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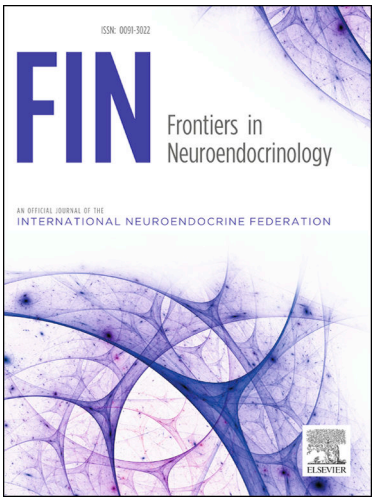

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You've Got Male: Sex and the Microbiota-Gut Brain Axis Across the Lifespan

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Abstract

Sex is a critical factor in the diagnosis and development of a number of mental health disorders including autism, schizophrenia, depression, anxiety, Parkinson's disease, multiple sclerosis, anorexia nervosa and others; likely due to differences in sex steroid hormones and genetics. Recent evidence suggests that sex can also influence the complexity and diversity of microbes that we harbour in our gut; and reciprocally that our gut microbes can directly and indirectly influence sex steroid hormones and central gene activation. There is a growing emphasis on the role of gastrointestinal microbiota in the maintenance of mental health and their role in the pathogenesis of disease. In this review, we introduce mechanisms by which gastrointestinal microbiota are thought to mediate positive health benefits along the gut-brain axis, we report how they may be modulated by sex, the role they play in sex steroid hormone regulation, and their sex-specific effects in various disorders relating to mental health.

Keywords: microbiota-gut-brain axis, microbiome, sex difference, mental disorders, antibiotic, neurological disorders, germ-free.

1 Introduction

Most major neuropsychiatric and neurological disorders exhibit skewed male to female prevalence ratio. While neurodevelopmental disorders such as autism spectrum disorder (ASD), attention deficit hyperactivity disorder, early onset schizophrenia, and Parkinson's disease are more common in males; mood disorders such as anxiety, depression, anorexia nervosa, post-traumatic stress disorder, and multiple sclerosis are more frequent in females [(McCarthy et al. 2017), Fig. 1]. In addition to factors such as genetic makeup, life experience, socioeconomic status, physiological state, and perceived stress levels, recent clinical and animal studies emphasize the role of gut microbiota in the neurobiology of mental disorders with specific differences observed in males and females (J. J. Chen et al. 2018a; Clarke et al. 2013; Huang et al. 2019; Jasarevic et al. 2017; Vemuri et al. 2019). While the contributions of many of these factors on neuropsychiatric and neurological disorders, including the gut microbiota and microbiota-gut-brain axis, have been extensively reviewed elsewhere (Cenit et al. 2017a; Jasarevic et al. 2016; Kelly et al. 2017a; Luczynski et al. 2016); the complex interaction between sex, microbiota-gut-brain communication and changes in mood and behaviour have been poorly addressed. In this review we will introduce mechanisms by which the microbiota can influence mood and behaviour, discuss the effect of sex on microbiota diversity and complexity in health and disease, and further interrogate how the microbiota can influence sex hormones and reciprocally how sex hormones can influence microbiota.

2 The Gastrointestinal Microbiota

On-going collaborative efforts including the Human Microbiome Project (HMP) (Human Microbiome Project 2012), MetaHIT (Qin et al. 2010), American Gut Project (McDonald et al. 2018), British Gut Project (Jackson et al. 2018), as well as important gut microbiome analyses (Falony et al. 2016; Zhernakova et al. 2016) have been instrumental in surveying and describing the gut microbiota at a population level. The human gastrointestinal tract harbours a complex and dynamic microbial ecosystem composed of bacteria, archaea, yeasts, single-celled eukaryotes, as well as helminthic parasites and viruses, including bacteriophage (Human Microbiome Jumpstart Reference Strains et al. 2010). Of the microbiota consortia, the bacteria are the most widely studied. Current combined HMP and MetaHIT data estimate that there are at least 2776 prokaryotic species that have been isolated from human faecal matter (Bilen et al. 2018). These have been classified into 11 different phyla with *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* comprising over 90% of the prokaryotic microbiome, while

Fusobacteria and *Verrucomicrobia* phyla are present in low abundance (Eckburg et al. 2005; Hugon et al. 2015; J. Li et al. 2014).

Further, everyone has a unique microbiota signature, established early in the first years of life, shaped by a number of factors including: mode of delivery (vaginal or C-section) (Fouhy et al. 2019; Wampach et al. 2018), whether breast-fed or formula-fed (Hill et al. 2017; Madan et al. 2016; Stewart et al. 2018), sex (Jasarevic et al. 2016; Markle et al. 2013; Wallis et al. 2016), genetics (Bonder et al. 2016), diet (Gentile and Weir 2018), medication (in particular antibiotic medication) (Cho et al. 2012; Cussotto et al. 2019; Le Bastard et al. 2018; Leclercq et al. 2017; Maier et al. 2018), exposure to viral or bacterial infections (Escobar et al. 2011; Estes and McAllister 2016; Karst 2016), and stress (Foster et al. 2017; Rea et al. 2016). Over millennia, we have co-evolved with our microbiota, becoming mutually co-dependent for survival (Bordenstein and Theis 2015; Gaulke et al. 2018). Under normal homeostatic condition, this microbial population plays a key role in metabolism, intestinal peristalsis, mucosal integrity, pH balance, immune priming and protection against invading pathogens (Hyland and Cryan 2016). Since our brains constantly receive signals from the gut, the relationship between the host and its microbiota along the microbiota-gut-brain axis from a general health and health maintenance perspective cannot be overlooked.

Genes within the human gut microbiota significantly outnumber host human genes 100:1 and are capable of producing a plethora of centrally acting compounds, influencing virtually all aspects of human physiology and biology (Goodrich et al. 2017; Rosenberg and Zilber-Rosenberg 2018). While our inherited genome is essentially stable for the lifetime of the host, the microbiome is immensely diverse, dynamic, and responsive to external input, enhancing its potential as a target for therapeutic intervention (Flowers and Ellingrod 2015).

2.1 Bioactive Microbial metabolites

The different bacteria in our gut are capable of synthesising and releasing neurotransmitters and neuropeptides including catecholamines, GABA, histamine, choline and acetylcholine, that may mediate local physiological effects (including influencing motility and permeability) or enter the bloodstream to potentially influence centrally mediated events (Lyte 2014; Mittal et al. 2017). Many bacteria secrete bacteriocins, antibiotic peptides that inhibit the growth of other bacteria in the vicinity, thus self-regulating and preventing over-domination of any one species (Derrien and van Hylckama Vlieg 2015; Kommineni et al. 2015). Bacteria residing in the colon utilise fibres indigestible by the host as a nutritional substrate resulting in the

production of branched chain amino acids or the short-chain fatty acids butyrate, propionate, lactate and acetate predominantly (Baxter et al. 2019). These fatty acids can readily penetrate the intestines to activate their free fatty acid receptors on gut, lymph, immune cells and tissues; while evidence suggests they can mediate epigenetic events, possess neuroactive properties, and can regulate microglia density, morphology and maturation (Dalile et al. 2019; Huuskonen et al. 2004; Paul et al. 2015). Bacteria also facilitate the breakdown of dietary compounds through bile acid (BA) metabolism (Just et al. 2018). BAs are best known for facilitating the absorption of dietary lipids and lipid-soluble vitamins from the gut lumen. Certain bacterial taxa possess bile salt hydrolase activity (*Lactobacillus*) to carry out deconjugation of primary BAs from taurine or glycine, while other bacteria (*Clostridium*) are capable of transforming unconjugated primary BAs into secondary BAs as a result of 7α-dehydroxylation activity (Jones et al. 2008; Ridlon et al. 2014). The bacteria in the lumen of the gut mediate all these effects, without any direct interaction with host cells.

2.2 Microbiota-Gut communication

The lining of the gut is composed of a single-cell layer of epithelial cells held together by tight junction protein complexes, and contains predominantly enterocytes, secretory cells and gut associated lymphoid tissue (GALT), all coated with a mucus layer to create a physical barrier that separates host cells from luminal microbiota (Chelakkot et al. 2018). This dynamic structure has many functions including regulating the absorption of nutrients and fluid from the lumen of the intestines and to serve as a physical barrier to existing commensal bacteria, invading pathogens or harmful substances (Chelakkot et al. 2018). The exchange of molecules through the mucous layer and epithelium (often through tight junction proteins) serve to facilitate communication between the gut and the immune system through the recognition of self and non-self antigens, and maintains a healthy resting inflammatory tone that facilitates communication along the gut-brain axis (Kelly et al. 2015). The enterocytes express innate immune receptors and can release cytokines and chemokines, while the GALT utilise lymphocytes to mount a more specific immune response to microbial molecules including cell wall components, such as peptidoglycans (Royet et al. 2011). Epithelial pattern recognition receptors (PRRs) recognise molecular patterns unique to bacteria and other microorganisms (pathogen associated molecular patterns, or PAMPs) of which the Toll-like receptor family are the most studied, and once activated can recruit inflammatory mediators, cytokine production and chemokine-mediated recruitment of acute inflammatory cells (Duerkop et al. 2009). The secretory cells are responsible for the release of mucus from goblet cells, control of bacterial

diversity by antimicrobial secretion from Paneth cells (Ehmann et al. 2019; Salzman 2010), and in neuroendocrine control via the release from enteroendocrine cells of substances such as ghrelin, somatostatin, cholecystokinin, peptide YY and serotonin amongst others (Thaiss et al. 2016).

Existing hypotheses suggest that these circulating cytokines, chemokines, endocrine messengers and microbial by-products can infiltrate the blood and lymphatic systems, or influence neural messages carried by the vagal and spinal afferent neurons to impact on centrally mediated events. Current research is involved in uncovering the underlying mechanisms by which these microbiome-influenced biomolecules impact brain, behaviour and mental health.

2.3 The Microbiota-Gut-Brain Axis

The microbiome-gut-brain axis is a complex bi-directional system including the brain, the sympathetic and parasympathetic divisions of the autonomic nervous system, the endocrine and immune systems, the enteric nervous system, and the gut microbiome (C. R. Martin et al. 2018). The microbiota can relay information about health status of the gut through endocrine (Cussotto et al. 2018), immune (Holzer et al. 2017; Soto et al. 2018; Thion et al. 2018b) and neuropeptide/neurotransmitter systems (Clarke et al. 2013; Lach et al. 2018; van Sadelhoff et al. 2019), the vagus nerve (Bravo et al. 2011; Fulling et al. 2019), and signalling molecules including short chain fatty acids (SCFA) (Dalile et al. 2019), branched chain amino acids (K. Gao et al. 2019b); bile moieties (Baars et al. 2018) and peptidoglycans (Arentsen et al. 2017). This in turn can profoundly impact on neuronal signalling in the brain and thus impact on emotional systems and behavioural response (C. R. Martin et al. 2018).

3 Sex difference in the Brain

Mammalian brain shows characteristic region-specific sex differences at the level of anatomy, circuitry, activity, molecular profiles, and epigenetic modifications (Bakker 2019; Cosgrove et al. 2007; Nugent et al. 2015). Some of these differences are present at birth but can also emerge during puberty and modulated in adulthood. While overall brain metabolism is comparable between males and females, males have greater brain volume while females have greater cortical thickness (Ritchie et al. 2018). Controlling for brain volume, males have greater percentage of white matter while females have greater percentage of grey matter and cerebral blood flow in humans (Cosgrove et al. 2007). It is important to point that, while these sex-

specific differences exist, a particular brain cannot be categorised as male or female brain, and any individual will have varying amounts of male and female brain characteristics (Joel 2011).

Females achieve peak cerebral volume at around 10 years while it is 14 years for males (Lenroot et al. 2007), which coincides well with average age of puberty indicating influence of sex steroid hormones. Sex hormonal contribution has been extensively studied in emergence of male and female physiological and behavioural traits in both, humans and animal models (Gillies and McArthur 2010; Juntti et al. 2010; Lu et al. 2019; Sato et al. 2004; Tsuda et al. 2014). Sex hormones role in modulating immune responses is also well characterised with a predominant pro-inflammatory effect of oestrogens as oppose to an anti-inflammatory effect of testosterone (Klein and Flanagan 2016). At birth, the testosterone level in males is fifty percent higher compared to females with comparable oestrogen levels, as measured in the human cord blood (Mitsui et al. 2019). There is a surge of sex hormones in the first 6 months of life, which is thought to contribute to sex differences in the brain [(McCarthy 2016), Figure 2].

Specific brain regions show sexual dimorphism at birth across several species, such as the sexually dimorphic nucleus of the preoptic area (SDN-POA) (larger in males, involved in male sexual behaviour), bed nucleus of stria terminalis (BNST) (larger in males, involved in sociosexual behaviour, mood, aggression, territoriality, and olfaction) and anteroventral periventricular nucleus (AVPV) (larger in females, regulate ovulatory cycle) (McCarthy et al. 2017). These differences emerge during development and involve testosterone-mediated inhibition or activation of apoptosis (E. C. Davis et al. 1996; McCarthy et al. 2017; Tsukahara et al. 2006). The role for sex hormones is evident form animal studies showing neonatal androgen exposure to be necessary and sufficient for masculinization of the brain (Sato et al. 2004; Wu et al. 2009). This is possibly due to increased aromatase activity responsible for conversion of testosterone to oestrogen, since oestrogen exposure to female neonates masculinizes their brain and behaviour (Wu et al. 2009). However, the direct role of androgens has also been reported using animal model with non-functional androgen receptor (Zuloaga et al. 2008). Oestrogens and androgens modulate synaptic plasticity and immune function, via the rapid response membrane receptors or cause transcriptional changes via the cytoplasmic receptor, thereby modulating neuronal circuits and behaviour [The following reviews can be referred to for more information on sex hormone receptor expression, proposed mechanisms, and sex differences in their function- (Baez-Jurado et al. 2019; Gillies and McArthur 2010;

McEwen and Milner 2017; Straub 2007)]. Postmenopausal oestrogen therapy in women have also shown promise with enhanced neuronal plasticity and improved cognition (Maki and Dumas 2009). While sex hormones drive a majority of these differences directly or indirectly, contribution of the sex chromosome at the cellular level in brain development has also been reported (Printzlau et al. 2017; Sekido 2014).

4 Sex differences in the Gut Microbiome across life

4.1 Human studies

Large population-wide studies fail to show any major sex-specific differences in the gut microbiota diversity, complexity or composition (Human Microbiome Project 2012; Mueller et al. 2006; Odamaki et al. 2016; Yatsunenko et al. 2012). However, based on community types categorised using clustering in stool samples from HMP consortium, females were more likely to show community type C (higher relative abundance of *Ruminococcaceae*, *Faecalibacterium* and *Alistipes*, lower relative abundance of *Bacteroides* and lack of *Prevotella*). Males were three times more likely to have community type D (higher relative abundance of *Prevotella* and lower relative abundance of *Bacteroides*) (Ding and Schloss 2014). The main findings of the articles reviewed on sex differences in microbiome composition is summarised in Table 1. There is now a growing interest in the role of sex differences in microbiome in the pathophysiology of a variety of diseases including cardiovascular diseases (Cross et al. 2018), diabetes (Markle et al. 2014), irritable bowel syndrome (Y. S. Kim and Kim 2018), obesity (Link and Reue 2017), and cancer (Jia and Xie 2018) among others.

Gut microbiota diversity increases in infancy and is stabilized by five years of age without any apparent sex-differences *per se* [(Avershina et al. 2014; Cheng et al. 2016), Figure 2]. At the taxa level, increased phylum *Firmicutes* and reduced *Bacteroidetes* profile is more common in girls along with increased colonization by *Lactobacillus* and *Bifidobacterium* in early infancy (Huda et al. 2019; R. Martin et al. 2016; Sordillo et al. 2017). This could disproportionately improve nutrient availability and gut barrier functions in females at this critical early window of brain development. Not many reports explore sex differences in gut microbiome profile during adolescence but in adults, alpha diversity increases slightly until middle age with greater microbiome diversity seen in females (de la Cuesta-Zuluaga et al. 2019). During middle-age, around the time of menopause, males and females have a steady alpha diversity up to 70 years after which there is a decline observed. There are no sex differences reported at this stage. The diversity rises in centenarians, which could be due to healthy ageing since frailty is associated

with reduce diversity (Claesson et al. 2012; van Tongeren et al. 2005). In adulthood, findings on microbiome composition report different taxa to be associated with sex but most studies agree with sex explaining a very small fraction of observed microbiome diversity at least in the healthy adult populations (Table 1). For example, comparisons at the species level in the Dutch cohort study report sex association with twelve microbial species (including *Bilophila*, *Lachnospiraceae*, and *Bifidobacterium* species) and 43 metabolic pathways (TCA cycle, gluconeogenesis, isoleucine biosynthesis, tryptophan degradation among others) (Sinha et al. 2019). After multiple corrections, relative abundance of only *Akkermansia muciniphila* was higher in females. This bacterium has been associated with leanness and protection against diabetes and obesity, thus is of interest as a probiotic (Zhang et al. 2019).

In parallel to above observations, menopausal status also affects gut microbiome, with premenopausal women having higher abundance of SCFA producing bacteria compared to postmenopausal women and age-matched men (Santos-Marcos et al. 2018; Santos-Marcos et al. 2019). The SCFA producing genera *Prevotella*, *Ruminococcus* and *Roseburia* are reported to depend on sex and hormonal status. More direct correlation with oestradiol levels suggests a positive correlation for *Gammaproteobacteria* class and unknown family from *Mixococcales* (*Proteobacteria*- Lipopolysaccharide (LPS) producers), while bacterial family *Prevotellaceae* was negatively correlated (d'Hennezel et al. 2017; Santos-Marcos et al. 2018). This could increase LPS production and inflammation by *Proteobacteria* species, in addition to reduced SCFA production by *Prevotellaceae* in females, thereby increasing risk to mental disorders in the pubertal and reproductive phases. Oestrogen replacement therapy given to postmenopausal women increases vaginal *Lactobacillus* abundance and protect against infection (Muhleisen and Herbst-Kralovetz 2016), and has promising effect on mood and cognition (LeBlanc et al. 2001; Zweifel and O'Brien 1997), but changes in gut microbiome needs to be addressed. Furthermore, oral contraceptives and ovariectomy are also associated with changes in gut microbiota (Sinha et al. 2019). Despite the role for female sex hormones in GI transit (Jung et al. 2003; Wald et al. 1981), clinical evidence for a change in gut microbiota composition across the menstrual cycle, or in response to contraceptive medication are very few. However, the vaginal microbiota is stable throughout the menstrual cycle in healthy Canadian women (Chaban et al. 2014). The human studies corroborate well with animal studies when it comes to sex-differences, but changes observed depend on species and strain [(Elderman et al. 2018), Table 1 and 2].

4.2 Animal studies

Sex-differences in the mouse microbiota composition arise during puberty with the males acquiring a distinct gut microbiota composition compared to prepubertal mice of both sexes (Baars et al. 2018; Jasarevic et al. 2016; Yurkovetskiy et al. 2013). One study does report subtle sex-differences in gut microbiome composition postnatally and during weaning [(Jasarevic et al. 2017), Table 2]. The gut microbiota composition of adult SPF females resembles that of the prepubertal mice of both sexes (Yurkovetskiy et al. 2013). Conventionalised mice are also reported to have higher *Firmicutes/Bacteroidetes* ratio (marker for obesity) in females compared to males (Baars et al. 2018), similar to observations in humans. Treatment of female rats with Letrozole, an aromatase inhibitor, reduces oestradiol and progesterone levels while increasing testosterone and Luteinizing hormone levels (Guo et al. 2016). This led to increased *Lactobacillus*, *Ruminococcus*, *Clostridium*, and *Prevotella* without altering *Bifidobacterium* and *Bacteroides*. Prenatal exposure of androgen in female rats increased faecal relative abundance of steroid hormone synthesizing bacteria- *Nocardiaceae* and *Clostridiaceae,* bacteria associated with synthesis and elongation of unsaturated SCFAs, and lower abundance of *Akkermansia, Bacteroides, Lactobacillus, and Clostridium* (Sherman et al. 2018). Gonadectomy along with high fat diet was also associated with increased genera of *Ruminococcaceae* family in male mice of three different strains, and reduced *Akkermansia* genus in female mice (Org et al. 2016). This suggests that interaction of diet and sex hormone is crucial in alterations and maintenance of gut microbiota composition. Castration in male mice abolishes sexual difference of gut microbiota composition, suggesting a critical role for testosterone in the complexity and diversity of virile males (Yurkovetskiy et al. 2013).

Oestrogens also regulate gut microbiota composition since beta diversity of ovariectomised (OVX) female mice clusters with male mice (Kaliannan et al. 2018). Further compositional analysis in the faecal microbiota of oestradiol treated males or OVX females show clustering with females, separate from male or OVX female cluster, suggesting the influence of oestrogen in modulating gut microbiota composition. Comparing the normal females to the OVX female or male groups revealed sex differences with lower *Proteobacteria*, reduced *Bacteroides* ratio and increased *Bifidobacterium* to *Enterobacteriaceae* ratio (Kaliannan et al. 2018). Some of these observations were recapitulated with 17-betaoestradiol treatments in males and OVX females. This study is conflicting with another report showing increased *Firmicutes/Bacteroidetes* ratio in adult female mice (Baars et al. 2018). Another study administering progesterone to OVX females report reduced anxiety and depressive-like

behaviour, along with increased *Lactobacillus* spp. in the gut (Sovijit et al. 2019). Both progesterone and *Lactobacillus* administration increased hippocampal *Bdnf* gene expression levels while antibiotic treatment abolished all these effects. In addition, in vitro analysis revealed positive effect of progesterone on the growth of *L. reuteri* (Sovijit et al. 2019). Thus, suggesting that sex hormones may directly influence specific gut microbial strains, which in turn modulate behaviour of the organism.

With regards to oestrus cycle phase, gut microbiota seem largely unaltered in the murine model. However, *Akkermansia* was the only genus to vary depending on the oestrus phase (Wallace et al. 2018). Subcutaneous oestradiol implants in adult female mice increased bacterial diversity in a week, with elevated levels of *Lactobacillaceae*, *Coriobacteriaceae* and unidentified family from the *Bacillales* (Benedek et al. 2017). Supplementing the above findings on sex hormones, oestrogen receptor beta (expressed in the adult colon) knockout mice are reported to modulate gut microbiome composition, specifically the taxa *Proteobacteria*, *Firmicutes* and *Bacteroidetes* (Menon et al. 2013). These data provide clear evidence that sex hormones manipulate microbiota and may contribute towards sex-dependent gut microbiota differences, and associated changes with endocrine, immune and neurotransmitter systems across the lifespan, which needs to be further explored.

While there exist sex differences in gut microbiota profile of adult humans and animals, the data is not unequivocal and deficient in early life and adolescent stages. In addition to assessing baseline sex differences on gut microbiome, it is crucial to uncover these differences in specific brain disorders, which will advance our understanding of sex biases in brain disorder prevalence, symptoms, and treatment. While several clinical studies associate gut microbiome changes in brain disorders, they often lack power to compare sex-differences and fewer animal studies exist in this regard, which are summarised below.

4.3 Gut Microbiota and Brain Disorders

Clinical studies assessing the gut microbiota of patients with neuropsychiatric and neurological disorders report largely a reduction in overall abundance and diversity although there is much variability in terms of quality of studies and findings seen. Refer to cited reviews for details on gut microbiome and brain disorders [ASD- (Mayer et al. 2014; Srikantha and Mohajeri 2019), ADHD- (Cenit et al. 2017b), Schizophrenia- (Codagnone et al. 2019; Severance and Yolken 2018), Parkinson's disease- (Lubomski et al. 2019; Miraglia and Colla 2019), Anxiety- (Rackers et al. 2018), Depression- (Liang et al. 2018; Winter et al. 2018), Anorexia nervosa-

(Roubalova et al. 2019), PTSD- (Leclercq et al. 2016), Alzheimer's disease- (Garcez et al. 2019), MS- (Zoledziewska 2019)]. One study in MDD report reduced *Bacteroidetes* in males and increased *Actinobacteria* in females (J. J. Chen et al. 2018a). Another study on atypical antipsychotic treatment to bipolar patients reported reduced alpha diversity in females with no change observed in males (Flowers et al. 2019). There is some disparity in gut microbiota compositional changes for specific mental disorders, which may be attributed to differences in demographics, patient recruitment characteristics, diet of patients and healthy controls, method used for acquiring composition data and its statistical analysis. In clinical studies involving brain disorders and microbiome analysis, though sex is equally represented within study groups, sex-comparisons are mostly underexplored or not reported.

A more convincing evidence for a causal role of gut microbiota in mental disorders comes from recapitulation of mental disorder symptoms in rodents by mere transfer of the gut microbiota. This has been done for ASD (Sharon et al. 2019), MDD (Kelly et al. 2016; Zheng et al. 2016), anxiety associated with irritable bowel syndrome (De Palma et al. 2017), schizophrenia (Zheng et al. 2019), and Parkinson's disease (Sampson et al. 2016). Limitations of these studies are that donor samples come solely from male human subjects and are then transferred into male animals, but no comparable study investigating the influence of human or animal faecal microbiota transplantation (FMT) from females has been conducted. In ASD gut microbiota transfer study, sex-differences only in behavioural outcome was assessed, wherein F1 male mice had greater detrimental effects on behaviour compared to females (Sharon et al. 2019). FMT from humans to animals does not exactly reproduce the gut microbiome of the human donor and bacterial species are colonized differently, thus questioning the ideal control and diseased human donor microbiome and the translatability of these studies.

Mouse models of mental disorders also report sex-specific alterations in the gut microbiota with pathological associations (Coretti et al. 2017; Jasarevic et al. 2017; Rincel et al. 2019). In the BTBR mouse model of ASD, *Bacteroides*, *Parabacteroides*, *Sutterella*, *Dehalobacterium* and *Oscillospira* genera were the main factors associated with behavioural alterations and enhanced intestinal permeability and inflammation (Coretti et al. 2017). Maternal separation with maternal unpredictable chronic stress in mice leads to sex-specific behavioural abnormalities and altered gut microbiome composition in adulthood of the offspring. The males exhibit social impairment along with abundance of taxa belonging to *Lachnospiraceae* and *Porphyromonadaceae* families unclassified *Firmicutes*, and *Bacteroides*, *Lactobacillus* and

Alloprevotella genera while females exhibit increased anxiety-like behaviour with limited gut microbiome alterations restricted to *Lactobacillus* and *Mucispirillum* genera (Rincel et al. 2019). Prenatal stress exposure disrupts composition structure of *Lactobacillus* and *Streptococcus* in both sexes. Stress during gestation is also reported to abolish sex differences in the offspring gut microbiome with greater alterations in male pups (increased abundance of bacteria from family, *Lachnospiraceae* and *Clostridiales*) than female pups from the stressed dam (Jasarevic et al. 2017).

Most clinical and animal studies lack sex comparisons of gut microbiota, and considering confounding factors arising due to sex; it is essential to understand the sex-dependent intricacies involved in mental disorders for efficacious sex-specific therapeutic approaches. The following section summarises studies addressing the effect of gut microbiome and sex on brain and behaviour (Table 3 and 4).

5 Sex- Microbiota-Gut-Brain interactions

It is clear that there can be much variability in microbiome studies in humans. This is often caused by several confounding factors such as genetics, diet, lifestyle, clinical history, hormonal levels, but studies are beginning to explore sex differences in gut microbiota-brain interactions (Table 3). Temperament in children and adults is associated with specific bacterial taxa, some of which depend on sex (Aatsinki et al. 2019; Christian et al. 2015; Taylor et al. 2019). In a New Zealand cohort, antibiotic use in first year of life but not maternal antibiotic treatment is associated with increased neurocognitive difficulty and depression score up to age eleven (Slykerman et al. 2017a), however, sex-specific analysis was not carried out. In case of animal models of early life adversity (Coretti et al. 2017; Jasarevic et al. 2017; Rincel et al. 2019; Thion et al. 2018b), males show greater emotional vulnerability over females, along with greater perturbation of the gut microbiota. The sex differences in the placental environment and inflammation status have been implicated in this bias (Kalisch-Smith et al. 2017; Thion et al. 2018a). Similarly, in a mouse model of multiple sclerosis, oestradiol treatment to female mice protected against autoimmune encephalomyelitis score and disease-induced gut microbiota and mucosal immune alterations (Benedek et al. 2017).

While it is difficult to tease apart specific contribution of gut microbiota, genetics and sex, studies involving gut microbiota transfer across different strains suggest that gut microbiota dominate over genetic influence on the cortical transcriptional profile as well as behavioural

response in animals (Gacias et al. 2016). Environment also has a greater influence over host genetics in determining the human gut microbiome (Rothschild et al. 2018), which was elegantly reported in US immigration study with loss of gut microbiome diversity (Vangay et al. 2018). Keeping these confounding factors in mind, we have summarised the literature on contribution of gut microbiota in sex-dependent consequences of behavioural and physiological (Table 3 and 4) outcomes, largely assessed in animals.

5.1 Behavioural analyses

5.1.1 Germ-free studies

Several germ-free rodent studies report a reduced or no effect on anxiety-like phenotype in males and females [Summarised in (Vuong et al. 2017)]. Conflicting studies report opposite effects on locomotor and rearing behaviour, increased in germ-free male and no change in female mice (Diaz Heijtz et al. 2011; Neufeld et al. 2011); increased in germ-free female and no change in male mice (Luk et al. 2018). The inconsistency may arise due to differences in mouse strain (Elderman et al. 2018) and lab-to-lab variation in the gut microbiota composition in the SPF mice. Germ-free males have no effect or increased self-grooming, suggestive of repetitive behaviour but the females have not been analysed (Arentsen et al. 2015; Desbonnet et al. 2014). Male mice show increased sociability index (Arentsen et al. 2015) but conflicting study also reports reduced sociability with reduced social novelty score in males and no significant effect observed in female mice (Desbonnet et al. 2014). Germ-free male mice have reduced immobility time on forced-swim test, tail-suspension test (TST) as well as reduced latency to feed in novelty suppressed feeding task, suggestive of decreased depressive-like behaviour (De Palma et al. 2015; Luo et al. 2018; Zheng et al. 2016). While another report shows no effect on TST immobility or percent sucrose preference, the trend is towards a lowered depressive phenotype in germ-free male mice (Campos et al. 2016). We did not find many reports on anhedonia or depressive-like behaviour in germ-free female rodents, but there seems to be no effect on TST in female germ-free mice (De Palma et al. 2015). Germ-free female mice have impaired cognition and memory, with reduced c-Fos and BDNF protein expression in the hippocampus (Gareau et al. 2011). Similar to females, germ-free male mice also have lower hippocampal BDNF expression (Sudo et al. 2004) and reduced percent alteration rate on Y-maze suggestive of impaired working memory (Zheng et al. 2016). Though the germ-free studies report conflicting findings possibly due to differences in the control gut microbiome across facilities, sex difference is a common them across these studies.

5.1.2 Antibiotic studies

The advantage of antibiotic treatment over germ-free studies is the temporal regulation of gut microbiota alterations or depletion, and translatability to humans. However, there may be differential findings based on the combination of antibiotics used, the dose and duration of the treatment as well as the route of administration and the age of the animals being tested. For this review, we will focus on behavioural sex-differences observed in control and antibiotic treated groups during specific life stage.

Preconception to gestation antibiotic treatment causes similar behavioural alteration in juvenile male and female offspring, with reduced social interaction and sensory-motor gating but no effect on anxiety-like or stereotypic behaviour (Degroote et al. 2016). Gestational antibiotic treatment reduces locomotor and rearing activity, and increases anxiety-like behaviour in the male offspring despite cross-fostering with control dams (Tochitani et al. 2016). Antibiotic administration to pregnant dams until weaning alters offspring behaviour in adolescence with reduced anxiety-like behaviour in males but not females (Leclercq et al. 2017). Male and female offspring of antibiotic-treated dams were comparable with unaltered locomotion and reduced sociability and social novelty, which were rescued by *Lactobacillus* administration to the dams (Leclercq et al. 2017). Postnatal oral gavage of antibiotics for 10 days increased visceral hypersensitivity only in adult male rats but did not alter anxiety-like behaviour or memory in males and female adult rats (O'Mahony et al. 2014). Chronic antibiotic administration post-weaning in male mice led to reduced anxiety-like behaviour, memory, and social interaction in adulthood, perturbed serum tryptophan metabolism, reduced hippocampal BDNF and Vasopressin levels (Desbonnet et al. 2015). Chronic antibiotic administration in late adolescence in male mice increased depressive-like behaviour, reduced social novelty without altering social interaction, nociception or working memory (Guida et al. 2018). These alterations were normalised and accompanied by increased alpha diversity of gut microbiota after suspension of antibiotics for around two weeks.

Adult chronic antibiotic treatment given twice daily for 11 days altered novel object recognition memory but not spatial discrimination, anxiety-like or depressive behaviour in male mice (Frohlich et al. 2016). Chronic antibiotic treatment in adult females also reduce hippocampal neurogenesis and memory in NOR (Mohle et al. 2016). One week oral but not intra-peritoneal administration of antibiotics reduced anxiety-like behaviour in male mice, which were unaltered by vagotomy and accompanied with increased hippocampal and decreased

amygdalar BDNF protein levels (Bercik et al. 2011). Chronic 13-week antibiotic treatment in adult male rats increased visceral pain sensitivity, depressive-like behaviour, perturbed memory in Morris water maze task but not novel object discrimination task and did not alter anxiety-like behaviour or somatic pain sensitivity (Hoban et al. 2016b). Similar to rodent studies, antibiotic treatment in adult zebrafish reduced anxiety-like behaviour and social cohesion recapitulating an ASD-like state (X. Wang et al. 2016b). In case of Drosophila, diet-induced mating preference was abolished with antibiotic treatment in both males and females, via altering the cuticular hydrocarbon sex pheromones (Sharon et al. 2010).

In summary, depletion of gut microbiota using antibiotics seems to have no effect or a detrimental effect on emotional and cognitive behaviour with the exception of reduced anxiety. Taken together, these data suggest that: a) antibiotic treatment in different windows of life differentially modulate behaviour, possibly by altering the gut microbiota, with specific sex differences, b) male and female brain develop and mature differently, gut microbiota perturbation at specific ages might end up affecting different circuits and thereby behaviour. While this concept of critical windows is not new, research is warranted to explore sex differences in microbiota-brain connection throughout development.

5.1.3 Probiotics, Prebiotics, and Diet

Meta-analysis of probiotic studies suggests a mood enhancing effect of probiotics in animal models but mixed findings for clinical studies (R. T. Liu et al. 2019b; Reis et al. 2018). In the past decade, probiotics including *Lactobacillus* and *Bifidobacterium* species have been assessed as a therapy for mental disorders such as ASD, anxiety, MDD, and to a lesser extent for schizophrenia, multiple sclerosis, Alzheimer's and Parkinson's disease. Prebiotic and probiotic treatment is effective in autism (Grimaldi et al. 2018) and has mood enhancing effects on patients with anxiety and depression (C. S. Kim and Shin 2019; R. T. Liu et al. 2019b; J. Liu et al. 2019a). However, other reports showed no effect, which may be due to dose and combination of probiotics used, study design and duration of treatment (Kelly et al. 2017b; Ng et al. 2018; Romijn et al. 2017). Prebiotic as well as faecal microbiota transfers in autism have beneficial effects on behaviour and gastrointestinal function of the patients (Kang et al. 2017). There is an increased interest in assessing clinical benefits of diet, prebiotic and probiotic treatments on other mental disorders.

Probiotic treatment for schizophrenia did not improve positive or negative symptoms but reduced the occurrence of bowel problems during the treatment phase (Dickerson et al. 2014).

Another study using fermented milk reported reduced constipation in patients with Parkinson's disease without altering dopaminergic therapy requirements, but motor function and sex differences were not evaluated (Barichella et al. 2016). However, increased dietary intake of polyunsaturated fats is inversely associated with Parkinson's disease, but an alteration in the gut microbiota has not been characterized (Kamel et al. 2014). *Lactobacillus rhamnosus* HN001 reduced anxiety and depression in postpartum depression (Slykerman et al. 2017b). Probiotic *Bifidobacterium longum* NCC3001 reduced depression symptoms in IBS patients (Pinto-Sanchez et al. 2017). Current research involving adjunct therapy consisting of probiotics with dietary components in treatment of brain disorders shows promising results (Burokas et al. 2017; Jamilian et al. 2018; Miyaoka et al. 2018; Ostadmohammadi et al. 2019; Raygan et al. 2018).

In animal model of ASD, maternal immune activation causes behavioural deficit, increase: gut permeability, relative abundance of *Clostridia* and *Bacteroidia*, and serum 4-ethylphenylsulfate, which were all rescued by *Bacteroides fragilis* treatment (Hsiao et al. 2013). In three different mouse models of autism (genetic, environmental, and idiopathic models), *L. reuteri* treatment alone reversed social deficits in males (Sgritta et al. 2019). This effect was modulated via the vagus nerve and oxytocin. Oxytocin levels are reduced in social disorder observed in rodent studies, and administration of *L. reuteri* seems to reduce social deficits and upregulate oxytocin levels in the PVN of the brain (Sgritta et al. 2019). Further *L. reuteri* also possess beta glucuronidase property that may enhance unconjugated oestrogen levels, which is known to regulate oxytocin levels (Acevedo-Rodriguez et al. 2015; Varian et al. 2017). It would have been interesting to see the effects in females since stress leads to greater colonic stress response with increased inflammation in females, an effect rescued by probiotic *Lactobacillus farciminis* (J. Y. Lee et al. 2017b).

Several preclinical and clinical studies associated with pre-/probiotic administration and FMT raise concerns about the ideal healthy host gut microbiota composition and ratios of key microbial species (Jayasinghe et al. 2016; Konig et al. 2017; Larsen et al. 2019; Suez et al. 2019). Thus, it is important to assess the gut microbiota composition prior to treatment and also determine the appropriate doses and combination of gut microbiota for an efficacious effect. Apart from bacteria, use of bacteriophage may also be considered to eliminate harmful gut microbiota species (Shkoporov et al. 2018; Sulakvelidze et al. 2001). This is a very nascent field and a lot of on-going work is addressing the co-influence of phage composition and mental

disorders (Bedarf et al. 2017; Severance et al. 2016). Further interrogation can delineate the role of sex hormones in behavioural and physiological outputs.

5.1.4 Other studies

Common food additives such as emulsifiers when administered orally to mice post-weaning, increased anxiety in males and reduced social novelty preference in females (Holder et al. 2019). The group had previously shown emulsifiers potentiate inflammation and directly alter gut microbiota composition using an *ex vivo* model of mucosal simulator of the human intestinal microbial ecosystem (Chassaing et al. 2017).

5.2 Inflammation, Metabolism and Neurotransmitters

Many brain disorders are stress- or inflammation-related disorders, which poises them to be modulated by the microbiota (C. R. Martin et al. 2018; Scott et al. 2017). Both the stress and immune responses are critical innate systems of healthy ageing and normal development, but dysfunctions can predispose individuals to a more vulnerable state leaving them more susceptible to mental disorders (Codagnone et al. 2019). Recent evidence implicates a role for the microbiota at the interface of the stress and immune system, which regulate host physiology to mediate effects in the brain (Foster et al. 2017; Rea et al. 2016; Thion et al. 2018a). As well as the role of the gastrointestinal microbiota in maintaining a healthy resting inflammatory tone at the level of the gut, the involvement of microbial-derived metabolites including neurotransmitters, neuropeptides and SCFAs in regulating the systemic immune and stress response has been reviewed extensively (Bruce-Keller et al. 2018; Morris et al. 2017; Russo et al. 2018).

Animal studies involving germ-free and other models, suggest sex-differences in the immune system (Fransen et al. 2017; Thion et al. 2018b) and metabolism (Baars et al. 2018; Clarke et al. 2013; H. Gao et al. 2019a; Kaliannan et al. 2018), which may play a role in determining which specific microorganisms are best suited to colonising the gut, which in turn may modulate overall physiology and behavioural outcome (Table 4). For example, GF females have increased intestinal type 1 interferon signalling compared to GF males that may colonize sex-specific microbiome (Fransen et al. 2017). Microglia are the predominant immune cells in the CNS and play an essential role in directing key processes including neurodevelopment, neurogenesis, synaptic pruning, and neuroinflammation (Jacobs et al. 2019; Tremblay et al. 2011). Germ-free mice show increased microglial density and excess ramification compared to SPF mice across different brain regions during development (Thion et al. 2018b). However,

one-week antibiotic treatment in adults had no effect on the density and morphology of microglia in either of the sexes (Thion et al. 2018b). Sex- and age-specific effects were also observed in the somatosensory cortex of GF mice compared to SPF mice, with greatly perturbed microglial density and transcription profile in male fetuses and adult females (Thion et al. 2018b). Trancriptomic analysis identified that in males the key signaling pathways involved were linked to the immune response whereas in females they were linked to transcriptional regulation. These microbiota-induced alterations may involve sex hormone regulations considering that the female sex hormone, oestrogen has a pro-inflammatory and male sex hormone testosterone has an anti-inflammatory effect (Klein and Flanagan 2016).

The GF male but not the GF female mice have elevated levels of hippocampal serotonin and its major metabolite, as well as circulating serum tryptophan levels compared to conventional sex-matched mice (Clarke et al. 2013). Restoring the gut microbiota in post-weaned GF male mice reversed reduced anxiety levels and peripheral tryptophan levels, but hippocampal serotonin levels were not affected (Clarke et al. 2013). These sex-specific microbiome profiles in turn may facilitate sex differences in physiology and behaviour. This idea is supported by the study where absence of gut microbiota diminished sex specific liver gene expression as well as lipid metabolic profile (Weger et al. 2019). Exposure to manganese in mice, which may occur through diet or environment, show sex differences in microbiome composition, amino acid metabolism, and reduced GABA-synthesizing enzyme expression only in males (Chi et al. 2017). It is unclear what role other macro and micronutrients may play in this context and whether their effects are sex dependent. *Lactobacillus* also regulates central GABA receptor expression via the vagus nerve in males (Bravo et al. 2011). This could change the levels of neurotransmitters and downstream signalling in specific brain regions, which also show sex-differences, thereby causing differential emotional responses. Sexual dimorphism is also reported for the neuropeptide Arginine Vasopressin (AVP), released from the hypothalamus, with increased expression in normal males (Fields et al. 2018). AVP regulates stress response and affect social and anxiety-like behaviour. Antibiotic administration decreases hypothalamic AVP expression, and AVP gene knockout and knockdown data suggests AVP influences gut microbiota composition in gene dose-dependent and sex-specific manner (Fields et al. 2018). Sex-differences have also been reported in the colonization of germ-free mice using human male microbiota, where female mice show greater diversity while male mice microbiota resembles more closely with the donor (J. J. Wang et al. 2016a). The study also reports specific sex-differences in OTUs and their interaction. Caesarean section mode of delivery in mice has

greater impact on adult female gut microbiome and body weight gain compared to males (Martinez et al. 2017). Collectively, these studies suggest important contribution of gut microbiota in sex-dependent metabolic effects, which may in turn regulate behavioural outcome.

5.3 Sex Hormone function

Metagenomic studies suggest that gut microbiota are capable of synthesizing steroids, modifying oestrogen, and expressing hydroxy-steroid dehydrogenases, enzymes responsible for regulating a balance between the active and inactive steroids (Wallis et al. 2016; Zou et al. 2019). The terms 'sterobiome' and 'estrobolome' have been used to represent the combination of enteric bacterial genes that can metabolize steroid or oestrogen respectively (Baker et al. 2017; Ridlon and Bajaj 2015). The gut microbiota in principle may regulate hormonal and steroidal levels that have a plethora of physiological effects due to widespread expression of oestrogen and androgen receptors. While oral contraceptives increase the gut permeability and risk of intestinal bowel disease, antibiotic treatment in turn is also speculated to affect oral contraceptive efficacy but thorough clinical research in these observations are lacking (Khalili 2016; Simmons et al. 2018).

In animal studies, female mice are reported to have lower serum 17-betaoestradiol levels post antibiotic treatment (Kaliannan et al. 2018). Similarly, in germ-free mice, females have reduced levels of oestradiol, progesterone and corticosterone (Kamimura et al. 2019), while males have higher oestradiol levels compared to SPF mice (Kubeck et al. 2016). In comparison, adult germ-free mice, serum testosterone levels were higher in females while lower in males compared to sex-matched SPF mice (Markle et al. 2013). Further, transfer of SPF adult male caecal content to pubertal female mice was sufficient to increase serum testosterone levels, and to masculinize microbiota composition, and alter the metabolic signature in adult female mice (Markle et al. 2013). These effects were blocked by an androgen receptor antagonist, flutamide suggesting an important role for testosterone signalling in mediating the consequences on male caecal microbiota transfer in females. The opposite study of transfer of adult female caecal content into pubertal males has not been done but considering no difference in pre-pubertal male or adult female caecal content, it may not have any drastic effect on gut microbiota composition. Interestingly, smaller litter size and problems in generating colonies are commonly reported in germ-free maintenance colonies, along with reduced progesterone levels during the start of dioestrus (Shimizu et al. 1998). Faecal microbiota transfer from

conventionalised animals attenuates these abnormalities. In humans, oestrogen levels correlate with faecal microbiome abundance and alpha diversity in the postmenopausal women (Vieira et al. 2017). However, an in-depth impact of human gut microbiota in sex hormonal signalling and fertility is a topic for future research. Oestrogen is inactive in the conjugated form, and beta-glucuronidase producing gut microbiota can convert the conjugated form to an unconjugated form, which is free to bind to the oestrogen receptor (Flores et al. 2012). Oestrogen have a plethora of physiological consequences throughout the body including several brain regions (McCarthy and Arnold 2011). Prolonged conjugated oestrogen supplementation causes reduction in gut microbiota beta-glucuronidase activity without modulating the gut composition (K. L. A. Chen et al. 2018b). Further androgens are converted to oestrogens via the aromatase enzyme, expressed locally in neurons and brain cells (Callard et al. 1980), thus developmental regulation of aromatase activity in males and females can also impact synaptic plasticity and neuronal circuit dynamics (Bender et al. 2017; Lu et al. 2019). It is thus interesting to ask whether aromatase activity may also be modulated by the gut microbiota.

5.4 Bile Acids and Dietary Hormone-Mimetic

Gut commensals are exposed to harsh environments, especially with presence of bile acids (BA) that possess bactericidal properties by destroying the bacterial lipid cell wall. The gut commensals have in-built resistance mechanisms such as expression of enzymes and efflux pumps capable of modifying steroids or releasing them out of the cells. Bioinformatics and microbiological analyses have determined several bacterial enzymes capable of modifying steroids including oestrogens and BA (Zou et al. 2019). Ketosteroid reductases, an oxidoreductase expressed by *Actinobacteria*, *Proteobacteria*, and *Firmicutes*, reversibly convert testosterone to androstenedione, and oestradiol to oestrone among other ketosteroids. Some of the enzymes within this group (enzyme required for 21-dehydroxylation) are exclusively expressed by the gut commensals (Ridlon and Bajaj 2015). In addition, pathogenic bacterial species belonging to *Streptococcus* and *Bacillus* genus express 5α-reductases, several other ketosteroid reductases that can act on BA and sex hormones. Bacteria such as *E. coli* and *Campylobacter jejuni* possess multidrug efflux pumps responsible for releasing BA from the bacterial cells (Garcia-Gomez et al. 2013; Lin et al. 2003). Antibiotic exposure in mice perturbed 87% of detected intestinal metabolites, with maximum change observed in steroid metabolism including BA and sex hormones (Baars et al. 2018). The authors also report the emergence of sex-specific BA composition in conventionalised male and female mice

compared to germ-free mice, with higher BA levels in females and more complex BA in conventionalised females (Baars et al. 2018). Germ-free mice have increased gall bladder size, increased density of BA transporters, higher BA concentration in serum, liver, and ileum (Selwyn et al. 2015). Moreover, BAs have been implicated as regulators of the gut brain axis in a mouse model of autism (Golubeva et al. 2017). BA acts as emulsifiers facilitating absorption of fats including short chain fatty acids (SCFA). Control female mice have higher SCFA-producing bacteria along with increased SCFA in colonic tissue compared to males (H. Gao et al. 2019a). Specific antibiotics have sex dependent effects on SCFA-producing bacteria, BA and SCFA levels (H. Gao et al. 2019a), which can regulate behavioural responses possibly via SCFA receptors or epigenetic modifications by butyrate (van de Wouw et al. 2018). Higher levels of SCFA and BA may give an advantage to females against brain disorders, but whether fluctuating hormonal levels modulate SCFAs thereby playing a detrimental role is unclear.

Environmental and dietary sources of oestrogens and steroid mimetics can have detrimental effects on the body. The gut commensals may interact with these factors thereby influencing the exogenous steroid metabolism and levels. One such example is the plant-derived phytoestrogens, such as lignans, isoflavones, and coumestans, which are structurally and functionally similar to oestrogens with higher affinity for ERβ, oestrogen receptor with anxiolytic property (Oyola et al. 2012; Patisaul and Jefferson 2010). Several studies have addressed the influence of phytoestrogens on neuropsychiatric symptoms especially during menopause [Reviewed in (Fattah 2017)]. Phytoestrogens modulate cognition in humans (Zhao and Brinton 2007). In animals, sex-distinct effects of phytoestrogens on anxiety and spatial memory are reported, where it decreases anxiety and enhances spatial memory in females, with opposite effects in males (Lund et al. 2001; Patisaul et al. 2005). The beneficial and detrimental effect of phytoestrogens is an active area of research with a potential to generate efficacious therapeutic interventions for physiological and neuropsychiatric disorders (Patisaul and Jefferson 2010; Rietjens et al. 2017).

6 Summary & Conclusion

Taking all together, the following picture emerges when one is considering the impact of sex on the microbiome-gut brain axis: 1) The male brain is particularly vulnerable to microbiota-based insults in early life. 2) Animal studies in a controlled environment show greater sex differences in microbiome-gut-brain axis than human studies where large cohorts are generally needed to unmask sex differences. 3) Absence of gut microbiota seems to diminish inherent

sex difference on a variety of physiological measures. 4) The microbiome alone or by modulating sex hormones can drive inflammatory and metabolic factors including SCFAs and neurotransmitters, thereby increasing sex differences. 5) The microbiome-regulated sex hormones along with the immune and metabolic factors can alter brain cells (microglia, oligodendrocyte, and neurons) modifying neuronal circuitry thereby impacting behavioural changes. 6) Male gut may also have an increased inflammatory tone affecting the microbial colonization early in life. 7) Compared to higher sex hormone drive in males during development, female ovaries do not secrete oestrogen, and this may underpin the fact that females are more frequently colonized by *Lactobacillus* and *Bifidobacterium* at birth, which may be beneficial to the developing brain. 8) In adolescence with the rise of sex hormones, males have a reduced testosterone-mediated inflammatory tone, and this may lead to divergence of gut microbiome profile as seen in case of *Prevotella* (more), and *Akkermansia* (less). 9) Diet-induced changes in the microbiome can aggravate oestrogens-induced modulation of synaptic plasticity, stress axis and behaviour. 10) Dietary components as well as drugs and dietary sex hormone mimetics can sex-dependently alter the microbiome, and thus modulate a cascade of physiological events leading to behavioural changes. 11) Further studies are critically needed in both animals and human cohorts to understand the relative bidirectional contribution and dynamics of sex hormones to microbiome-induced effects. Likewise, there is a pressing need to understand the sex-dependent compensatory changes with diet, drugs, probiotics, and other factors at the level of microbiome and host.

Host genetic microbiome interactions are also being characterised with the possibility of heritable microbiome (Goodrich et al. 2014; Goodrich et al. 2017; Zhernakova et al. 2016) and it would be interesting to check for sex difference in the context of brain health (Corella et al. 2018). Because of genetic- and environmental- differences in the microbiome composition, it is clear that the microbiome field is at the heart of precision medicine (Kashyap et al. 2017; Zmora et al. 2018) and precision nutrition approaches (Bashiardes et al. 2018; Mills et al. 2019; Zeevi et al. 2015). Whether different tailored microbiome-based interventions should be generated across both sexes warrants much further exploration.

To conclude, it is clear that although there is enough evidence to suggest an interaction between the sex, sex hormones and the gut microbiome on brain and behavioural outcomes much research is still needed to unravel its contribution. Given the sex bias that exists for the prevalence of mental disorders (Fig. 1), further studies into the interaction of sex hormones and gastrointestinal microbiota on the onset, prevalence and progression of these debilitating

neurological disorders are warranted. It cannot be stressed enough that many mental disorders are often associated with metabolic or immunological problems; and while the early life environmental factors and genetics including sex, play a huge role, the focus needs to shift on perturbing the most amenable factor, the gut microbiota, for a potential sex-dependent therapy.

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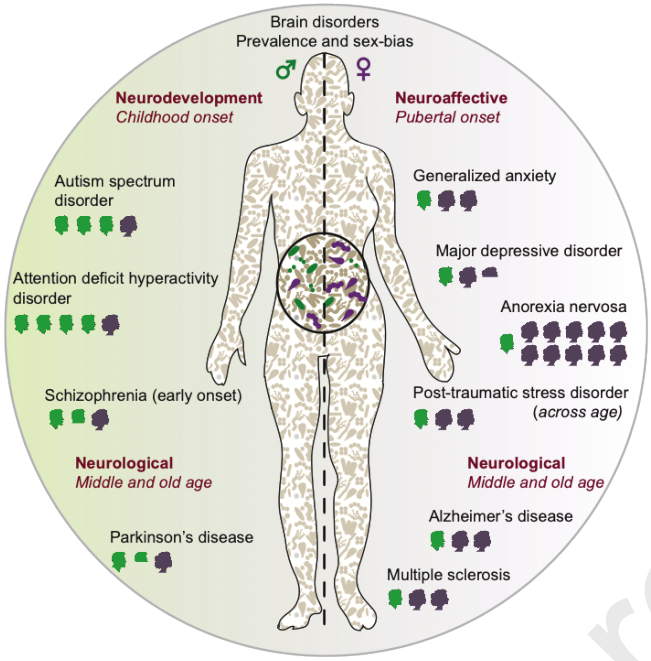

Figure 1. Sex-bias in several brain disorders and their likely onset. The disorders shown on left show higher prevalence in males, and the ones on right show greater prevalence in females (males-green, females- purple). Vast majority of gut bacteria are equipresent in males and females, while some bacterial taxa show sex-difference in abundance, irrespective of health status. Male to female ratio and data source for mental disorders: ASD (3:1)- (Loomes et al. 2017), ADHD (4:1)- (Erskine et al. 2013), Schizophrenia (1.7:1)- (Jongsma et al. 2019), Parkinson's disease (1.48:1)- (Picillo et al. 2017), generalized anxiety (1:1.9)- (Remes et al. 2016), MDD (1:1.95)- (Salk et al. 2017), Anorexia nervosa (1:10, high variation 1:3 to 1:12)- (Raevuori et al. 2014), PTSD (1:2)- (Galea et al. 2005), Alzheimer's disease (1:1.18)- (G. B. D. D. Collaborators 2019a), MS (1:2)- (G. B. D. M. S. Collaborators 2019b).

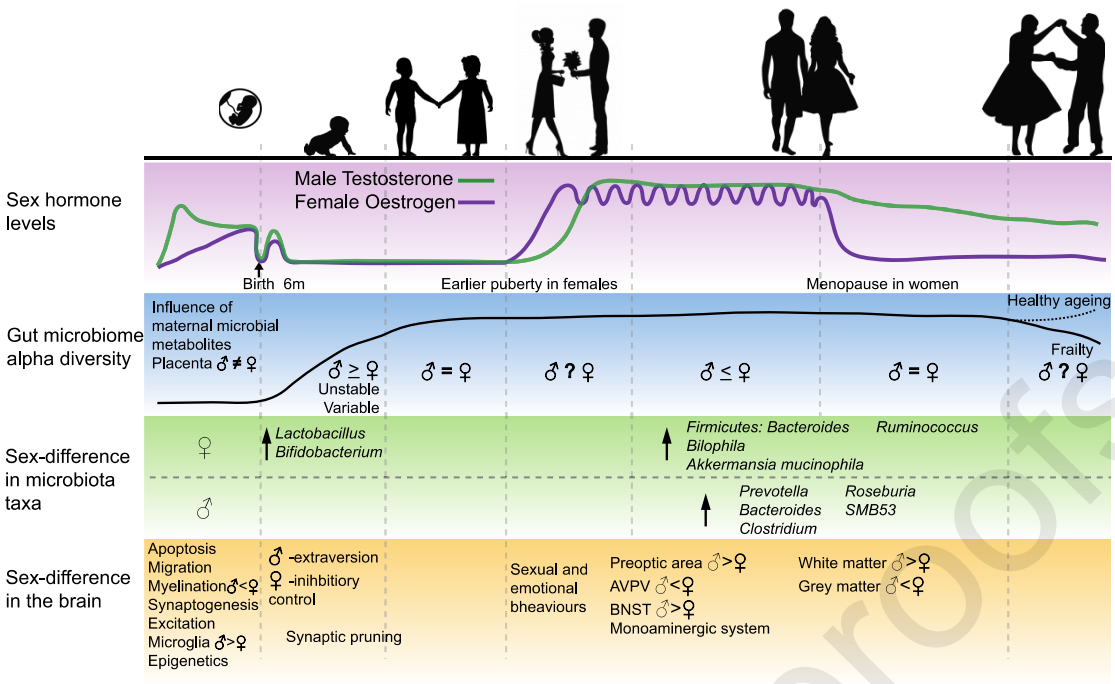

Figure 2. An overview of sex differences in sex hormonal levels, gut microbiome profiles and neurological readouts across lifespan. Gestation- oestrogen levels are comparable between males and females, while testosterone levels increase in male foetus with development of the gonads. At this stage, the foetal microbiome is largely non-existent, but the maternal gut microbial metabolites may influence foetal development. This is a critical stage as the brain is still developing and maternal stress, inflammation, hormones and oher microbial factors may influence neuronal circuits and impact behaviour. After birth, there is a surge of sex hormones observed, which is thought to play a role in sexual differentiation of brain and behaviour. While most studies on infants do not report sex- differences in alpha diversity, one study does point to a greater alpha diversity in males along with increased extraversion, while female infants display greater inhibitory control. Genera that metabolize milk oligosaccharides, Lactobacillus and Bifidobacterium are better colonized in female gut in infancy, this could improve gut barrier and enhance nutrient availability, bile acids and short chain fatty acids, protecting the females against earlier environmental insults. Most studies at prepubertal stage, characterized by minimum levels of sex hormones, do not report sex differences in alpha diversity. Puberty arises earlier in females along with emergence of sex-specific sexual and emotional behaviours. In adulthood, males have a steady testosterone level, which gradually declines with age, while females show a cyclical pattern of hormones through menstruation with menopause leading to a sharp decline in oestrogen levels. The alpha diversity of gut microbiome is higher in reproductive females with no difference observed in middle-age adults. Adult females seem to have higher Firmicutes/Bacteroidetes ratio along with other differences in microbiome taxa. It

is unclear if sex differences exist in ageing population, but higher alpha diversity seems to be a hallmark for healthy ageing. In adults, neuroimaging and biochemical studies in humans and animals report anatomical and neurochemical (monoamine pathways) differences in the brain such as: increased white matter in males and grey matter in females, increased volume of preoptic area and bed nucleus of stria terminalis (BNST) in males, while larger Anteroventral Periventricular nucleus of hypothalamus (AVPV) in females. These regions are responsible for sexual behaviours and are modulated by sex hormones. While several sex differences exist in the brain, there is a large overlap and brain cannot be categorized as masculine or feminine but consists of mosaics.

Table 1. Sex differences in gut microbiota composition in healthy humans across lifespan.

Age	Cohort/Strain	Males (M)	Females (F)	Reference
Birth- 3 months	Antwerp, Belgium		F>M *L. ruminis, L. gasseri, and L. reuteri*	(R. Martin et al. 2016)
1.5-24 months	Bangladesh	Vitamin A- ↑*Bifidobacterium*	↑*Bifidobacterium* until 11 weeks. No effect of Vit. A.	(Huda et al. 2019)
2.5 month	FinnBrain Birth Cohort Study, Finland, temperament at 6 months	M=F α-diversity, different bacterial clusters (community types)		(Aatsinki et al. 2019)
3-6 months	US ethnically diverse VDAART study (Vitamin D Antenatal Asthma Reduction Trial)	M=F α-diversity	↑community type (↑*Firmicutes* (Family- *Lachnospiraceae* and *Clostridiales U.*) ↓*Bacteroides*	(Sordillo et al. 2017)
18-27 month	Columbus, Ohio, US cohort	M=F α- or β- diversity.		(Christian et al. 2015)
18-40 years	Human Microbiome Project	M>>>F Community type D: ↑*Prevotella* ↓*Bacteroides*	Community type C: ↑*Ruminococcaceae*, *Alistipes*, *Faecalibacterium* and ↓*Bacteroides* and absence of *Prevotella*	(Ding and Schloss 2014)
Young adult (20-45 years), middle age (46-69 years)	American gut project- citizen science initiative- US and UK cohort	α-diversity: F>M, less pronounced in middle age. Sequence variant (SV) richness: US- M=F, UK young adult cohort- F>M		(de la Cuesta-Zuluaga et al. 2019)
	Columbia cohort	α-diversity: F>M, less pronounced in middle-age. SV richness in young adult: F>M		
	China cohort	M=F α-diversity		
18-81 years	Dutch, LifeLines-DEEP	12 microbial species associated with sex, including *Bilophila*, *Lachnospiraceae*, and *Bifidobacterium spp.*	↑α-diversity, *Akkermansia muciniphila*. Anti-androgen oral contraceptive- ↑*Bacteroides caccae*, *Coprobacillus spp.*	(Sinha et al. 2019)
20-75 years	Spanish, COR- DIOPREV study (CORonary Diet Intervention with Olive oil and cardiovascular PREVention study)	↑*Clostridium*, *Roseburia*, and *SMB53*.	↑*Bilophila*, *Ruminococcus*, *Parabacteroides*.	(Santos-Marcos et al. 2019)
30-83 years	Control from colorectal cancer study, Washington D.C., US	↑*Bacteroidetes*	Obese F-↓*Bacteroidetes*	(Dominianni et al. 2015)
20-50, >60 years	European (France, German, Italy, Sweden)	↑*Bacteroides-Prevotella*		(Mueller et al. 2006)
Premenopause (46 years)	Spanish ONCOVER study (volatile compounds detection system for the early cancer diagnosis)	↓*Actinobacteria* ↑*Sutterella* genus	↑*Bilophila*, *Ruminococcus* (*Lachnospiraceae*), and *Prevotella*	(Santos-Marcos et al. 2018)
Postmenopause (56 years)			↑*Firmicutes/Bacteroidetes* ratio	

Menopause effect		No change	↑*Firmicutes* ↓*Actinobacteria* ↑*Roseburia*, *Lachnospira*, ↓*Parabacteroides*, *Prevotella*, *Bilophila*	
55-70 years	Spanish, COR- DIOPREV study	↑*Veillonella* and *Methanobrevibacter*	↑*Bilophila*	(Haro et al. 2016)
		BMI>33 -↓*Bacteroides* compared to BMI<33	No difference	

Table 2. Sex influences gut microbiome in male and female rodents. (Bolded words indicate congruous findings across two or more studies.)

Species and Strain	Age	Treatment	Males (M)	Females (F)	Reference
Mouse C57BL/6J:129S1/SvImJ F1 hybrid	P2	None	↑*Haemophilus,* ↑*Sphingobium*	↑*Streptococcus*	(Jasarevic et al. 2017)
Mouse NOD/Jsd	3 weeks	None	No sex difference in gut microbiota.		(Markle et al. 2013)
Mouse C57BL6/J (APPPS1-21)	3 weeks	None	No sex difference in gut microbiota.		(Dodiya et al. 2019)
Mouse C57BL/6J:129S1/SvImJ F1 hybrid	4 weeks	None	↑***Dehalobacterium*** ↑*Flexispira*	↑*Mucispirillum*, *Odoribacter*, and *Desulfovibrio*	(Jasarevic et al. 2017)
Mouse NOD/ShiLtJ	4 weeks	None	No sex difference in gut microbiota.		(Yurkovetskiy et al. 2013)
Mouse NOD/Jsd	6 weeks	None	Sex difference in gut microbiota.		(Markle et al. 2013)
Mouse C57BL6/J	6 weeks	None		***Akkermansia*** associated with oestrus phase.	(Wallace et al. 2018)
Mouse C57BL6/J (APPPS1-21) model of Aβ amyloidosis.	7 weeks	None	↑*Mollicutes RF39* (***Tenericutes***).	Not significant.	(Dodiya et al. 2019)
Mouse NOD/ShiLtJ	2.5-3 months	None	Composition variability across experiments: ↑*Peptococcaceae*, *Proteobacterium,* ***Enterobacteriaceae***; ↑*Veillonellaceae, Porphyromonadaceae*, *Kineosporiaceae spp.*	↑Shannon α-diversity**,** Microbiome: F= pre-pubertal mice, M is distinct.	(Yurkovetskiy et al. 2013)
Mouse NOD/Jsd	3 months	None	↑*Roseburia*, *Bilophila*, *Coprococcus I.*	↑*Parabacteroides*, *Incertae sedis* (*Lachnospiraceae*).	(Markle et al. 2013)
Mouse C57BL6/J	3 months	None	↑**Shannon α-diversity**, ***Dehalobacterium***, *AF12*, ***Bacteroides***, *Dorea*, *Mucispirillum*, *Roseburia*, *Adlercreutzia*, ***Ruminococcus***, *Anaerostipes*. Colonic content: ↑Pheylalanine and Tyrosine.	↑***Lactobacillus***, *Prevotella*, *Sutterella*. Colon: ↑SCFA.	(H. Gao et al. 2019a)

			Colon: ↑uracil and inosine.		
Mouse C57BL/6JRccHsd	3-4 months	None	↑**Shannon α-diversity**, ***Bacteroides***, *Alistipes*, *Clostridiales*, ***Ruminococcus***, and *Rikenellaceae*.	↑*Firmicutes*, ↑*Firmicutes/Bacteroidetes* ratio, and ***Lactobacillus***.	(Baars et al. 2018)
Mouse C57BL/6	3.5-4 months	None	↑**and = α-diversity**. ↑*Proteobacteria, Escherichia* and *Shigella*, ↑*Clostridia*, *Butyricimonas*, *Peptococcus*.	↓*Firmicutes*/*Bacteroidetes* ratio, ↑*Bifidobacterium*/ ***Enterobacteriaceae*** (B/E) ratio, ↑***Akkermansia***.	(Kaliannan et al. 2018)
Mouse 89 different inbred strains	4 months	None	↑*Actinobacteria* and ***Tenericutes*** phyla, genera-*Allobaculum*, *Anaeroplasma*, and *Erwinia*.	↑*SMB53* (*Colstridiaceae*) and 3 members of family *Lachnospiraceae* (*Dorea*, *Coprococcus* and *Ruminococcus*)	(Org et al. 2016)
Mouse NOD/ShiLtJ	2.5-3 months	GF colonized with SPF F at birth	↑*Cytophagaceaee* and *Bacteroidaceae* (*Bacteroidetes*), ↑*Peptostreptococcaceae* (*Firmicutes*).	Sex-difference in microbiome clustering.	(Yurkovetski y et al. 2013)
Mouse NOD/Jsd	2.5 months	FMT: Adult M→SPF F weanling		↑*Roseburia*, *Blautia*, *Coprococcus I*, ↓*Peptococcus* compared to SPF F (not at 34 weeks). Microbiome distinct from SPF M & F.	(Markle et al. 2013)
Mouse C57BL6/J (APPPS1-21)	P22 and 7 weeks	ABX gavage P14-P21, continue 1:50 in drinking H2O.	P22- No sex difference in gut microbiota.	7 weeks- ↑***Akkermansia muciniphila*** and an *Allobaculum spp*. cluster.	(Dodiya et al. 2019)
Mouse C57BL/6J, C3H/HeJ, and DBA/2J	4 months	GDX+ High fat-high sucrose diet (HFD)	• GDX + chow diet: Altered microbiome, reversed by testosterone except for DBA2J. • GDX + HFD: ↑*Ruminococcaceae*	• GDX + HFD: Altered microbiome ↓***Akkermansia***	(Org et al. 2016)
Mouse NOD/ShiLtJ	2.5-3 months	♂ Castration at 4 weeks	• Gut microbiome clusters overlap with F. • ↓*Lactobacillaceae* compared to M, and =F.		(Yurkovetski y et al. 2013)
Mouse C57BL/6	3.5-4 months	Ovariectomised F (OVX)		• Gut microbiome composition = M. • ↑*Proteobacteria* and its members (genus *Escherichia/Shigella*) • ↓***Akkermansia.***	(Kaliannan et al. 2018)

Mouse C57BL/6	3.5-4 months	11-17 week: E2 (oestradiol)	• E2: ↓*Proteobacteria*, gut microbiome closer to F and distinct from M/OVX. • M+E2: ↑***Akkermansia***.	• OVX+E2: ↑***B/E* ratio**, ↑*Bilophila*, *Alistipes*, ***Bacteroides*** and *Eubacterium* compared to M/OVX.	(Kaliannan et al. 2018)
Mouse C57BL6/J (F)	3 months	OVX + progesterone (P4) or *Lactobacillus reuteri* at 10 w.	• OVX+P4: ↑***Lactobacillus***, ↓anxiety on OFT not EPM, ↓immobility on FST compared to OVX. Blocked by ABX. • OVX+P4/ *L. reuteri*: ↑hippocampus BDNF and ↓immobility on FST. • P4: ↑*Lactobacillus reuteri* growth *in vitro*.		(Sovijit et al. 2019)
Mouse C57BL6 (F)	3 months	s.c. E2- 1 week prior to myelin protein immunization.	• E2 protect against autoimmune Encephalomyelitis (EAE) in animal model. • ↑diversity, *Lactobacillaceae*, *Coriobacteriaceae* and unidentified family from the *Bacillales* order. • ↑Breg cells, IL-10+ B cells, & anti-inflammatory macrophages in Mesenteric lymph nodes.		(Benedek et al. 2017)
Rat Sprague-Dawley (F)	2.5 months	PCOS model 6-9 week: letrozole (aromatase inhibitor).	• ↓ ***Lactobacillus***, *Ruminococcus* and ***Clostridium***, ↑*Prevotella*. • ↑Androgens, ↓Oestrogens, perturbed oestrus cycle. • Rescued by 2-week *Lactobacillus reuteri* or FMT from healthy F.		(Guo et al. 2016)
Rat Wistar (F)	4-8 months	GD20: s.c. Testosterone cypionate	• ↓***Akkermansia***, ***Bacteroides***, ***Lactobacillus***, ***Clostridium***. • ↓Leutinizing hormone, ↑body weight, hypertension, adipokines mRNA in white adipose tissue. • ↑bacteria associated with steroid hormone synthesis (*Nocardiaceae* and *Clostridiaceae*), and SCFA synthesis.		(Sherman et al. 2018)

Table 3. Summary of human studies reporting sex differences associated with gut microbiome in behaviour and mental health.

Cohort	Age	Method	Male (M)	Female (F)	Reference
FinnBrain Birth Cohort Study	2.5 months	16S Illumina MiSeq. 2.5-month stool sample. 6-month Infant Behaviour Questionnaire.	• *Bifidobacterium* associated with surgency. • ↑*Bifidobacterium* and *Clostridiaceae*, ↓*Veillonella*- ↑regulation.	• ↑*Veillonella*- ↓fear reactivity.	(Aatsinki et al. 2019)
Columbus, Ohio, U.S.	18-27 months	16S Roche 454 FLX Pyrosequencing. Early Childhood Behaviour Questionnaire.	• ↑ Motor activation, high intensity pleasure, extraversion. • ↑ Phylogenetic diversity and Shannon index- ↑sociability and high intensity pleasure. • ↑*Ruminococcaceae* genus and *Parabacteroides*– ↑sociability. • ↑*Rikenellaceae* genus and *Dialister*- ↑high intensity pleasure. • *Ruminococcaceae* ↑with age.	• ↑ Inhibitory control, soothability, and effortful control. • ↑ Shannon index- ↓effortful control. • ↑*Rikenellaceae* genus- ↑fear. • *Ruminococcaceae* not changed with age.	(Christian et al. 2015)
ASD with and without GI symptoms. Shenzhen, China.	2-8 years	Shotgun Illumina Hiseq4000. Candidate (immune system) gut microbiota associated epitope (ME).	• ME diversity • ASD > non-ASD.	• ME diversity • ASD = non-ASD.	(Wang et al. 2019)
ChiBS study, Belgium	8-16 years	16S Illumina Miseq. Coddington Life Events Scale for Children, self-test for emotion.	• No sex difference in gut microbiota diversity or composition.	• ↑emotional problems	(Michels et al. 2019)
Kangbuk Samsung Health Study, Korean, Cross-sectional study personality traits and gut microbiome	23-78 years	16S Illumina MiSeq. Revised NEO personality inventory	• ↑Neuroticism- ↑*Haemophilus*. • ↑Conscientiousness- ↑*Lachnospiraceae*.	• ↑Neuroticism- ↑*Peptostreptococcaceae* and *Gammaproteobacteria* but not significant post FDR correction.	(Kim et al. 2018)

Bipolar Illness Onset Study. Denmark	22-41 years	16S Illumina MiSeq. Bipolar disorder patient (BD), Healthy relatives and individuals.		• *Flavonifractor* associated with BD F>M. (*Flavonifractor*-may induce oxidative stress and inflammation)	(Coello et al. 2019)
Illinois, U.S.	25-45 years	16S Illumina MiSeq2000. Depression, anxiety, and stress scales-42 (DASS).	↑ DASS scores: • ↑*Butyricimonas*, and *Phascolarctobacterium*, • ↓*Dorea*, *Rikenellaceae*, *Ruminococcus*, and *Blautia*.	↑ DASS scores: • ↑ *Lactobacillaceae*, *Lactobacillus*, *Dialister*, *Paraprevotella*, *Bacteroide*s, and *Bacteroidaceae*, • ↓*Proteobacteria*, *Anaerostipes*, and *Parabacteroides*.	(Taylor et al. 2019)
First episode drug naïve MDD and healthy control (HC), China	25-60 years	16S Roche 454 FLX pyrosequencing. 17-item Hamilton Depression Rating Scale (HDRS-17)	MDD: • ↓*Bacteroidetes*. • ↑21 OTUs and ↓53 OTUs. • ↑ *Atopobium*, *Bacteroides*, *Veillonella*, *Erysipelotrichaceae incertae sedis*, and and ↓*Anaerovorax*, *Gordonibacter*, and *Pyramidobacter*. • ↑HDRS score- ↑*Collinsella*, ↓*Veillonella*.	MDD: • ↑*Actinobacteria*. • ↑29 OTUs and ↓28 OTUs. • ↑ *Atopobium*, *Actinomyces*, *Bifidobacterium*, *Blautia*, *Roseburia*, *Desulfovibrio*, *Eubacterium*, among others. • ↓*Howardella*, *Sutterella*, and *Pyramidobacter*. • ↑HDRS score- ↓*Clostridium XIVa*, *Erysipelotrichaceae incertae sedis*, and *Streptococcus*.	(Chen et al. 2018)
Spnish cohort from on-going FLORINASH Project (multi-institution)	30-65 years	16S Roche 454 FLX pyrosequencing. Diffusion tensor imaging of brain white and grey matter. Trail making test for cognition.	• Obesity: ↓ Bacterial diversity.	• Obesity: X Bacterial diversity. • ↑Age, intra-abdominal fat- ↓*Actinobacteria*.	(Fernandez-Real et al. 2015)
Australia, Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome (ME/CF)	6-81 years	Bacterial culture and MALDI-TOF MS analysis. Cross-sectional. Bioscreen Patient Questionnaire	• ↑ME/CF symptom-↑*Streptococcus* and *Lactobacillus*. • (Possible detrimental effect of ↑ D-lactate).	• ME/CF- ↑ behavioural impairment. • ↑ME/CF symptom- ↑*Clostridium*, ↓*Bifidobacterium* and *Streptococcus*.	(Wallis et al. 2018)

Table 4. Summary of animal studies reporting sex differences associated with microbiota-gut-brain axis.

Species, strain	Age	Method	Sex-Specific Effects in Males (M)	Sex-Specific Effects in Females (F)	Ref.
Animal models, Behaviour and gut microbiota					
Mouse C57BL/6J:129S1/SvImJ F1 hybrid	P2, P6, P28	**Prenatal stress** GD1-7: chronic variable stress. Offspring microbiome. 16S Illumina Miseq.	• P28: ↓β-diversity and *Flexispira*, ↑*Desulfovibrio*, *Dehalobacterium*, *Clostridiales*, *Lachnospiraceae*	• P28: No change • Stress abolished sex difference in microbiota composition	(Jasarevic et al. 2017)
Mouse CF-1	2 months	**Chronic stress** Alternate day restraint and FST for 19 days at 6 weeks. 16S Illumina Miseq.	• OFT: No effect on rearing • EPM: ↑ total distance (M>F) • ↓*Ruminococcus gnavus*	• OFT: ↑ rearing in centre • ↑*Ruminococcus gnavus*, ↓*Sarcina*	(Tsilimigras et al. 2018)
Mouse C3H/HeNRj	3-6 months	**MIA + MS + Maternal CUS** GD17: LPS. P2-P14: MS+CUS. 5month: faecal sample. 16S Illumina MiSeq.	• ↓ Pup USV • ↓ Adult social interaction COR ↓*Clostridium XIVa* ↓*Lachnospiraceae* ↑*Lactobacillus* and ↑*Bacteroides* • X EPM, Marbles buried	• USV: mild impairment • X social interaction. • ↑ EPM anxiety COR ↓*Clostridium XIVa*, ↓*Lachnospiraceae* • ↑ Marbles buried COR ↓*Clostridium XIVa*, ↓*Lactobacillus*	(Rincel et al. 2019)
Rat Sprague Dawley	6 weeks	**MIA + ABX** P3, P5: LPS i.p. P35- P39: ABX once daily oral gavage. (Neomycin, polymyxin B and metronidazole). 16S Illumina HiSeq 2500.	• ↓ Social preference towards LPS-treated rats (partially rescued with ABX) • LPS: ↑Hypothalamus oxytocin receptor gene expression	• LPS /ABX: No sex difference in microbiome • LPS: X PFC/Hypothalamus oxytocin or its receptor	(Kentner et al. 2018)
Rat Long-Evans	6-7 weeks	**MIA + Propionate** GD12-16: s.c. daily propionate or GD15-16: s.c. daily LPS. P10-18: Twice on alternate days s.c. propionate.	• LPS: ↑acoustic startle	• Prenatal propionate: ↓prepulse inhibition. • Prenatal +Postnatal propionate: impaired habituation and sensitized acoustic startle	(Foley et al. 2015)
Ferret *Mustela putorius furo*.	5-6 months	**MIA** GD30: PolyI:C. Offspring microbiome and behaviour. 16S Illumina MiSeq.	• ↑ Adult amphetamine-induced locomotion	• ↓ Social preference • Larger alteration in β-diversity	(Li et al. 2018)

Mouse Swiss Webster	6-7 weeks	**GF/ Con / *Bifidobacterium*** P1: gavage GF dam +SPF bedding. GF postnatal colonization with *Bifidobacterium* (human infant type). 16S Illumina MiSeq.	• GF: X EPM, OFT, ↓ motor performance (rescued by *Bifid.*) • *Bifid*: ↑EPM anxiety, X OFT	• GF: ↓ EPM, OFT anxiety (rescued by *Bifid)*, ↑OFT locomotion and ↓Social Preference (not rescued)	(Luk et al. 2018)
Siberian hamster *Phodopus sungorus*	>2 months	**ABX** 1 week enrofloxacin (Microbiome), 1 week enro, 1 week break, 1 week enro, 1 week post treatment monitor (Behaviour), 16S Illumina MiSeq	• ↓*Cyanobacteria*, *Proteobacteria*, *Elusimicrobia*, and *Euryarchaeota* • ↑*TM7* • ↓Aggression on 2nd treatment	• ↓*Cyanobacteria*, *Proteobacteria*, and *Tenericutes* • ↓ Aggression after 1st and 2nd ABX	(Sylvia et al. 2017)
Mouse C57BL/6	7 weeks	**Diazinon treatment** 13-week diazinon in drinking water (agriorganophosphate insecticide). Faecal metabolomics, Illumina- 16S MiSeq, shotgun NextSeq.	• Gut microbiome: ↑9 genera, ↓10 genera Altered *Bacteroides*, *Coprobacillus*, *Butyrivibrio*, *Staphylococcus*, *Lachnospiraceae_Johnsonella* • Altered bacterial genes: potassium and neurotransmitter metabolism, chemotaxis • ↓faecal taurine and glycine M>F	• Gut microbiome: ↑3 genera, ↓10 genera Altered *Lachnospiraceae, Roseburia*, *Johnsonella*, *Ruminococcus* • Altered bacterial genes: photosynthesis and metabolism of aromatic compounds	(B. Gao et al. 2017)
Rat Wistar	2.5 months	**Exercise + Diet Preference** P49- 1-week voluntary wheel running (run)/ sedentary (sed). 2-5 week run/sedentary + diet preference (high-fat/high-sucrose /high corn-starch). 16S Illumina MiSeq.	• ↓ Body weight • ↓ High-fat preference • ↑ High sucrose and corn-starch preference • ↑ Chao1 diversity • Microbiome altered	• Run distance F>M • X Body weight, dietary preference • ↑ Calorie intake • Microbiome unaltered • ventral striatum: ↑preproenkephalin and μ-opioid receptor 1 mRNA	(Lee et al. 2017)
Metabolism, Neurotransmitter and immune factors					
Mouse: C57BL/6J:129S1/SvImJ F1 hybrids	P2	**Prenatal stress** GD1-7: Chronic variable stress, vaginal proteomics. Pup colon and plasma metabolomics. Brain amino acids. 16S Illumina MiSeq.	• ↑*Bacteroides* and *Clostridium* compared to control, similar to control ♀ pup • PVN: ↓free amino acid levels	• ↑*Bifidobacterium*, similar to control ♂ pup • PVN**:** ↑or no change in free amino acid levels	(Jasarevic et al. 2015)

Mouse: C57BL/6J:129S1/SvImJ F1 hybrids	E18.5, P2, 2 months	**Prenatal stress + Vaginal microbiota transfer** C-section offspring from control or stress dam colonized with control (C) or stress (S) vaginal microbiota at birth. 16S Illumina MiSeq. Shotgun Illumina NextSeq500.	• Colonization with stressed microbiome: ↓ Body weight (not at P70) ↑ Stress-induced corticosterone • EPS: ↑gut inflammatory signatures at E18.5	• E18.5 controls: ↑ gut inflammatory monocytes and neutrophils • EPS: ↓ sex-differences in several parameters	(Jasarevic et al. 2018)
Mouse C3H/ HeNRj	2.5-6 months	**MIA + MS + Maternal CUS** GD17: LPS, P2-P14: daily MS+CUS. Medial PFC RNA microarray. 5-month: faecal sample. 16S Illumina MiSeq.	• X stress-induced corticosterone • 2.5 months- ↑paracellular permeability (not at 5.5 months) • ↑33 ↓59 OTUs, ↑*Lactobacillus*, *Bacteroides*, *Alloprevotella* • mPFC: ↑18 ↓138 genes • ↑ Social behaviour COR ↑*Arc*	• Impaired stress-induced corticosterone recovery • Visceral hyposensitivity, and no effect on intestinal permeability • ↑27 ↓37 OTUs, ↓*Lactobacillus* and *Mucispirillum* • mPFC: ↑91 ↓17 genes • ↑ Anxiety COR ↑*Arc, Fosb*, *Junb* and *Gadd45b*	(Rincel et al. 2019)
Mouse CD1	2 months	**MIA+ Probiotic** 5-7 week: Kefir (probiotic), at 6 week- single LPS injection.	LPS only: ↑*Proteobacteria* Kefir + LPS: • ↓*Proteobacteria* and LPS sickness score • X body weight • Serum: slight ↓ IL levels • Brain: ↓ IL-6 mRNA	LPS only: ↓*Firmicutes* ↑*Bacteroidetes* Kefir + LPS: • Faster improvement in LPS sickness score. • rescue ↓ body weight and ↓cytokine levels (PFC>Hpc>Hypothalamus)	(Murray et al. 2019)

Mouse C57BL6/J	6-7 months	**Chronic stress + PUFA** 28 days social isolation, dietary supplement of docosahexaenoic acid (DHA). 16S Illumina MiSeq.	Social isolation: • ↑*Allobaculum*, *RF39*, *Lactobacillus*, member of *Rikenellaceae* family • KEGG pathways: ↑methane and dibasic acid, aromatic amino acid metabolism DHA supplementation: • ↓ EPM anxiety and anhedonia • ↓*Lactococcus* and *Leuconostoc* ↑*Streptococcus* and *Helicobacter* • KEGG pathways: ↑tyrosine and glutathione metabolism, and unsaturated fatty acid biosynthesis • ↑*Allobaculum*, ↓*Ruminococcus* COR ↑open arm entry and sucrose consumption	Social isolation: • ↑*Clostridia* class (*Clostridium*, *Ruminococcus*, *Coprococcus*, *Anaerotruncus*) • KEGG pathways: ↑bacterial chemotaxis and fatty acid metabolism DHA Supplementation: • No effect	(Davis et al. 2017)
Mouse C57BL/6J	8 weeks	**GF Microglia** RNA microarray Illumina BeadArray Scanner 500GX. RNAseq-Illumina HiSeq 2000.	GF compared to SPF: • E18.5 SSC: 1216 DEGs, mostly ↓ • E18.5 neocortex: ↑microglia • P60 SSC: 26 DEGs, ↑ microglia *#No sex-difference in human mid-trimester foetal microglia.*	• E18.5 control F vs M: ↑ DEGs associated with inflammation, apoptosis, LPS response GF compared to SPF: • E18.5 SSC: 20 DEGs • P20 neocortex: ↑microglial • P60 SSC: 433 DEGs (associated with ↓ inflammatory response)	(Thion et al. 2018)
Mouse C57BL/6J	8 weeks	**ABX Microglia** 1-week ABX in sterile drinking water (ampicillin, colistin, streptomycin, amphotericin). RNAseq- Illumina NextSeq 500.	• 92 microglia genes regulated (63 immune response) • No effect on microglial density or morphology	• 40 microglia genes regulated (11 transcription regulation)	(Thion et al. 2018)

Mouse Swiss Webster	1-2 months	**GF + Colonization at P21 (ExGF)** Hpc: MicroRNA microarray, Exiqon. PFC: RNAseq Illumina NextSeq500.	GF: X body weight • ↓LD anxiety (rescued with colonization) • Plasma: ↑Trp, ↓Kynurenine/Trp (rescued) • Hpc: ↓*Bdnf*, ↑ serotonin, 5-HIAA • PFC: ↑miR-294-5p (rescued) • PFC: 190 DEGs, ↑ myelination- and activity-related genes ExGF vs GF PFC: 15 DEGs, some myelination genes normalized.	GF: • ↓body weight • Plasma: ↓kynurenine/ Trp • Hpc: ↓*5-HT$_{2C}$* • PFC: X miR-294-5p • No change in brain myelination. ExGF vs Con • PFC: ↑miR-294-5p	(Clarke et al. 2013) (Moloney et al. 2017) (Hoban et al. 2016b)
Mouse C57BL/6N, C57BL/6, Pglyrp2 knockout (KO)	P3- 2 months and 15 months	**GF/ABX** GD14-P3-Ampicillin ABX in drinking water. Peptidoglycan sensing molecule gene in controls: • P3 PFC: F>M • P3 Striatum: M>F • P14 Striatum: F>M • GF/ABX: ↓PGN, Region-specific	KO: • P3: ↓striatal *Bdnf* • Juvenile: ↑social interaction ↑ striatal *c-Met* • 15 months: ↓anxiety-like behaviour ↓amygdalar α-synuclein	• GF P3: ↑striatal synaptophysin KO: • P3 PFC: ↑ synaptophysin • P3 Striatum: ↑synaptophysin ↓*c-Met* • Juvenile Amygdala: ↑*c-Met* • 15 months: ↑anxiety-like behaviour (OFT, EPM, LDB) ↑motor coordination (rota-rod) Spinophilin: ↑FC, ↓amygdala	(Arentsen et al. 2017; Arentsen et al. 2018)
Mouse C57BL6/J	2 months	**ABX** 2-week vancomycin or ciprofloxacin-metronidazole in drinking water at 8 weeks of age. 16S Illumina HiSeq2500.	Vancomycin: • ↑*Morganella*, *Sutterella*, *Proteus* • Colonic content: ↑lactate, ↓tyrosine Ciproflaxin-metronizodale: • Colonic content: ↓ lactate • X Shannon index, SCFA	Vancomycin: • Colonic content: ↓SCFA F>M ↓branched-chain amino acids ↓tyrosine and alanine Ciproflaxin-metronizodale: • Similar effects to vancomycin • Colonic content: ↓ lactate	(H. Gao et al. 2019a)

Mouse C57BL6/J	5 months	**Manganese exposure** 8- 21 week- Manganese exposure ($MnCl_2$ in drinking water). 16S Illumina MiSeq. Metagenomic Illumina NextSeq. Faecal metabolomics.	• ↓ Sex difference in bacterial genes • Gut microbiota: ↑*Firmicutes*, *Tenericutes* ↓*Bacteroidetes*, *Verrucomicrobia* • ↓ Microbiota genes: GABA, phenylalanine, and lipid synthesis genes, antibiotic resistance, Mn transporter and oxidation genes.	• Gut microbiota: ↓*Firmicutes*, ↑*Verrucomicrobia* ↑*Bifidobacterium*, *Akkermansia* • ↑ Microbiota genes: Phenylalanine, LPS, lipid synthesis genes, DNA repair genes, antibiotic resistance, Mn transporter and oxidation genes.	(Chi et al. 2017)
Mouse C57BL6/J	10 months	**HFD + Arachidonic Acid** 4-week old: High fat diet (HFD) for 10 weeks, and continued HFD +/- Arachidonic acid (AA) for 15 weeks. 16S Illumina MiSeq.	• HFD**:** ↑caecal SCFA content HFD+AA: • Microbiota COR ↑obesity, inflammation • Hypothalamus: ↑inflammation and microglia, ↑*AGRP*, *NPY* ↓*POMC*, *LEPR* • Caecum: ↑acetate ↓butyrate	• HFD: ↓caecal SCFA content HFD+AA: • ↑*Proteobacteria* ↓*Verrucomicrobia* phylum, shifts microbiota composition towards protective functions, ↓inflammation, ↑SCFA producers • Hypothalamus: ↓*AGRP*, ↑*LEPR* and *GRP109A* • Caecum: ↓acetate ↑butyrate	(Zhuang et al. 2017)
Mouse BTBR (T + tf/J), C57BL/6J	12 months	**ASD mouse model** BTBR compared to C57BL6/J. 16S Illumina MiSeq.	• 167 OTUs different • ↑*Lactobacillus*, ↓*Desulfovibrio* • Colon: ↑IL-6 and Cd11c gene expression	• 245 OTUs different • ↑*Proteobacteria* (LPS producing), *Prevotella*, *Sutterella*, *Akkermansia* • ↓*Oscillospira*, *TM7*	(Coretti et al. 2017)
Mouse APPSWE/PS1L 166P (mouse model of Aβ amyloidosis)	7 weeks	**Aβ amyloidosis + ABX** ABX (kanamycin, metronidazole, colistin, gentamicin, vancomycin): P14-P21 gastric gavage and P21-1:50 in drinking water. Rescue: Control FMT-gavage daily P25 till 7 weeks + 1:50 ABX. 16S Illumina MiSeq.	Cortex of model alone: • Aβ deposition, microglia size M>F ABX: • Plasma: ↓pro-inflammatory markers, ↑anti-inflammatory/ neuroprotective markers • Cortex: ↓Aβ deposition ↑TGFβ pathway genes • Several effects rescued by FMT	ABX: • ↑*Akkermansia muciniphila* and an *Allobaculum spp.* cluster, ↑pro-inflammatory pathways (F>M) • Plasma: ↑pro-inflammatory markers • Cortex: No effect on Aβ deposition till 3 months	(Dodiya et al. 2019)

Mouse NOD/ShiLtJ (Type 1 Diabetes model)	2.5-3 months	**SPF, GF and F1 Gnotobiotic** F1 Gnotobiotic mice from GF: Colonized with VSL3*, SFB: segmented filamentous bacteria, SECS: *Proteobacterium* sequence similar to *E. coli /Shigella* isolated from ♂. 16S Roche 454 FLX Pyrosequencing.	• SFB: ↓T1D markers • Con, SFB, SECS compared to GF: Serum: ↑ testosterone Pancreatic lymph nodes: ↑activated macrophage ↑IFNγ (T-cells) and IL-1 • Heat-inactivated SECS exposed macrophages: ↑T-cell IFNγ	• SPF insulitis: F>M • GF/ ♂castration: attenuates sex-difference T1D • Gnotobiotic: No changes seen in any groups **VSL3 mix: Bifidobacterium breve, B. longum, B. infantis, Streptococcus thermophilus, Lactobacillus bulgaricus L. acidophilus, L. plantarum, L. casei*	(Yurkovetskiy et al. 2013)
Sex hormone, Bile Acid (BA) and Hormone-mimetic					
Mouse NOD/Jsd (Type 1 Diabetes model) NMRI (ASF colonized)	2-3 months	**GF/ ASF- colonized** Serum metabolite kit: AbsoluteIDQ® p180 Kit, Biocrates Life Science AG. **FMT:** Adult M→SPF weaned F + **Androgen receptor (AR) antagonist** (flutamide) *Microbiome: regulate testosterone, anti-inflammatory T cells and protects against T1D in adult life*.	• SPF M<F Insulitis Serum: ↑ lysine, Trp amino acid • GF: attenuates sex-difference T1D Serum: ↑several serum amino acids (greater difference than between ♀) ↓testosterone No effect on 17β-oestradiol • GF+ASF: Restores sex-bias in T1D	• GF: ↑ serum testosterone • FMT: Serum: ↑testosterone, ↓sphingolipid ↓glycerophospholipid ↓T1D relative to F→F recipients (not observed at 34 weeks) • FMT + AR antagonist: Blocked FMT effects	(Markle et al. 2013)
Rat Sprague Dawley	Pubertal onset	**MS + *Lactobacillus*** P2-P14 maternal separation (MS) and *Lactobacillus* in water.	MS: delayed puberty reversed by probiotics	MS: enhanced puberty reversed by probiotics	(Cowan and Richardson 2018)
Siberian hamster	4 weeks, 2.5-3 months	**LPS + ABX** P3, P5- LPS i.p. P72-78-ABX enrofloxacin. Faecal samples- P24, pre-ABX P71, during ABX P78, post-ABX P85. 16S Illumina MiSeq.	• LPS: No effect on male reproductive physiology, adult microbiome • ABX: ↓12 families- *Campylobacteraceae*, *Rhodospirillaceae* ↓Nose-nose investigation duration • LPS+ABX: ↑grooming	• LPS: ↓ovarian mass ↓% regular oestrus cycling F ↓*Corynebacteriaceae*, ↑*Ruminococcaceae* • ABX: ↓16 families- *Brachyspiraceae, Bacteroidaceae, Desulfovibrionaceae*	(Sylvia et al. 2018)

Mouse C57BL/6J	GF: 3-3.5 months Con: 3.5-4 months	**GF male versus female** Serum BA, ileum gene microarray. 16S Illumina MiSeq.	• GF: ↑ BA transporter gene ↓ BA binding protein ↓ Basolateral BA transporter • Con: ↑ LDL receptor gene	• GF: ↓sex difference in ileal genes • GF and Con: ↑total and 1˚ BA • Con: ↑total and specific 2˚ BA	(Baars et al. 2018)
Mouse C57BL/6J	3.5 months	**Gonadectomy (GDX) + HFD** HFD: high-sucrose-high-fat diet, gall bladder bile composition.	• ↑BA (M>F) • ↑a- and b-Murincholic acid • ↓c-Murincholic acid	• ↓a- and b-Murincholic acid • ↑c-Murincholic acid	(Org et al. 2016)
Mouse C57BL/6J	3.5, 6 months	**WD + ABX** Western diet: wean-20 weeks. ABX (ampicillin, vancomycin, neomycin, metronidazole): 20-26 weeks (2x/ day) oral gavage.	WD: ↑Metabolic syndrome (MeS) • ↑ obesity • ↑ Intestinal permeability • ↑ Glucose intolerance • Serum: ↑LPS,LBP,TNFα,IL1β,IL6 All Rescued by ABX	• WD: MeS less severe compared to ♂ • WD+ABX: Only impaired glucose tolerance Alleviated sex difference in MeS	(Kaliann an et al. 2018)
Mouse C57BL/6J	4-7 months	**WD+ Ovariectomy+ Oestradiol** Western diet: wean-20 weeks. 10-week- ovariectomy. 11-17-week oestradiol in water.	• WD+Oestradiol: Rescued MeS	• WD+OVX: ↑obesity, MeS markers • (Rescued by Oestradiol) • At 3 weeks single WD ♂to ♀ FMT: ↑obesity and MeS	(Kaliann an et al. 2018)
Mouse C57BL/6J	7-8 months	♂ **WD + Oestrogen mimetic** 4-month male: 5-week isoflavones (ISO, oestrogen mimetic) genistein and daidzein. 16S Illumina MiSeq.	• Rescue MeS • Gut: ↑ERα mRNA levels, ↑alkaline phosphatase activity • Microbiome:↓*Proteobacteria*, ↑*Akkermansia*, *Bifidobacterium*, *Bacteroides* • Altered bacterial gene pathways: ↓LPS synthesis, ↑amino acid metabolism		Kaliannan et al. 2018)

5-HIAA- 5-hydroxyindole acetic acid, AA- Arachidonic acid, Aβ- Amyloid beta, ABX- Antibiotic, AGRP- Agouti-related peptide, AR- Androgen receptor, ASF- Altered Schaedler's flora (two *Lactobacilli*, one *Bacteroides*, one spiral bacteria of *Flexistipes*, and four obligate anaerobic *Fusobacterium species*), BA- Bile acid, Bdnf- Brain derived neurotrophic factor, c-Met- tyrosine-protein kinase Met/ hepatocyte growth factor receptor (proto-oncogene), COR- correlated, Con- Conventional, CUS- Chronic unpredictable stress, DEGs- Differentially expressed genes, E- Embryonic day, EPM- Elevated-plus maze test, F/B- *Firmicutes* to *Bacteroidetes* ratio, FMT- Faecal microbiota transfer, FST- Forced-swim test, GABA- γ-amino-butyric acid, GD- Gestation day, GDX- Gonadectomised, GF- Germ-free, GRP109A- G-protein-coupled receptor 109A, Hpc- Hippocampus, IFN-Interferon, IgA- Immunoglobulin A, IL-Interleukin, i.p.- Intraperitoneal, LD- Light dark box test, LDL- low density lipoprotein, LEPR- Leptin receptor gene, LPS- Lipopolysaccharide, MeS- Metabolic syndrome, MIA- Maternal immune activation, MS- Maternal separation, NPY- Neuropeptide Y gene, OFT- Open-field test, OTUs- operational taxonomic units, OVX- Ovariectomised, P-Postnatal day, PFC- Prefrontal cortex, Pglyrp- Peptidoglycan recognition protein, PGN- Peptidoglycan, POMC- Pro-opiomelanocortin, PVN- Paraventricular nucleus of hypothalamus, s.c.- Subcutaneous, SCFA- Short chain fatty acid, SECS- *Proteobacterium* sequence similar to *E. coli /Shigella*, SFB- segmented filamentous bacteria, SPF- Specific pathogen free, T1D- Type 1 diabetes, TNF-α- Tumour necrosis factor α, WD- Western diet.

Highlights

1) Male brain is particularly vulnerable to microbiota-based insults in early life.
2) Subtle differences in male and female gut microbiome.
3) Microbiota influences sex hormone conjugation and bile acid levels.
4) Microbiota and sex hormones can regulate inflammatory and metabolic factors.
5) Gut microbiota enhances sex difference on a variety of physiological measures.