota across both male and female donors. However, the extent and

timing of these changes can vary, highlighting the need for further research to understand and optimize the influence of sex hormones on microbial profiles.

4.2. Impact of Sex Hormones on Microbiome Diversity

Our study revealed distinct shifts in microbiota influenced by sex hormones. The study's α-diversity metrics showed lower diversity in females than in males, as previously mentioned, but also in controls, with a significant reduction in Simpson's index. The lack of difference between the microbiota in the bioreactors without hormones and those with testosterone indicates that testosterone might not significantly alter the α-diversity. In a recent study, Moadi et al. (2024) found that testosterone supplementation led to significant increases in α-diversity and shifts in microbial composition in pre-pubertal mouse samples, indicating a pronounced effect before puberty. However, they also reported that testosterone had no significant effect on α-diversity, β-diversity, or differentially abundant taxa in samples collected after puberty, suggesting that the influence of testosterone on the microbiome may diminish or vary with the developmental stage (Moadi et al., 2024).

In our study, the distinct differences observed in female hormones suggest that estrogen and progesterone have a more pronounced effect on microbial diversity. This lower diversity in females could be attributed to hormone concentrations, which are known to influence microbial ecosystems (Yoon & Kim, 2021). In the real-world context, such changes are known to potentially increase women's susceptibility to conditions linked to microbial imbalances, such as inflammatory bowel disease or metabolic syndrome (Santos-Marcos et al., 2023; S. Y. So & Savidge, 2021).

Differential abundance analysis at the genus level further highlights the sex-specific influence of hormones. For example, the female bioreactor exhibited enrichment of *Eubacterium coprostanoligenes*, *Agathobacter, Sutterella*, and *Acidaminococcus*, whereas *Parasutterella* was significantly enriched in the control group. These findings align with those of previous studies highlighting the influence of estradiol on microbial composition. Ortiz-Alvarez De La Campa et al. (2024) observed that increased estradiol levels were significantly associated with *Eubacterium coprostanoligenes* in mice, supporting our observation of its enrichment in the female bioreactor (Ortiz-Alvarez De La Campa et al., 2024). Similarly, Zhou et al. (2020) reported a positive correlation between *Sutterella, Agathobacter*, and estradiol levels in obese women with PCOS (L.

Zhou et al., 2020). However, Wu et al. (2022) found a positive correlation between *Parasutterella* and estradiol, which is in contrast to our results showing a higher abundance in the control group (Wu et al., 2022).

In every phase of the cycle, female hormones caused sharper increases in Bacteroidota and more dramatic declines in Proteobacteria and Firmicutes compared to the control, which maintained a more gradual and steady bacterial profile. Notably, the female bioreactor demonstrated higher peaks in Bacteroidota and a decline in Firmicutes during phases 2 (E+P-) and 3 (E+P+), when estrogen levels were elevated. These findings suggest that hormonal fluctuations play a critical role in driving specific shifts in the bacterial composition. This aligns with Shin et al. (2019), who reported that higher estradiol levels in healthy women correlated with increased Bacteroidetes abundance and reduced Firmicutes abundance (J.-H. Shin et al., 2019), reinforcing the idea that sex hormones significantly influence the gut microbiome dynamics, particularly throughout the menstrual cycle. Interestingly, despite significant differences in α-diversity, β-diversity analysis revealed no discernible differences between groups or cycle phases. This suggests that although the overall diversity of bacterial species varied, the community composition remained relatively similar across the different study groups.

In the female bioreactor, distinct patterns of bacterial association with SCFAs emerged at different time points. At 6h, Bacteroidaceae were positively associated with acetate, whereas Enterobacteriaceae were linked to propionate. By 12h, Bifidobacteriaceae and Veillonaceae were positively correlated with propionate and butyrate, whereas Oscillospiraceae and Ruminococcaceae were negatively associated with these SCFAs. By 48h, Bifidobacteriaceae and Coriobacteriaceae were positively associated with propionate and butyrate. Despite these associations, SCFA concentrations in the female bioreactor were lower than those in all other treatment groups, with a notable decline in every cycle phase, particularly in phase 3 (E+P+), which exhibited the most pronounced decrease. This decline in SCFA levels may reflect hormonal effects on microbial metabolic activity and highlight the potential disruptions in gut health due to hormonal fluctuations (Acharya et al., 2023).

4.3. Interaction of Psychotropic Medication with Gut Microbiota

We observed that psychotropic medication significantly affected gut microbiota, leading to increased microbial diversity and evenness within the bioreactor. This finding aligns with a recent meta-analysis by Minichino et al. (2024), who reported that treatment with antipsychotics and antidepressants is associated with significant changes in gut microbiome diversity indices, taxonomy, and functionality (Minichino et al., 2024). They also found that baseline gut microbiome parameters were linked to subsequent therapeutic responses, highlighting the importance of the initial microbial profiles in predicting treatment outcomes (Minichino et al., 2024). The instability in the relative abundance of major phyla, particularly the increase in Bacteroidota and decrease in Firmicutes and Actinobacteria, suggests that psychotropic medications may selectively inhibit the growth of certain bacterial populations. This observation is consistent with the findings of Pan et al. (2022), who studied longitudinal changes in the microbiome of children during treatment with atypical antipsychotics including aripiprazole. Their research also indicated changes in the composition of microbiota over time (Pan et al., 2022).

Furthermore, Flowers et al. (2017) reported significant differences in gut microbiota communities between patients treated with atypical antipsychotics (including aripiprazole) and those who were not (Flowers et al., 2017). In their study, treated women exhibited decreased species diversity compared to the non-treated group, whereas men showed no significant differences between treatment groups (Flowers et al., 2017). This sex-specific response to antipsychotic treatment reinforces our findings of higher α-diversity in the psychotropic group, particularly during the early fermentation phases, despite the suppression of taxa, such as Enterococcus.

A notable observation from our study is the impact of psychotropic medication on SCFA production, which is a crucial aspect of the gut microbiome function. In our study, acetate levels increased initially but declined after 24h, while propionate and butyrate levels steadily increased throughout the fermentation period. This shift in SCFA profiles may have important implications for gut health. SCFAs such as acetate, propionate, and butyrate are vital metabolites produced by gut bacteria through fermentation of dietary fibers and resistant starches. These SCFAs contribute to energy metabolism, regulate immune responses, and maintain the integrity of the gut barrier (Mann et al., 2024).

Our results are consistent with the findings of Hua et al. (2022), which highlighted that ketamine and its metabolites can enhance antidepressant effects by promoting microbiota associated with SCFAs (Hua et al., 2022). Lu et al. (2023) also demonstrated that antidepressant medications exert therapeutic effects by interacting with the gastrointestinal microbiome and its metabolites (Y.-L. Lu et al., 2023). Furthermore, Singh et al. (2022) reviewed evidence showing that antipsychotic drugs can disturb the intestinal barrier, intensify gut-associated lymphoid tissue activity, and reduce SCFA synthesis (Singh et al., 2022), which aligns with our observation of SCFA profile changes.

4.4. Implications for Personalized Treatment

Aripiprazole, a partial dopamine agonist, is widely prescribed for conditions such as schizophrenia, bipolar disorder, and, in some cases, MDD (Tuplin & Holahan, 2017). These conditions, particularly in women, often intersect with hormonal fluctuations that can modulate both psychiatric symptoms and treatment responses (Brand et al., 2022; Lin et al., 2024). Estrogen and progesterone are known to influence not only the dopaminergic pathways but also (Hwang et al., 2020; Wieczorek et al., 2023), as observed in this and past studies, the gut microbiota, which together play critical roles in mental health (D. Li et al., 2023; Sovijit et al., 2021). This interaction raises important considerations for women with psychotic disorders, a significant clinical population taking aripiprazole. For these individuals, the effects of aripiprazole on the gut microbiota may be compounded by sex hormone-driven changes in the gut-brain axis. These results suggest that psychotropic medications may need to be tailored based on the sex-specific microbiome and hormonal status of the patients (Lotter & Altfeld, 2019).

The observed differences between male and female gut microbiomes, often referred to as the "microsexome" (Mulak et al., 2022), indicate that treatments effective for one sex may have unintended or less effective outcomes for the other. The "microsexome" not only influences gut composition but also plays a crucial role in regulating immune processes and driving sex differences in disease manifestation (Vemuri et al., 2019). For instance, the reduced abundance of beneficial SCFA-producing bacteria in females undergoing psychotropic treatment raises concerns about the long-term impact of these medications on gut health, potentially leading to dysbiosis or metabolic complications (Hua et al., 2022; Singh et al., 2022). As a result, treatments such as psychotropic medications or those aimed at manipulating the microbiome, including fecal

microbial transplants, may need to be carefully adjusted based on the patient's sex to optimize therapeutic outcomes.

4.5. Limitations

Although fermentation bioreactor models are valuable tools for studying the gut microbiome, they have certain limitations. A significant challenge is the inability to fully replicate the complexities of the human gut environment. For instance, these models do not capture key physiological factors such as the movement of the gut (peristalsis), protective mucus layers, or direct interactions between gut microbes and host tissues (Costa & Ahluwalia, 2019). These limitations indicate that while the model offers controlled conditions for studying microbial dynamics, it may not account for all aspects of gut function and its interaction with the microbiota.

Despite these limitations, the in vitro fermentation model offers several advantages that make it an effective tool for studying the gut microbiome. This system allows for the controlled manipulation of different variables, enabling researchers to simulate the specific physiological conditions of both male and female guts (Isenring et al., 2023). The use of immobilized fecal microbiota enhances stability and microbial viability, closely mimicking the environment of the colon (Dieni & Ustadi, 2020). Additionally, the continuous mode of operation and parallel reactor design ensure reproducibility and enable long-term studies, providing valuable insights into microbial community dynamics and functional outputs, such as SCFA production (Isenring et al., 2023).

5. Conclusion

This study provides compelling evidence for the need to consider sex differences in the microbiome when prescribing psychotropic medication. Future research should focus on understanding the molecular mechanisms by which sex hormones and psychotropic drugs interact with the microbiome. Moreover, the significant role of SCFAs underscores the potential consequences of microbiome disruption, particularly in females. The loss of beneficial SCFA-producing microbes in females could indicate the need for tailored therapeutic strategies, such as incorporating probiotics or dietary modifications to maintain SCFA production and gut health during psychotropic treatment. Additionally, hormonal fluctuations, especially those related to the

menstrual cycle or menopause, may further influence microbial responses, thereby emphasizing the importance of hormone-informed treatment strategies.

Clinical studies are needed to confirm these findings in human populations and explore how personalized interventions targeting the gut microbiome may improve psychiatric treatment outcomes. By considering microbiome composition, hormonal status, and sex-specific responses, future psychiatric treatments could achieve more precise and effective results, reducing the risk of gut dysbiosis and improving overall mental health outcomes.

Authorship Contributions

Luana Leao: Literature acquisition and review, drafting, data collection, and performing experiments. Galal Esmail: Performed experiments, data analysis, drafting, and reviewing the manuscript. Saba Miri: Performed experiments, data collection, and critical revision of the manuscript. Walid Mottawea: Performed experiments and data collection. Riadh Hammami: Conceptualization, drafting, and critical revision of the manuscript. The authors would like to thank Dr. Ayman ElSayed for his assistance in the gas chromatography analyses, which significantly contributed to the study.

CHAPTER 3:

General conclusion and perspectives

This thesis explored the complex interactions between the gut microbiome, psychotropic medications, and sex steroid hormones. Through a combination of in vitro fermentation models and microbiome analysis, this research has helped decipher the crucial role of sex-specific factors in shaping gut microbiota and the effects of a psychotropic drug. These findings provide significant insights into the "microsexome", underscoring how sex hormones influence not only the composition and diversity of gut bacteria, but also the functional outcomes related to gut health, including SCFA production and microbial stability.

The results of this study suggest that sex hormones play a critical role in determining the gut microbial composition and diversity. The simulated male and female colons exhibited distinct microbial profiles in response to hormonal manipulation and psychotropic medications, with the female model showing a trend toward reduced microbial diversity and alterations in key microbial taxa. This reduction in diversity, particularly in SCFA-producing bacteria, highlights the potential vulnerabilities in females related to gut health, metabolic regulation, and immune responses when exposed to psychotropic treatments (Lu et al., 2023; Shin et al., 2019)

The limitations of the in vitro bioreactor model, while acknowledged, does not diminish the value of the findings. The controlled environment enabled precise observation of microbial dynamics and the interplay between hormones, medication, and microbial communities. However, future work is necessary to validate these findings in vivo, as human gut physiology introduces complexities that are not fully captured by in vitro systems, such as the influence of host-microbiota interactions, immune responses, and hormonal fluctuations during life stages such as menstruation and menopause (Krog et al., 2022).

The implications of this study extend beyond the study of microbiome-drug interactions. This work opens avenues for more personalized approaches to psychiatric treatment by revealing the sex-specific effects of psychotropic drugs on the gut microbiota. Sex differences in microbiome composition and response to medication suggest that future therapeutic strategies should consider the hormonal status and microbiome profiles of patients. Tailoring psychotropic drug treatments based on sex could optimize therapeutic outcomes while minimizing the adverse effects related to gut health and metabolic functions (X. Gao et al., 2018). Moreover, this study emphasizes the

potential of integrating microbiome-modulating therapies, such as probiotics, prebiotics, and fecal microbiota transplantation, into treatment plans for psychiatric conditions. Particularly, for women undergoing psychotropic treatment, interventions aimed at preserving or enhancing SCFA-producing bacteria may offer protective benefits against dysbiosis and related complications. Hormonal fluctuations, including those occurring during the menstrual cycle or menopause, can further exacerbate these microbial shifts (Vemuri et al., 2019).

In conclusion, this thesis contributes to the growing body of evidence highlighting the importance of the microbiome in mental health and the need for sex-specific approaches to psychiatric care. The insights gained from this research lay the foundation for future investigations into the "microsexome" and its implications for precision psychiatry, ultimately aiming to improve treatment outcomes and patient well-being.

Future directions in this area of research would be:

i. Expand the donor pool by replicating this study with a larger number of fecal donors to capture greater diversity in terms of age, health status, and hormone levels ensures that the results are generalizable to a wider population.
ii. Test different psychotropic medications in addition to aripiprazole, comparing their effects on gut microbiota composition, and determining how different classes of these drugs affect the microbiome.
iii. Conduct *in vivo* studies using animal models to validate these findings, particularly focusing on the gut-brain axis and its role in mood disorders while assessing the long-term effects of both hormones and psychotropic drugs on microbiome health.
iv. Undertake clinical trials involving both healthy and mentally ill individuals undergoing psychotropic treatment should be undertaken to assess how changes in gut microbiota correlate with treatment outcomes and overall mental health improvement.

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