Multi-Omics Mapping of Gut Microbiota's Role in Progesterone Metabolism

Guo-Jie Brandon-Mong[1,#], Mei-Jou Chen[2,3,#], Ronnie G Gicana[1], Hurng-Yi Wang[4,5], Chi-Ying Li[6], Tien-Yu Wu[1], Chia-Hung Chou[2], Yi-An Tu[2], Yu-Yuan Chang[2], Yi-Li Lai[1], Po-Ting Li[2], Yu-Lin Tseng[2], Tsun-Hsien Hsiao[7,8,*], and Yin-Ru Chiang[1,9,*]

[1]Biodiversity Research Center, Academia Sinica, Taipei 115, Taiwan;

[2]Department of Obstetrics and Gynecology, National Taiwan University Hospital and College of Medicine, National Taiwan University, Taipei 100, Taiwan;

[3]Livia Shan-Yu Wan Chair Professor of Obstetrics and Gynecology, National Taiwan University, Taipei 100, Taiwan;

[4]Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei 100, Taiwan;

[5]Institute of Ecology and Evolutionary Biology, National Taiwan University, Taipei 106, Taiwan;

[6]School of Pharmacy, Tzu Chi University, Hualien 97004, Taiwan;

[7]School of Medicine, National Tsing Hua University, Hsinchu 300, Taiwan;

[8]Institute of Molecular and Cellular Biology, National Tsing Hua University, Hsinchu 300, Taiwan

[9]Department of Agricultural Chemistry, National Taiwan University, Taipei 106, Taiwan.

[#]Equal contribution

*Correspondence. E-mail: thhsiao@life.nthu.edu.tw (Tsun-Hsien Hsiao); E-mail: yinru915@gate.sinica.edu.tw (Yin-Ru Chiang)

Competing Interest Statement: The authors declare no conflict of interest.

Highlights

- The microorganisms in the human gut can convert progesterone into several neurologically active steroids.
- Progesterone is primarily converted into pregnanolone by the gut microbiota in women with infertility.
- Members of the bacterial families Enterobacteriaceae and Veillonellaceae play key roles in the pregnanolone production.
- Genes involved in pregnanolone production are prevalent in the genomes of Enterobacteriaceae and Veillonellaceae.
- The abundances of Enterobacteriaceae and Veillonellaceae differ significantly between women who experienced healthy pregnancies and those who experienced miscarriages.

Abstract

Background

The female reproductive hormone, progesterone, is crucial in regulating the menstrual cycle and maintaining pregnancy. Despite known correlations between gut microbiota, sex hormones, and host phenotypes, the metabolic fates of progesterone in the human gut remain unclear.

Results

Here, an integrated multi-omics approach was employed to investigate gut microbiota-mediated progesterone metabolism. Fresh fecal samples were collected from infertile women with progesterone treatment (n = 14). Progesterone (1 mM) was amended into fecal cultures to enrich progesterone-metabolizing gut microbes, and the key players were identified through culturomics-based metagenomics analysis. Microbial functions were predicted by tracking temporal changes in metabolite profiles and microbial abundances. The prevalence of progesterone metabolism genes among gut microbiota was explored through functional genomics analysis. Pregnanolone was identified as the primary microbial metabolite of gut progesterone. Members of Enterobacteriaceae and Veillonellaceae were identified as crucial pregnanolone producers; progesterone metabolism genes were abundant in these bacterial families. Moreover, these bacterial families showed significant abundance differences among healthy women (n = 19) and women with miscarriages (n = 41).

Conclusions

This study provides potent therapeutic targets for improving progesterone bioavailability and demonstrates stereoselective production of neurosteroids using characterized bacterial strains and their corresponding metabolic pathways.

Keywords: gut microbiome, infertility, low progesterone bioavailability, Enterobacteriaceae, progesterone, pregnanolone, Veillonellaceae

INTRODUCTION

Progesterone is a key hormone that regulates critical physiological processes, including fertilization, implantation, embryogenesis, menstrual cycling, and pregnancy maintenance [1,2](Goletiani et al., 2007; Kolatorova et al., 2022). In healthy individuals, plasma progesterone concentrations follow characteristic patterns - remaining below 1 ng/mL during the follicular phase, rising to 10-40 ng/mL in early pregnancy, and reaching 100-200 ng/mL by late pregnancy [3](Sykes and Bennett, 2018). Insufficient progesterone levels or progesterone resistance (endometrial progesterone unresponsiveness) have been implicated in numerous pathological conditions, including endometriosis, premenstrual dysphoric disorder, chronic endometritis, and infertility due to impaired endometrial receptivity [4–7](Marquardt, et al., 2023; Melo et al., 2021; Coomarasamy et al., 2020; Toffoletto et al., 2014). Insufficient progesterone levels or progesterone resistance are also associated with increased miscarriage risk [8–10](Doldi et al., 1998; Unfer et al., 2005; Piltonen, 2016). Understanding the factors that contribute to progesterone deficiency or progesterone resistance is therefore crucial for developing targeted therapeutic strategies for these disorders.

While progesterone supplementation during the luteal phase is essential for successful embryo implantation and pregnancy maintenance, its bioavailability varies significantly among individuals, particularly when administered orally (approximately 10%) [11](Ciampaglia et al., 2015). Both endogenous and exogenous progesterone undergo enterohepatic circulation between the liver and gut, with reabsorption primarily occurring in the small intestine [12,13](Rogers and McLellan, 1951; Lu et al., 2001). In the gut, common progestin medicines, especially progesterone, can be metabolized by microbial activities, affecting their efficacy, bioavailability, and safety [14](Nuriel-Ohayon et al., 2019). Understanding progesterone metabolism by the gut microbiota is therefore crucial both for therapeutic applications and for monitoring maternal health during pregnancy.

The human gut harbors over 1,000 bacterial species [15–17](Qin et al., 2010; Rajilić-Stojanović and de Vos, 2014; Costea et al., 2018), forming intricate microbe-microbe and host-

microbe interactions within human holobionts [18](Bordenstein et al., 2015). Research has revealed significant associations between gut bacterial community structures, sex hormone profiles, and host phenotypes [19,20](Hsiao et al., 2023; Chen et al., 2024). For example, elevated androgen levels in women with polycystic ovary syndrome (PCOS) correlate with a decrease in gut microbial diversity [21](Thackray, 2019). Moreover, oral progesterone administration can reshape gut microbial composition, notably increasing *Lactobacillus reuteri* abundance [22](Sovijit et al., 2021). While humans can modulate their gut microbiota through the immune [23](Brown et al., 2013) and hormonal systems [24](Kaliannan et al., 2018), the gut microbiota in turn influences host physiology and plays critical roles in various health conditions and diseases [25](McCurry et al., 2024). For example, the *Escherichia*/*Shigella* subgroup (Enterobacteriaceae family) in the gut microbiota is associated with menstrual disorders, although the underlying mechanisms remain unclear [26](Yao et al., 2024). These reciprocal interactions between humans and their gut microbiota appear fundamental to maintaining hormonal homeostasis.

Gut microbiota play a crucial role in regulating progestogenic levels and profiles in humans [22,27](Coombes et al., 2020; Sovijit et al., 2021). While the liver primarily produces conjugated steroids that enter the gut through enterohepatic circulation, more than 90% of steroids found in human feces are unconjugated [28,29](Eriksson et al., 1970; Arp et al., 2024). During late pregnancy, substantial amounts of C_{21} steroids—predominantly pregnanediol isomers (see **Figure 1** for individual structures)—are excreted through feces, with pregnant individuals showing daily excretion levels of approximately 33 mg [12,28](Rogers and McLellan, 1951; Eriksson et al., 1970). These findings suggested microbial involvement in gut progesterone metabolism. Using mouse models, we demonstrated that *Clostridium innocuum* reduces plasma progesterone levels and metabolizes progesterone into epipregnanolone through the microbial enzyme 5β-dihydroprogesterone reductase, resulting in arrested ovarian follicular development [20](Chen et al., 2024). Furthermore, the gut bacterial species *Ruminococcus gnavus* and *Bacteroides acidifaciens* have been shown to transform pregnenolone and hydroxypregnenolone into dehydroepiandrosterone and testosterone, respectively

[30](Pernigoni, 2021).

The transformation of circulating progesterone into neurosteroids occurs in both peripheral organs and the brain [31](Sundström-Poromaa, 2020). In females, this process involves specific enzymes: 3α-hydroxysteroid dehydrogenase converts progesterone to allopregnanolone in the brain, while 3β-hydroxysteroid dehydrogenase produces isopregnanolone in the liver [32–34](Trauger, 2002; Anderson and Odegard, 2004; Liang and Rasmusson, 2018). Allopregnanolone serves crucial functions, offering protective and mood-stabilizing effects during pregnancy and the postpartum period [35–37](Bixo et al., 1997; Kanes et al., 2017; Osborne et al., 2017), as well as demonstrating well-documented analgesic properties [38,39](Fujita et al., 2018; Diviccaro et al., 2022). This led to the FDA's approval of synthetic allopregnanolone (Brexanolone) for treating postpartum depression (https://www.fda.gov/news-events/pressannouncements/fda-approves-first-treatment-post-partum-depression). While allopregnanolone was long thought to be produced exclusively by human enzymes, recent research has revealed that gut bacteria, specifically actinobacterial species from the genera *Gordonibacter* and *Eggerthella*, can produce allopregnanolone from gut glucocorticoids [25](McCurry et al., 2024). This discovery suggests that gut microbiota may influence circulating neurosteroid profiles and levels.

Neurosteroids with a pregnane skeleton, including allopregnanolone and its stereoisomers pregnanolone and epipregnanolone, exhibit distinct interactions with neuronal γ-aminobutyric acid ($GABA_A$) receptors. While allopregnanolone acts as a positive modulator of $GABA_A$ receptors, 5β-progestogens (such as epipregnanolone and pregnanolone) function as negative modulators and have been implicated in stress-related disorders and depression [40,41](Wang et al., 2002; Zorumski et al., 2013). Although allopregnanolone has been extensively characterized, other neurosteroids remain poorly understood. Investigation of bacterial contributions to host neurosteroid profiles faces two key challenges: the scarcity of relevant bacterial species in the gut microbiota (< 0.1%) [20,25](Chen et al., 2024; McCurry et al., 2024), and the intricate interplay between human and microbial enzymes in neurosteroid production. Current knowledge of the major bacterial species, their microbial products,

and physiological significance thus remains limited.

To address these knowledge gaps, we employed an integrated multi-omics approach to investigate gut microbiota-mediated progesterone metabolism. We amended progesterone (1 mM) into fecal cultures to enrich progesterone-metabolizing gut microbes, which were identified on a PacBio platform through culturomics-based metagenomics analysis [42](Li et al., 2023). We predicted microbial functions by tracking temporal changes in metabolite profiles and microbial abundances. We further explored the prevalence of progesterone metabolism genes among gut microbiota using functional genomics. Additionally, we utilized a culturomics approach [43](Lagier et al., 2018) to isolate and characterize strict anaerobes capable of neurosteroid production. This integrated multi-omics strategy enabled the identification of major microbial products and corresponding bacterial species involved in gut progesterone metabolism.

MATERIALS AND METHODS

Chemicals

Progesterone was purchased from Sigma-Aldrich (St. Louis, MO, USA). Four neurosteroids were purchased from Steraloids (Newport, RI, USA): 3α-hydroxy-5α-pregnan-20-one (allopregnanolone), 3β-hydroxy-5α-pregnan-20-one (isopregnanolone), 3α-hydroxy-5β-pregnan-20-one (pregnanolone), and 3β-hydroxy-5β-pregnan-20-one (epipregnanolone). All other chemicals were of analytical grade and were sourced from Mallinckrodt Baker (Phillipsburg, NJ, USA), Merck Millipore (Burlington, VT, USA), and Sigma-Aldrich, unless otherwise specified.

Participants, protocols of hormone treatment, and sample collection

Human fecal sample collection was performed as described in [20]Chen et al. (2024), and the procedure was approved by the local ethics committee (Clinical Trial /Research Approval NTUH-REC no.:202103046RINB). This study included infertile female patients who underwent hormonal preparation of the endometrium before frozen-thawed embryo transfer. Endometrial preparation

involved administration of exogenous estrogen and progesterone. Hormone replacement therapy began on day 3 of the menstrual cycle. Estradiol valerate was administered in increasing doses: 4 mg/day (days 3-8), 8 mg/day (days 9-11), and 12 mg/day (from day 12) until optimal endometrial thickness was achieved for embryo transfer. Two days before the scheduled embryo transfer, transvaginal progesterone gel (8% Crinone; Merck) was administered at 90 mg/day for 2 days, then increased to 180 mg/day for 14 days. Oral progesterone (Utrogestan, 600 mg/day) was initiated on the day of embryo transfer. Participants provided fecal samples one day before starting progesterone treatment. Serum samples were collected at the following time points: one day before oral progesterone treatment, 2 days after starting the progesterone treatment, 1 week after starting treatment, and if pregnancy was confirmed, 1 week after confirmation.

Identification of microbial progestogenic metabolites in fecal cultures

Fresh fecal samples were collected from infertile women aged 32–43 years (see Table S1 for detailed information) who underwent oral progesterone therapy for endometrial preparation and thawed embryo transfer. The samples (approximately 0.5 g each) were anaerobically incubated with progesterone (1 mM) in either a chemically defined medium containing mineral salts (DCB-1, 100 mL) or Brain Heart Infusion (BHI) medium (100 mL) at 37°C in darkness. Subsequently, 17α-ethinylestradiol (final concentration: 50 μM), which is not metabolized by gut microbiota, was added to the fecal cultures as an internal control. Daily samples were extracted from the progesterone-enriched fecal cultures, and progesterone-derived microbial products were isolated from these culture samples (1 mL) through dual extraction with ethyl acetate. After complete solvent evaporation, the residue was reconstituted in 50 μL of methanol, and progestogenic metabolites were identified using ultraperformance liquid chromatography–atmospheric pressure chemical ionization–high-resolution mass spectrometry (UPLC–APCI–HRMS). To determine temporal changes in bacterial community structures within the fecal cultures, bacterial cells were collected by centrifugation (10,000 × *g* for 10 min at 4°C). Bacterial DNA was extracted from the pellet using a QIAamp PowerFecal Pro DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration was

determined using either a NanoDrop ND-1000 spectrophotometer or a Qubit dsDNA Assay kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Bacterial 16S rRNA was amplified by PCR, and the resulting amplicons were sequenced using the PacBio platform (described below).

UPLC–APCI–HRMS

Progestogenic metabolites were detected and analyzed using UPLC-HRMS, consisting of a UPLC system coupled to an APCI-MS system. Metabolite separation was performed on a reversed-phase C_{18} column (ACQUITY UPLC BEH C_{18}; 1.7 μm, 100 × 2.1 mm; Waters, Milford, MA, USA) at a flow rate of 0.45 mL/min with the column oven maintained at 50°C. The mobile phase comprised two solutions: solution A (0.1% formic acid [v/v] in 2% acetonitrile [v/v]) and solution B (0.1% formic acid [v/v] in acetonitrile [v/v]). The separation was achieved using a gradient elution where solution B was increased from 40% to 55% over 9 minutes, followed by an increase to 100% within 1 minute. Mass spectrometric data were collected in positive ionization mode with a parent scan range of 100-500 *m*/*z*. The capillary and APCI vaporizer temperatures were set to 120°C and 395°C, respectively. The sheath, auxiliary, and sweep gas flow rates were set to 40, 5, and 2 arbitrary units, respectively. The source voltage was maintained at 6 kV with a current of 15 μA. Individual adduct ion compositions were determined using Xcalibur Software (Thermo Fisher Scientific).

Isolation of neurosteroids-producing gut microbes through a culturomic approach

Progesterone-metabolizing bacteria were isolated from human fecal samples using culturomics. Fecal samples (5 g) were suspended in anaerobic DCB-1 broth (100 mL) and shaken vigorously. The suspensions were serially diluted (up to 10^{-6}), and 100 μL aliquots from the 10^{-3} to 10^{-6} dilutions were spread on selective agar plates designed for various bacterial taxa. The plates were then incubated in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA). Several selective media were used for bacterial isolation: Brain Heart Infusion (BHI), Trypticase Soy Agar (TSA), Reinforced Clostridium Medium (RCM), and Gifu Anaerobic Medium (GAM; BD-Difco, Sparks, MD, USA) were used to isolate gut anaerobes, including members of the Family Clostridiales. *Clostridium butyricum* isolation medium (BIM) and iron sulfite agar (HiMedia,

Mumbai, India) were used to isolate *C. butyricum* and related species. *Bacteroides fragilis* selective medium (BFS) was used to isolate members of the phylum Bacteroidetes. Raffinose-*Bifidobacterium* (RB) Medium and BFM selective medium were used to isolate Bifidobacteria and related taxa [44] (Nebra and Blanch, 1999). All molten agars were supplemented with 2 mM sterile sodium sulfide before pour plating. De Man-Rogosa-Sharpe (MRS) medium (BD-Difco) was used to isolate lactic acid bacteria and related taxa. Progesterone (1 mM) was added to all growth media. The media were prepared using the Hungate technique, and bacterial cultures were grown on agar in an anaerobic chamber. All glassware, media, petri dishes, and reagents were maintained under anaerobic conditions. After 2-5 days of anaerobic incubation at 37°C, colonies were observed and isolated. Individual colonies were inoculated into 800 µL of GAM broth in 48-well plates and incubated at 37°C in an anaerobic chamber with agitation (100 rpm) for 3 days. Active cultures ($OD_{600nm} > 0.1$) were used as pre-cultures for subsequent high-throughput screening, as described below.

Screening and characterization of neurosteroid-producing gut microbes

Individual colonies growing on agar plates were picked using sterile toothpicks and inoculated in 1 mL GAM broth containing progesterone (1 mM) in a 48-well titer plate. The plates were placed in an anaerobic box with AnaeroPack™ (bioMerieux, France) and incubated at 37°C with shaking (100 rpm) for 5 days. For high-throughput screening of progesterone metabolism, 100 µL aliquots from each well were transferred to 96-well microtiter plates. Equal volumes (100 µL) of DNPH reagent (0.4 g 2,4-dinitrophenylhydrazine, 3 mL concentrated H_2SO_4, 35 mL absolute ethanol, and 12 mL MQ water) were added to each well. Progesterone metabolic activity was indicated by the formation of a brick-red precipitate after 30 minutes of anaerobic incubation. Colonies showing positive results were further purified and cultured in GAM broth (10 mL) containing 1 mM progesterone. Their progesterone metabolic activity was confirmed using thin-layer chromatography (TLC), and the resulting neurosteroids (major microbial products) were identified using UPLC-APCI-HRMS.

General molecular biological methods

Bacterial genomic DNA was extracted with a Presto Mini gDNA Bacteria Kit (Geneaid, New Taipei City, Taiwan). PCR amplification was performed in 25-μL reactions containing nuclease-free H_2O, Invitrogen Platinum Hot Start PCR 2X Master Mix (Thermo Fisher Scientific), 200 nM of each primer, and 10-30 ng template DNA. PCR products were verified by 1.5% TAE-agarose gel electrophoresis with SYBR Green I nucleic acid stain (Invitrogen, Thermo Fisher Scientific). The PCR products were purified using a GenepHlow Gel/PCR Kit (Geneaid).

PacBio sequencing of bacterial 16S rRNA gene amplicons

Bacterial genomic DNA was extracted using a QIAamp PowerFecal DNA Kit (Qiagen) and quantified with a Qubit 4.0 Fluorometer (Thermo Scientific). The full-length 16S rRNA gene (V1-V9 regions) was amplified using barcoded universal primers. Each primer contained a 5′-phosphorylated buffer sequence (GCATC), a 16-base barcode, and degenerate 16S-specific sequences: Forward: 5′Phos/GCATC-[16-base barcode]-AGRGTTYGATYMTGGCTCAG-3; Reverse: 5′Phos/GCATC-[16-base barcode]-RGYTACCTTGTTACGACTT-3′; (where M = A/C; R = A/G; Y = C/T). PCR amplification was performed using KAPA HiFi HotStart ReadyMix (Roche) with 2 ng template DNA. Cycling conditions were: 95°C for 3 min; 20-30 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 60 s; followed by 72°C for 5 min. PCR products were verified by 1% agarose gel electrophoresis. Samples showing distinct ~1,500 bp bands were purified using AMPure PB Beads for PacBio library preparation. Raw PacBio fastq files were deposited in NCBI SRA: DCB samples under BioProject PRJNA1088978 (accession numbers SRR30505914-SRR30505967) and BHI samples under BioProject PRJNA1196623 (accession numbers SRR31672780-SRR31672827).

PacBio read quality filtering and taxonomic classification

Raw PacBio reads were processed using QIIME2-DADA2 pipeline (version 2023.5). Sequences shorter than 1,000 bp or longer than 1,600 bp, including primers and chimeric sequences, were removed. Dereplicated sequences were used to generate amplicon sequence variants (ASVs) with single-nucleotide resolution. ASVs were classified to species level using bacterial 16S rRNA RefSeq sequences from the NCBI nucleotide database

(https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/Bacteria). Taxonomic data were retrieved using the NCBI Datasets Command Line Tool (https://github.com/ncbi/datasets). Classification thresholds were: species (≥98.7%), genus (≥94.5%), family (≥86.5%), order (≥82.0%), class (≥78.5%), and phylum (≥75.0%) (Barco et al., 2020; Chun et al., 2018; Yarza et al., 2014). *Erysipelatoclostridium* in this study is listed as [*Clostridium*] in the NCBI database. Samples were rarefied to 10,340 reads and ASV counts were converted to percentages.

Bacterial community structure analysis of progesterone-treated gut microbiota

Fecal samples from 14 female patients were anaerobically cultured in BHI or DCB-1 broth with 1 mM progesterone. Bacterial community structure differences were analyzed and visualized using NMDS. Differential abundance of bacterial genera between initial (Day 0) and final stages (Day 3 for BHI, Day 6 for DCB) was determined using DESeq2 [45](Love et al., 2014).

Correlation analysis between bacterial genera and neurosteroid abundance

SparCC networks were generated with 1,000 permutations using centered log-ratio transformed data and Spearman correlations [46](Friedman et al., 2012) in R Studio with SpiecEasi package [47](Kurtz et al., 2015). Four time points were used for both BHI (Days 0, 1, 2 and 3) and DCB (Days 0, 2, 4, and 6) samples. Bacterial genera showing at least one positive correlation with neurosteroids were visualized in a bubble correlogram. Interactions were represented by red (positive), blue (negative), or white (none) bubbles. Asterisks (*) denote genera with validated progesterone-metabolizing function through culturomics. In the DCB correlogram, black text indicates genera present in both BHI and DCB conditions, while grey text indicates genera unique to DCB.

Functional Genomic Analysis

Bacterial genomes from Veillonellaceae (n = 14) and Enterobacteriaceae (n = 22) were retrieved from the NCBI database. RefSeq bacterial genomes were used for all species except *Megasphaera vaginalis*. *Negativicoccus massiliensis* was excluded due to genome contamination and lack of RefSeq data. Genomes were analyzed by local BLAST against characterized and putative 5β-

reductase and 3α-HSD protein sequences. Reference sequences included: *Clostridium innocuum* old yellow enzyme (COG1902) encoding 5β-reductase (UniProt: N9V7B0_CLOIN) (Arp et al., 2024); *Comamonas testosteroni* putative 5β-reductase (WP_012837513.1) and characterized 3α-HSD (WP_012837515.1) [48](Feller et al., 2021); *Rhodococcus jostii* 5β-reductase (WP_011597925.1) (Feller et al., 2021); *Pseudomonas stutzeri* putative 3α-HSD (EF530217) [48](Feller et al., 2021); *Homo sapiens* 5β-reductase (NP_005980.1) and 3α-HSD (AAB41916.1). The *Salmonella enterica* protein sequence (NP_460634.1) showing highest identity to human 5β-reductase was selected as a reference. BLAST results were visualized in a heatmap showing protein sequence identity between bacterial genomes and reference sequences. Color gradient (blue-white-red) indicates sequence identity. Protein sequences with >88% coverage are shown in bold text.

Quality filtering and taxonomic classification for comparative meta-analysis

V3-V4 16S rRNA sequences from fecal samples of healthy females and those with recurrent miscarriage (<14 weeks gestation) were obtained from NCBI SRA (PRJNA700829) [49](Liu et al., 2021). Analysis was restricted to genus level due to V3-V4 region length limitations. Full-length 16S rRNA sequences were trimmed to V3-V4 regions (~440 bp) using mothur [50](Schloss et al., 2009). Sequence processing in mothur included: 1) File merging (merge.files); 2) Dereplication (unique.seqs); 3) Count table generation (count.seqs); 4) Removal of sequences <400 bp or >450 bp and chimeras; 5) MAFFT alignment (Katoh et al., 2002); 6) ASV clustering (diffs=15) using precluster. ASVs were classified against bacterial 16S rRNA RefSeq sequences from NCBI (https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/Bacteria). Taxonomic data were retrieved using NCBI Datasets Command Line Tool (https://github.com/ncbi/datasets). Classification thresholds were: genus (≥94.5%), family (≥86.5%), order (≥82.0%), class (≥78.5%), and phylum (≥75.0%) [51–53](Barco et al., 2020; Chun et al., 2018; Yarza et al., 2014). Samples were rarefied to 11,835 reads and bacterial abundances were converted to percentages.

Comparative meta-analysis

NMDS was used to analyzed and visualized bacterial community structures in fecal samples from

healthy Shanghai women, women with miscarriage [49](Liu et al., 2021), and progesterone-treated women. DESeq2 determined differential abundance of bacterial genera and families between healthy controls versus miscarriage and progesterone-treated groups [45](Love et al., 2014).

Statistical analyses

All experiments were performed at least in triplicate. Bacterial community structure changes were assessed by PERMANOVA (adonis) using R package vegan. Differential abundance analysis used DESeq2 with Benjamini-Hochberg adjusted p-values to reduce false discovery rate [45](Love et al., 2014).

RESULTS

Characterization of key metabolites produced by gut microbiota during progesterone metabolism

Gut bacteria can metabolize progesterone in two ways: as a carbon source [54](Kornman and Loesche, 1982) or as a terminal electron acceptor [20](Chen et al. 2024). To investigate microbial progesterone metabolism, we collected fresh fecal samples from female patients with infertility who had received oral progesterone (**Table S1**). We incubated these samples under two conditions: anaerobically in Brain Heart Infusion (BHI, an electron-rich growth medium) and DCB-1 (a chemically defined mineral medium) [55](He et al., 2003), with 1 mM progesterone serving as either a terminal electron acceptor or major carbon source, respectively. Using ultraperformance liquid chromatography (UPLC)–atmosphere pressure chemical ionization (APCI)–high-resolution mass spectrometry (HRMS), we analyzed residual progesterone and its metabolites (**Figure 2**). In the electron-rich BHI medium, gut microbiota metabolized over 90% of progesterone within 3 days, leaving only 0.1 mM unchanged. This bioprocess generated several neurosteroids: allopregnanolone (0.05 mM), epipregnanolone (0.06 mM), isopregnanolone (0.07 mM), and pregnanolone (0.65 mM) (**Figure 2A**; left panel). In contrast, when progesterone was amended in the electron-poor DCB-1

medium, approximately 0.7 mM (approximately 30%) remained unchanged after 6 days, with pregnanolone emerging as the primary metabolic product (**Figure 2A**; right panel).

Patient-specific analysis revealed distinct patterns of neurosteroid production (**Figure 2B**). Most fecal samples showed higher neurosteroid production in BHI compared to DCB-1 conditions. Individual variations were notable: Patient 11's microbiota produced only pregnanolone; Patient 1's microbiota generated epipregnanolone, isopregnanolone, and pregnanolone but not allopregnanolone; and Patients 10 and 14's microbiota primarily produced allopregnanolone and pregnanolone. Despite these variations, pregnanolone consistently emerged as the dominant metabolite across all samples. These findings demonstrate that gut microbiota preferentially use progesterone as a terminal electron acceptor rather than a carbon source, primarily converting it to pregnanolone—a pattern consistently observed across our cohort of female patients with infertility.

Progesterone-induced changes in gut microbial composition

To identify the major bacterial species involved in anaerobic progesterone metabolism, we analyzed bacterial community structures in progesterone-amended fecal cultures through DNA extraction, 16S rRNA gene amplification, and PacBio sequencing. Non-metric multidimensional scaling (NMDS) analysis based on Bray-Curtis dissimilarity revealed distinct temporal changes in the progesterone-treated gut microbiota. In the electron-rich BHI broth, initial samples (Day 0) clustered at the top right of the coordinate, while samples from the final incubation stage (Day 3) clustered at the bottom (**Figure 3A**, left panel). Permutational multivariate analysis of variance (PERMANOVA) confirmed significant differences (global $R^2 = 0.34$; $P = 0.001$) between original and progesterone-enriched gut microbiota (**Figure 3A**, left panel). The initial bacterial community comprised Bacillota (57.9%), Bacteroidota (27.0%), Actinomycetota (11.9%), and Pseudomonadota (2.2%). After 3 days of anaerobic incubation with progesterone, we observed significant increases in Bacillota (synonym Firmicutes; 69.1%; $p < 0.05$) and Pseudomonadota (synonym Proteobacteria; 11.3%; $p < 0.01$) (**Figure 3B**).

In DCB-1 broth with progesterone (1 mM) as the primary carbon source, initial samples (Day 0)

clustered at the top left coordinate, while final samples (Day 6) clustered on the right (**Figure 3A**, right panel). PERMANOVA showed a modest but significant difference (global $R^2 = 0.11$; $P = 0.029$) between original and progesterone-enriched communities (**Figure 3A**, right panel). After 6 days of anaerobic incubation in this electron-poor medium, three bacterial phyla showed significant increases: Actinomycetota (synonym Actinobacteria; $p < 0.05$), Thermodesulfobacteriota (synonym Thermodesulfobacteria; $p < 0.01$), and Synergistota (synonym Synergistetes; $p < 0.05$) (**Figure 3B**).

Progesterone treatment enriches specific bacterial genera in the gut microbiome

PacBio sequencing of bacterial 16S rRNA genes yielded DNA fragments averaging 1.5 kb in length, enabling species-level identification of gut microbes. NMDS analysis showed distinct clustering of gut microbiota between initial and final incubation stages across both growth media (SI **Figure S1**). PERMANOVA confirmed significant differences between original and progesterone-enriched gut microbiota from 14 female patients (BHI: global $R^2 = 0.14$, $P = 0.001$; DCB-1: global $R^2 = 0.11$, $P = 0.002$) (SI **Figure S1**). In BHI cultures, 25 bacterial genera showed significant enrichment ($p < 0.05$), predominantly from Bacillota (84%, 21/25) and Pseudomonadota (12%, 3/25) (**Figure 4**, top panel). The most enriched genera were *Anaerotignum* (ranking: 1) and *Bittarella* (ranking: 2) (Bacillota), *Shigella* (ranking: 3) and *Escherichia* (ranking: 5) (Pseudomonadota), and *Fusobacterium* (ranking: 4) (Fusobacteriota). In DCB-1 mineral cultures, significantly enriched genera showed a different pattern, though still dominated by Bacillota (79%, 15/19). The top enriched genera in DCB-1 were *Cloacibacillus* (ranking: 1) and *Pyramidobacter* (ranking: 3) (Synergistota), *Bilophila* (ranking: 2) (Pseudomodota), and *Anaerotruncus* (ranking: 4) and *Christensenella* (ranking: 5) (Bacillota) (**Figure 4**, bottom panel).

Mapping key bacterial species that synthesize specific neurosteroids

Using Sparse Correlations for Compositional data (SparCC), we identified the major bacterial species involved in neurosteroid production. Under BHI growth conditions, bacteria from the phylum Bacillota dominated progesterone metabolism, accounting for approximately 79% (71/90) of the responsible genera (**Figure 5**). Within this phylum, *Anaerotignum* and *Bittarella* showed a balanced

conversion of progesterone into all four neurosteroids. In contrast, genera including *Butyricicoccus*, *Dorea*, *Intestinimonas*, *Lentihominibacter*, *Oscillibacter*, and *Vescimonas* primarily produced 5α-progestogens (allopregnanolone and isopregnanolone). Actinobacterial genera such as *Bifidobacterium* and *Collinsella* also contributed to 5α-progestogen production. Members of the Veillonellaceae family (*Allisonella*, *Anaeroglobus*, *Dialister*, *Megasphaera*, and *Veillonella*) specifically produced 5β-progestogens (epipregnanolone and pregnanolone). Similarly, proteobacterial genera from the Enterobacteriaceae family, particularly *Escherichia* and *Shigella*, mainly produced 5β-progestogens, especially pregnanolone. Most of these bacterial genera were also identified in DCB-1 cultures (**Figure 6**). Notably, under electron-poor conditions, *Bilophila* (Phylum Verrucomicrobiota), *Cloacibacillus* (Phylum Thermodesulfobacteriota), and *Oscillibacter* (Phylum Bacillota) emerged as the primary genera responsible for pregnanolone production.

Culturomics analysis confirms the role of specific gut microbes in progesterone metabolism

To functionally validate gut microbes capable of anaerobic progesterone metabolism, we employed a culturomics approach. While microbial steroid metabolism has been well-studied in actinobacteria and proteobacteria [56–58](Chiang et al., 2020; Hsiao et al., 2020, 2021), we focused on isolating strictly anaerobic gut microbial species. We cultured fresh fecal samples from 14 female patients in two types of media, all supplemented with sodium sulfide (2 mM) as a strong reducing agent: (1) rich media containing progesterone as the terminal electron acceptor, including brain heart infusion (BHI), Gifu anaerobic medium (GAM), Trypticase Soy Broth (TSB), Reinforced *Clostridium* medium (RCM), *Bacteroides fragilis* selective medium (BFS), Raffinose-Bifidobacterium selective medium (RB), and *C. butyricum* isolation medium (BIM); and (2) chemically defined mineral media with progesterone as the sole carbon source (denitrifying, Fe^{3+}-reducing, and sulfate-reducing media; Shih et al., 2017)[59]. We successfully isolated 92 strains capable of anaerobic progesterone metabolism, representing species from Bacillota (8 genera), Bacteroidota (3 genera), and Pseudomonadota (1 genera). When cultured in BHI medium with progesterone (1 mM), most firmicutes isolates produced epipregnanolone as their primary product

(**Table 1**). Some firmicutes species, such as *Enterococcus gallinarum* and *Hungatella effluvi*, predominantly produced pregnanolone, whereas others, like *Erysipelatoclostridium innocuum* and *E. ramosum*, produced pregnanolone as a minor product. Additionally, another firmicutes species, *Holdemania filiformis*, primarily produced isopregnanolone. Notably, we did not isolate any bacterial species capable of converting progesterone to allopregnanolone. Our SparCC analysis predicted that 6 of the 13 characterized genera (54.5%) - *Anaerostipes*, *Blautia*, *Enterococcus*, *Erysipelatoclostridium*, *Hungatella*, and *Parabacteroides* - were involved in neurosteroid production (**Figure 5**). None of the 92 bacterial strains grew with progesterone in mineral media under denitrifying, iron-reducing, or sulfate-reducing conditions, indicating that these gut bacteria utilize progesterone as a terminal electron acceptor rather than a carbon source for anaerobic respiration.

Progesterone-Metabolizing Genes Across Gut Bacterial Genomes

Metabolomic analysis of the progesterone-amended fecal cultures identified pregnanolone as the predominant microbial metabolite of progesterone. SparCC analysis (**Figures 5** and **6**) indicated that members of two bacterial families - Enterobacteriaceae (particularly *Escherichia* and *Shigella*) and Veillonellaceae (including *Allisonella*, *Anaeroglobus*, *Dialister*, *Megasphaera*, and *Veillonella*) - are primarily responsible for pregnanolone production. We identified the genes encoding two enzymes involved in the biotransformation of progesterone to pregnanolone: 5β-steroid oxidoreductase [60](Chen and Penning, 2014) and 3α-hydroxysteroid oxidoreductase [61,62](Maser et al., 2000, 2001). Both enzymes catalyze reversible reactions [56](Chiang et al., 2020; **Figure 7A**). These oxidoreductase genes have been previously identified across diverse organisms, including humans [60](Chen and Penning, 2014), firmicutes [29](Arp et al., 2024), actinobacteria [63](Bergstrand et al., 2016), and proteobacteria (mainly betaproteobacteria and gammaproteobacterial) [48,56,64](Holert et al., 2018; Chiang et al., 2020; Feller et al., 2021). Using these known progesterone metabolism genes as queries, we investigated their distribution in bacterial genomes from Enterobacteriaceae and Veillonellaceae (**Figure 7B**). The 5β-steroid oxidoreductase genes are widely distributed across these genomes and show the highest sequence similarity to

gammaproteobacterial enzymes (up to 100% identity) and moderate similarity to animal enzymes (up to 40%). These proteobacterial and animal enzymes belong to the aldo-keto reductase superfamily [60](Chen and Penning, 2014), whereas the actinobacterial 5β-steroid oxidoreductases are members of the old yellow enzyme superfamily [63](Bergstrand et al., 2016). For 3α-hydroxysteroid oxidoreductase, characterized variants include the aldo-keto reductase superfamily in animals [65](Degtiar and Kushlinsky, 2001) and the short-chain dehydrogenase/reductase family in betaproteobacteria [62](Maser et al., 2001). We identified homologs of both variants in Enterobacteriaceae and Veillonellaceae members (**Figure 7B**). The Enterobacteriaceae enzymes show greater similarity to betaproteobacterial 3α-hydroxysteroid oxidoreductases (up to 38% identity), while the Veillonellaceae enzymes are more similar to human variants (up to 36% identity).

Gut microbiota profiles in healthy women and women with miscarriage

Analysis of progesterone-metabolizing gut microbiota, particularly Enterobacteriaceae and Veillonellaceae, was conducted using a previously established case-control cohort of women with unexplained miscarriages versus those with normal pregnancies [49](Liu et al., 2021). The cohort pairs were matched after excluding confounding factors including abnormal karyotypes, age, BMI, lifestyle, education, autoimmune conditions, infections, and gynecological disorders [49](Liu et al., 2021). All subjects were East Asian women aged< 40 years. Analysis of gut bacterial community structures using NMDS and PERMANOVA revealed significant differences (P< 0.05) among the two groups at the Family level (global R^2 = 0.022) (**Figure 8A**). We observed that healthy women had a significantly higher abundance (P< 0.05) of Enterobacteriaceae (mean 5.0%) (**Figures 8B** and **S2**). At the genus level, *Escherichia* was predominant, comprising over 90% of this family across the two groups (**Figures 8B** and **S2**). Conversely, Veillonellaceae showed an opposite pattern, with a significantly lower abundance (P< 0.05) in healthy women (approximately 0.4%) (**Figures 8B** and **S2**). This pattern extended to the genus level, where women with miscarriage showed significantly higher abundances of *Dialister*, *Megasphaera*, and *Veillonella* (P< 0.05) compared to healthy women (**Figure 8B**). We also observed significantly higher abundances of Veillonellaceae family

members, particularly *Megasphaera* and *Veillonella* genera, in the gut microbiota of women treated with progesterone (**Figure S2**).

DISCUSSION

While microbial steroid metabolism has been well-studied, research has primarily focused on steroid catabolism (biodegradation), in which microorganisms break down steroids for carbon and energy [56](Chiang et al., 2020). Notable examples include *Mycobacterium tuberculosis* (phylum Actinomycetota), which requires host cholesterol for survival [66](Pandey and Sassetti, 2008), and *Thauera* spp. (phylum Pseudomodota), which degrades gut testosterone in male mice [19](Hsiao et al., 2023). However, our current data reveal that human gut microbiota show minimal progesterone degradation. Instead, we observed substantial conversion of progesterone to reduced progestogens (neurosteroids), particularly in electron-rich media like BHI.

Our study employed a multi-omics approach to explore the diversity and physiological significance of gut microbes that metabolize progesterone. Through MS-based metabolomic analysis, we discovered that human gut microbiota convert progesterone into at least four neurosteroids: two 5α-progestogens (allopregnanolone and isopregnanolone) and two 5β-progestogens (epipregnanolone and pregnanolone). Among them, isopregnanolone has shown promise in clinical trials for treating Tourette's syndrome (https://clinicaltrials.gov/ct2/show/NCT05434546). Using SparCC analysis, we identified multiple species within the Bacillota phylum—including *Anaerostipes*, *Butyricicoccus*, *Doera*, *Holdemania*, *Intestinimonas*, and *Oscillibacter*—that likely contribute to isopregnanolone production. We confirmed this computational prediction through isolation and characterization of several gut microbes capable of producing isopregnanolone, notably *Holdemania filiformis* (phylum Bacillota), which we found produces isopregnanolone as its sole metabolic product from progesterone. The SparCC analysis suggested the important role of Bacillota (e.g., *Butyricicoccus*, *Intestinimonas*, and *Oscillibacter*) and Actinomycetota (e.g., *Bifidobacterium* and *Collinsella*) in

converting progesterone to allopregnanolone, another 5α-progestogen. This bioprocess requires two enzymatic steps: Δ4(5α)- and 3α-hydroxyl reduction reactions, mediated by 5α-steroid reductase and 3α-hydroxysteroid reductase, respectively [20,56](Chiang et al., 2020; Chen et al., 2024). However, we could not isolate individual gut microbes capable of allopregnanolone production from the human fecal samples. The transformation may be performed by yet-uncultivated gut microbes possessing both enzymes, or alternatively, through synergistic metabolism—a common phenomenon in anaerobic environments where multiple microbes cooperatively transform complex organic compounds [67](Wei et al., 2021).

Our metabolomics analysis identified pregnanolone as the primary microbial metabolite of progesterone. SparCC analysis revealed that gut microbes from Enterobacteriaceae (gamma-proteobacteria) and Veillonellaceae (firmicutes) play crucial roles in pregnanolone production. Insufficient progesterone levels are a major causal factor in menstrual disorders [20](Chen et al., 2024). The reported association between menstrual disorders and gut microbes from the *Escherichia*/*Shigella* subgroup (Enterobacteriaceae family) [26](Yao et al., 2024) may be partially explained by these bacteria's unique ability to metabolize progesterone, resulting in decreased circulating progesterone levels. Functional genomic analysis demonstrated that steroidal A-ring oxidoreductases are prevalent among Enterobacteriaceae (including *Escherichia*, *Salmonella*, and *Shigella*) and Veillonellaceae (including *Allisonella*, *Dialister*, *Megasphaera*, and *Veillonella*). The sequence homology of progesterone metabolism enzymes between humans and these bacterial species indicates extensive host-microbe interactions. However, the evolutionary origin of these microbial genes requires further investigation at the molecular level. Notably, genes involved in ring-cleavage of steroidal substrates—such as *kshAB* and *hsaAB* for aerobic degradation [63](Bergstrand et al., 2016) and *atcABC* for anaerobic pathways [68](Yang et al., 2016)—are scarce in the genomes from the Enterobacteriaceae and Veillonellaceae families. Together, these results indicate that gut microbiota preferentially use progesterone as a terminal electron acceptor rather than a carbon source.

Although both Enterobacteriaceae and Veillonellaceae require progesterone for growth, their competitive dynamics in the human colon are influenced by oxygen availability and redox conditions. In our study, we added sodium sulfide, a strong reducing agent, to the growth media to create highly reducing conditions (redox potential < -200 mV). These conditions preferentially support strictly anaerobic bacteria (e.g., Bacillota and Bacteroidota) while limiting the growth of facultative anaerobes like Proteobacteria (e.g., *Escherichia*). The human gut naturally maintains similar reducing conditions (-200 to -300 mV) [69](Panigrahi et al., 2022), creating an environment where strict anaerobes like Veillonellaceae can thrive. Under these conditions, Veillonellaceae likely outcompete facultative anaerobic Enterobacteriaceae, particularly in the colon. This competitive advantage may explain the significant increase in Veillonellaceae abundance observed in the gut microbiota of women with progesterone treatment.

This study reveals the relationship between gut microbiota and host progesterone metabolism and bioavailability. Using genomic, metabolomic, and culturomic approaches, we identified bacterial genera, functional genes, and metabolites involved in bacterial progesterone metabolism. We found that members of the Veillonellaceae family convert progesterone to pregnanolone, which shows minimal binding affinity for the progesterone receptor [70](Khan et al., 2024). Our reanalysis of a cohort study [49](Liu et al., 2021) using the NCBI database demonstrated significant enrichment of Veillonellaceae genera—*Dialister*, *Megasphaera*, and *Veillonella*—in women who experienced miscarriage. Given that adequate progesterone levels are crucial for pregnancy maintenance and embryo implantation, the bacterial taxa and genes identified in this study may serve as biomarkers for monitoring progesterone bioavailability and optimizing supplementation during pregnancy and in vitro fertilization

Conclusions

The interplay between gut microbiota and immune function is well established [49,71](Liu et

al., 2021; Hall and Klein, 2017). However, the role of gut microbes in progesterone metabolism and their impacts on immunity and pregnancy outcomes requires further investigation. Gut microbiota influence pregnancy outcomes through two mechanisms: direct effects on progesterone metabolism and independent activation of maternal immune responses. Our study revealed distinct differences in progesterone-metabolizing gut microbiota between women with unexplained miscarriages and those with normal pregnancies. These findings suggest that dysbiosis leading to increased progesterone-metabolizing bacteria may contribute to miscarriage through multiple mechanisms: reduced progesterone levels, compromised endometrial receptivity, increased uterine contractility, and disrupted immune tolerance [72](Saccone et al., 2017). This is particularly significant given that progesterone's systemic immunosuppressive effects, including inhibition of macrophage and dendritic cell activation [73–75](Zwahlen and Stute, 2024; Polikarpova et al., 2019; Xu et al., 2011), are essential for maintaining pregnancy by preventing excessive immune responses that could trigger miscarriage [76](Tan et al., 2015). The dual role of progesterone and its metabolites in pregnancy maintenance and maternal mental health underscores the importance of understanding these microbial interactions for addressing both pregnancy loss and postpartum mood disorders.

Microbial progesterone metabolism affects both pregnancy outcomes and produces neuroactive metabolites that influence maternal physiology during and after pregnancy. Allopregnanolone, a FDA-approved neurosteroid for treating postpartum depression, has limited clinical use due to high production costs. Our findings suggest an alternative therapeutic strategy through in situ microbial synthesis of allopregnanolone in the human gut. The high stereospecificity of microbial steroid-transforming enzymes may reduce unwanted stereoisomer formation compared to traditional synthetic methods. Isolation and characterization of these gut microbes and their corresponding enzymes present opportunities for therapeutic development and biotechnology applications.

Acknowledgements

We thank the Small Molecule Metabolomics core facility sponsored by the Institute of Plant and Microbial Biology, Academia Sinica (Taiwan), for its UPLC–HRMS analysis (AS-CFII-111-218).

Author contributions

M.-J.C., T.-H.H., and Y.-R.C. designed the research; G.-J.B.-M., R.G.G., H.-Y.W., C.-Y.L., T.-Y.W., C.-H.C., Y.-A.T., Y.-Y.C., C.-Y.L, Y.-L.L., P.-T.L., and Y.-L.T. conducted the research; M.-J.C., and C.-Y.L. contributed new reagents and analytic tools; G.-J.B.-M., M.-J.C., T.-H.H., and Y.-R.C. analyzed the data; and G.-J.B.-M., M.-J.C., T.-H.H., and Y.-R.C. composed the manuscript.

Declaration of competing interests

The authors declare that they have no competing interests related to this work.

Data availability

Raw PacBio fastq files were deposited in NCBI SRA: DCB samples under BioProject PRJNA1088978 (accession numbers SRR30505914-SRR30505967) and BHI samples under BioProject PRJNA1196623 (accession numbers SRR31672780-SRR31672827).

Ethics approval

Human fecal sample collection was approved by the Clinical Trial /Research Approval NTUH-REC No.:202103046RINB. This animal study conducted in accordance with the requirements of the Taiwan Animal Protection Law on Scientific Application of Animals and the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University College of Medicine (IACUC No. 20220423).

Funding

This study was supported by the Ministry of Science and Technology of Taiwan (112-2314-B-001-014-, 110-2311-B-001-033-MY3, 109-2314-B-002-125-MY3, 110-2811-B-002-562, 110-2222-E-008-002, and 112-2314-B-002-306), Academia Sinica Career Development Award (AS-CDA-110-L13), and Start-up subsidy of National Tsing Hua University (113Q2417E1).

References

1. Goletiani NV, Keith DR, Gorsky SJ. Progesterone: review of safety for clinical studies. Exp Clin Psychopharmacol. 2007;15:427–44.

2. Kolatorova L, Vitku J, Suchopar J, Hill M, Parizek A. Progesterone: A steroid with wide range of effects in physiology as well as human medicine. Int J Mol Sci. 2022;23:7989.

3. Sykes L, Bennett PR. Efficacy of progesterone for prevention of preterm birth. Best Pract Res Clin Obstet Gynaecol. 2018;52:126–36.

4. Marquardt RM, Tran DN, Lessey BA, Rahman MS, Jeong J-W. Epigenetic dysregulation in endometriosis: Implications for pathophysiology and therapeutics. Endocr Rev. 2023;44:1074–95.

5. Melo P, Chung Y, Pickering O, Price MJ, Fishel S, Khairy M, et al. Serum luteal phase progesterone in women undergoing frozen embryo transfer in assisted conception: a systematic review and meta-analysis. Fertil Steril. 2021;116:1534–56.

6. Coomarasamy A, Devall AJ, Brosens JJ, Quenby S, Stephenson MD, Sierra S, et al. Micronized vaginal progesterone to prevent miscarriage: a critical evaluation of randomized evidence. Am J Obstet Gynecol. 2020;223:167–76.

7. Toffoletto S, Lanzenberger R, Gingnell M, Sundström-Poromaa I, Comasco E. Emotional and cognitive functional imaging of estrogen and progesterone effects in the female human brain: a systematic review. Psychoneuroendocrinology. 2014;50:28–52.

8. Doldi N, Gessi A, Destefani A, Calzi F, Ferrari A. Polycystic ovary syndrome: anomalies in progesterone production. Hum Reprod. 1998;13:290–3.

9. Unfer V, Casini ML, Marelli G, Costabile L, Gerli S, Di Renzo GC. Different routes of progesterone administration and polycystic ovary syndrome: a review of the literature. Gynecol Endocrinol. 2005;21:119–27.

10. Piltonen TT. Polycystic ovary syndrome: Endometrial markers. Best Pract Res Clin Obstet Gynaecol. 2016;37:66–79.

11. Ciampaglia W, Cognigni GE. Clinical use of progesterone in infertility and assisted reproduction. Acta Obstet Gynecol Scand. 2015;94 Suppl 161:17–27.

12. Rogers J, McLELLAN F. Isolation of pregnanediol from human bile after oral administration of progesterone. J Clin Endocrinol Metab. 1951;11:246–50.

13. Lu K, Lee MH, Patel SB. Dietary cholesterol absorption; more than just bile. Trends Endocrinol Metab. 2001;12:314–20.

14. Nuriel-Ohayon M, Neuman H, Ziv O, Belogolovski A, Barsheshet Y, Bloch N, et al. Progesterone increases *Bifidobacterium* relative abundance during late pregnancy. Cell Rep. 2019;27:730–6.e3.

15. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464:59–65.

16. Rajilić-Stojanović M, de Vos WM. The first 1000 cultured species of the human gastrointestinal microbiota. FEMS Microbiol Rev. 2014;38:996–1047.

17. Costea PI, Hildebrand F, Arumugam M, Bäckhed F, Blaser MJ, Bushman FD, et al. Enterotypes in the landscape of gut microbial community composition. Nat Microbiol. 2018;3:8–16.

18. Bordenstein SR, Theis KR. Host biology in light of the microbiome: Ten principles of holobionts and hologenomes. PLoS Biol. 2015;13:e1002226.

19. Hsiao T-H, Chou C-H, Chen Y-L, Wang P-H, Brandon-Mong G-J, Lee T-H, et al. Circulating androgen regulation by androgen-catabolizing gut bacteria in male mouse gut. Gut Microbes.

2023;15:2183685.

20. Chen M-J, Chou C-H, Hsiao T-H, Wu T-Y, Li C-Y, Chen Y-L, et al. *Clostridium innocuum*, an opportunistic gut pathogen, inactivates host gut progesterone and arrests ovarian follicular development. Gut Microbes. 2024;16:2424911.

21. Thackray VG. Sex, microbes, and polycystic ovary syndrome. Trends Endocrinol Metab. 2019;30:54–65.

22. Sovijit WN, Sovijit WE, Pu S, Usuda K, Inoue R, Watanabe G, et al. Ovarian progesterone suppresses depression and anxiety-like behaviors by increasing the *Lactobacillus* population of gut microbiota in ovariectomized mice. Neurosci Res. 2021;168:76–82.

23. Brown EM, Sadarangani M, Finlay BB. The role of the immune system in governing host-microbe interactions in the intestine. Nat Immunol. 2013;14:660–7.

24. Kaliannan K, Robertson RC, Murphy K, Stanton C, Kang C, Wang B, et al. Estrogen-mediated gut microbiome alterations influence sexual dimorphism in metabolic syndrome in mice. Microbiome. 2018;6:205.

25. McCurry MD, D'Agostino GD, Walsh JT, Bisanz JE, Zalosnik I, Dong X, et al. Gut bacteria convert glucocorticoids into progestins in the presence of hydrogen gas. Cell. 2024;187:2952–68.e13.

26. Yao L, Cooper A, Lau CH-F, Wong A, Blais BW, Carrillo CD. Strain-specific recovery of S. sonnei from artificially contaminated baby carrots: Enhancing food-safety investigations with a customized *Shigella* detection method based on genomically predicted antibiotic resistance traits. J Food Prot. 2024;87:100300.

27. Coombes Z, Yadav V, McCoubrey LE, Freire C, Basit AW, Conlan RS, et al. Progestogens are metabolized by the gut Microbiota: Implications for colonic drug delivery. Pharmaceutics. 2020;12:760.

28. Eriksson H, Gustafsson JA, Sjövall J. Excretion of steroid hormones in adults. C19 and C21 steroids in faeces from pregnant women: C_{19} and C_{21} steroids in Faeces from pregnant women. Eur J

Biochem. 1970;12:520–6.

29. Arp G, Jiang A, Dufault-Thompson K, Levy S, Zhong A, Wassan JT, et al. Gut bacteria encode reductases that biotransform steroid hormones [preprint]. bioRxiv. 2024;10.04.616736. Available from: https://doi.org/10.1101/2024.10.04.616736

30. Pernigoni N, Zagato E, Calcinotto A, Troiani M, Mestre RP, Calì B, et al. Commensal bacteria promote endocrine resistance in prostate cancer through androgen biosynthesis. Science. 2021;374:216–24.

31. Sundström-Poromaa I, Comasco E, Sumner R, Luders E. Progesterone - friend or foe? Front Neuroendocrinol. 2020;59:100856.

32. Trauger JW, Jiang A, Stearns BA, LoGrasso PV. Kinetics of allopregnanolone formation catalyzed by human 3 alpha-hydroxysteroid dehydrogenase type III (AKR1C2). Biochemistry. 2002;41:13451–9.

33. Anderson GD, Odegard PS. Pharmacokinetics of estrogen and progesterone in chronic kidney disease. Adv Chronic Kidney Dis. 2004;11:357–60.

34. Liang JJ, Rasmusson AM. Overview of the molecular steps in steroidogenesis of the GABAergic neurosteroids allopregnanolone and pregnanolone. Chronic Stress (Thousand Oaks). 2018;2:2470547018818555.

35. Bixo M, Andersson A, Winblad B, Purdy RH, Bäckström T. Progesterone, 5alpha-pregnane-3,20-dione and 3alpha-hydroxy-5alpha-pregnane-20-one in specific regions of the human female brain in different endocrine states. Brain Res. 1997;764:173–8.

36. Kanes S, Colquhoun H, Gunduz-Bruce H, Raines S, Arnold R, Schacterle A, et al. Brexanolone (SAGE-547 injection) in post-partum depression: a randomised controlled trial. The Lancet. 2017;390:480–9.

37. Osborne LM, Gispen F, Sanyal A, Yenokyan G, Meilman S, Payne JL. Lower allopregnanolone during pregnancy predicts postpartum depression: An exploratory study. Psychoneuroendocrinology. 2017;79:116–21.

38. Fujita M, Fukuda T, Sato Y, Takasusuki T, Tanaka M. Allopregnanolone suppresses mechanical allodynia and internalization of neurokinin-1 receptors at the spinal dorsal horn in a rat postoperative pain model. Korean J Pain. 2018;31:10–5.

39. Diviccaro S, Cioffi L, Falvo E, Giatti S, Melcangi RC. Allopregnanolone: An overview on its synthesis and effects. J Neuroendocrinol. 2022;34:e12996.

40. Wang M, He Y, Eisenman LN, Fields C, Zeng C-M, Mathews J, et al. 3beta -hydroxypregnane steroids are pregnenolone sulfate-like GABA(A) receptor antagonists. J Neurosci. 2002;22:3366–75.

41. Zorumski CF, Paul SM, Izumi Y, Covey DF, Mennerick S. Neurosteroids, stress and depression: potential therapeutic opportunities. Neurosci Biobehav Rev. 2013;37:109–22.

42. Li D, Sun T, Tong Y, Le J, Yao Q, Tao J, et al. Gut-microbiome-expressed 3β-hydroxysteroid dehydrogenase degrades estradiol and is linked to depression in premenopausal females. Cell Metab. 2023;35:685–94.e5.

43. Lagier J-C, Dubourg G, Million M, Cadoret F, Bilen M, Fenollar F, et al. Culturing the human microbiota and culturomics. Nat Rev Microbiol. 2018;16:540–50.

44. Nebra Y, Blanch AR. A new selective medium for Bifidobacterium spp. Appl Environ Microbiol. 1999; 65:5173-5176.

45. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15:550.

46. Friedman J, Alm EJ. Inferring correlation networks from genomic survey data. PLoS Comput Biol. 2012;8:e1002687.

47. Kurtz ZD, Müller CL, Miraldi ER, Littman DR, Blaser MJ, Bonneau RA. Sparse and compositionally robust inference of microbial ecological networks. PLoS Comput Biol. 2015;11:e1004226.

48. Feller FM, Holert J, Yücel O, Philipp B. Degradation of bile acids by soil and water bacteria. Microorganisms. 2021;9:1759.

49. Liu Y, Chen H, Feng L, Zhang J. Interactions between gut microbiota and metabolites modulate

cytokine network imbalances in women with unexplained miscarriage. NPJ Biofilms Microbiomes. 2021;7:24.

50. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009;75:7537–41.

51. Barco RA, Garrity GM, Scott JJ, Amend JP, Nealson KH, Emerson D. A genus definition for Bacteria and Archaea based on a standard genome relatedness index. MBio. 2020;11(1):e02475-19.

52. Chun J, Oren A, Ventosa A, Christensen H, Arahal DR, da Costa MS, et al. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. Int J Syst Evol Microbiol. 2018;68:461–6.

53. Yarza P, Yilmaz P, Pruesse E, Glöckner FO, Ludwig W, Schleifer K-H, et al. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nat Rev Microbiol. 2014;12:635–45.

54. Kornman KS, Loesche WJ. Effects of estradiol and progesterone on *Bacteroides melaninogenicus* and *Bacteroides gingivalis*. Infect Immun. 1982;35:256–63.

55. He J, Ritalahti KM, Yang K-L, Koenigsberg SS, Löffler FE. Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. Nature. 2003;424:62–5.

56. Chiang Y-R, Wei ST-S, Wang P-H, Wu P-H, Yu C-P. Microbial degradation of steroid sex hormones: implications for environmental and ecological studies. Microb Biotechnol. 2020;13:926–49.

57. Hsiao T-H, Chen Y-L, Meng M, Chuang M-R, Horinouchi M, Hayashi T, et al. Mechanistic and phylogenetic insights into actinobacteria-mediated oestrogen biodegradation in urban estuarine sediments. Microb Biotechnol. 2021;14:1212–27.

58. Hsiao T-H, Lee T-H, Chuang M-R, Wang P-H, Meng M, Horinouchi M, et al. Identification of essential β-oxidation genes and corresponding metabolites for oestrogen degradation by actinobacteria. Microb Biotechnol. 2022;15:949–66.

59. Shih C-J, Chen Y-L, Wang C-H, Wei ST-S, Lin I-T, Ismail WA, et al. Biochemical mechanisms and microorganisms involved in anaerobic testosterone metabolism in estuarine sediments. Front Microbiol. 2017;8:1520.

60. Chen M, Penning TM. 5β-Reduced steroids and human Δ(4)-3-ketosteroid 5β-reductase (AKR1D1). Steroids. 2014;83:17–26.

61. Maser E, Möbus E, Xiong G. Functional expression, purification, and characterization of 3alpha-hydroxysteroid dehydrogenase/carbonyl reductase from *Comamonas testosteroni*. Biochem Biophys Res Commun. 2000;272:622–8.

62. Maser E, Xiong G, Grimm C, Ficner R, Reuter K. 3alpha-Hydroxysteroid dehydrogenase/carbonyl reductase from *Comamonas testosteroni*: biological significance, three-dimensional structure and gene regulation. Chem Biol Interact. 2001;130-132:707–22.

63. Bergstrand LH, Cardenas E, Holert J, Van Hamme JD, Mohn WW. Delineation of steroid-degrading microorganisms through comparative genomic analysis. MBio. 2016;7:e00166.

64. Holert J, Cardenas E, Bergstrand LH, Zaikova E, Hahn AS, Hallam SJ, et al. Metagenomes reveal global distribution of bacterial steroid catabolism in natural, engineered, and host environments. MBio. 2018;9(1):e02345-17.

65. Degtiar WG, Kushlinsky NE. 3alpha-Hydroxysteroid dehydrogenase in animal and human tissues. Biochemistry (Mosc). 2001;66:256–66.

66. Pandey AK, Sassetti CM. *Mycobacterial* persistence requires the utilization of host cholesterol. Proc Natl Acad Sci U S A. 2008;105:4376–80.

67. Wei ST-S, Chen Y-L, Wu Y-W, Wu T-Y, Lai Y-L, Wang P-H, et al. Integrated multi-omics investigations reveal the key role of synergistic microbial networks in removing plasticizer Di-(2-ethylhexyl) phthalate from estuarine sediments. mSystems. 2021;6:e0035821.

68. Yang F-C, Chen Y-L, Tang S-L, Yu C-P, Wang P-H, Ismail W, et al. Integrated multi-omics analyses reveal the biochemical mechanisms and phylogenetic relevance of anaerobic androgen biodegradation in the environment. ISME J. 2016;10:1967–83.

69. Panigrahi MK, Kaliaperumal V, Akella A, Venugopal G, Ramadass B. Mapping microbiome-redox spectrum and evaluating Microbial-Redox Index in chronic gastritis. Sci Rep. 2022;12:8450.

70. Khan SH, Dube N, Sudhakar N, Fraser O, Villalona P, Braet SM, et al. Ancient and modern mechanisms compete in progesterone receptor activation. RSC Chem Biol. 2024;5:518–29.

71. Hall OJ, Klein SL. Progesterone-based compounds affect immune responses and susceptibility to infections at diverse mucosal sites. Mucosal Immunol. 2017;10:1097–107.

72. Saccone G, Ciardulli A, Baxter JK, Quiñones JN, Diven LC, Pinar B, et al. Discontinuing oxytocin infusion in the active phase of labor: A systematic review and meta-analysis. Obstet Gynecol. 2017;130:1090–6.

73. Zwahlen M, Stute P. Impact of progesterone on the immune system in women: a systematic literature review. Arch Gynecol Obstet. 2024;309:37–46.

74. Polikarpova AV, Levina IS, Sigai NV, Zavarzin IV, Morozov IA, Rubtsov PM, et al. Immunomodulatory effects of progesterone and selective ligands of membrane progesterone receptors. Steroids. 2019;145:5–18.

75. Xu Y, He H, Li C, Shi Y, Wang Q, Li W, et al. Immunosuppressive effect of progesterone on dendritic cells in mice. J Reprod Immunol. 2011;91:17–23.

76. Tan S-Y, Hang F, Purvarshi G, Li M-Q, Meng D-H, Huang L-L. Decreased endometrial vascularity and receptivity in unexplained recurrent miscarriage patients during midluteal and early pregnancy phases. Taiwan J Obstet Gynecol. 2015;54:522–6.

Table 1. Neurosteroid-producing gut bacteria isolated from infertile female patients. Progesterone metabolic activity of individual bacterial isolates was examined in BHI broth containing 1 mM of progesterone.

Bacterial identities	Strains	Phylum	Family	Progesterone as sole carbon source	Major neurosteroids production*				Unidentified metabolite
					AlloP (5α3α)	IsoP (5α3β)	Preg (5β3α)	EpiP (5β3β)	
Anaerostipes caccae	15	Bacillota	Lachnospiraceae	-	-	-	+	++	-
Enterococcus gallinarum	1	Bacillota	Enterococcaceae	-	-	-	+++	+	-
Enterococcus faecalis	1	Bacillota	Enterococcaceae	-	-	-	-	-	+
Erysipelatoclostridium innocuum	23	Bacillota	Erysipelotrichaceae	-	-	-	+	+++	-
Erysipelatoclostridium ramosum	2	Bacillota	Erysipelotrichaceae	-	-	-	++	+	-
Holdemania filiformis	1	Bacillota	Erysipelotrichaceae	-	-	+++	-	-	-
Hungatella effluvii	6	Bacillota	Clostridiaceae	-	-	-	+++	+	+
Robinsoniella peorensis	29	Bacillota	Lachnospiraceae	-	-	-	++	+++	-
Bacteroides kribbi	2	Bacteroidota	Bacteroidaceae	-	-	-	+	++	-
Bacteroides ovatus	1	Bacteroidota	Bacteroidaceae	-	-	-	+	++	-
Alistipes putridinis	1	Bacteriodota	Rikenellaceae	-	+	+	+	+	-
Alistipes shashii	1	Bacteriodota	Rikenellaceae	-	+	+	+	-	-
Parabacteroides distasonis	8	Bacteroidota	Tannerellaceae	-	-	-	+	++	-
Escherichia coli	1	Pseudomonadota	Enterobacteriaceae	-	-	-	-	-	+
TOTAL	**92**								

*, the anaerobic neurosteroids production of individual species was examined in the BHI or GAM broth containing 1 mM of progesterone.

+++Over 80% of the substrate was transformed into corresponding product. ++ Over 50% (but less than 80%) of the substrate was transformed into corresponding products. +, Over 10% (but less than 50%) of the substrate was transformed into corresponding products.

Figure legends

Figure 1. Two- and three-dimensional structural representations of progesterone and its neurosteroid derivatives. The structures include pregnanolone, allopregnanolone (5α3α), isopregnanolone (5α3β), pregnanolone (5β3α), and epipregnanolone (5β3β). The 5α-progestogens (allopregnanolone and isopregnanolone) and 5β-progestogens (pregnanolone and epipregnanolone) are distinguished by their A/B-ring conformations. Key carbon atoms are numbered on the progesterone structure and its derivatives according to the standard steroid numbering system.

Figure 2. Metabolomic analysis of fecal cultures from progesterone-treated infertility patients under anaerobic conditions. Fecal samples were collected from 14 infertile patients receiving progesterone treatment and cultured in electron-rich (BHI) or electron-poor (DCB-1) media supplemented with 1 mM progesterone. Patient characteristics are detailed in **Table S1**. (**A**) Quantification of residual progesterone and microbial metabolites in BHI (left) and DCB-1 (right) cultures. Values represent mean ± SE (n = 14). (B) Distribution of major microbial metabolites across individual patient samples (P1-P14) in progesterone-supplemented cultures. Statistical significance was determined using unpaired nonparametric *t*-test; *p<0.05.

Figure 3. Temporal changes in bacterial community structure following progesterone treatment, analyzed at the phylum level using PacBio sequencing. Fecal samples from 14 female patients were incubated anaerobically with 1 mM progesterone in either BHI media (left) or DCB-1 media (right). (**A**) NMDS analysis showing shifts in bacterial communities over time. (**B**) Bacterial phyla significantly enriched by progesterone treatment. Values represent mean ± SE (n = 14). Statistical significance determined by unpaired nonparametric *t*-test: *p<0.05, **p<0.01, ***p<0.001."

Figure 4. Heatmaps showing bacterial genera that significantly increased (p<0.05) in relative abundance when fecal cultures were treated with progesterone. Fecal samples from 14 patients (P1-P14) were cultured anaerobically with 1 mM progesterone in two different media, namely BHI (top panel) and DCB-1 (bottom panel), for 3 and 6 days, respectively. The normalized abundance of each bacterial genus is represented by Z-scores. Genera are arranged vertically by their adjusted *p*-values,

with the most significantly enriched genus at the top (rank 1).

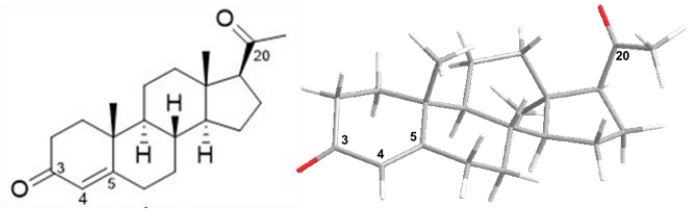
Figure 5. Correlations between gut bacterial genera and neurosteroid production in progesterone-supplemented BHI broth. Correlation coefficients were calculated using SparCC with 1000 bootstrap replicates. Circle colors indicate correlation strength and direction (red: positive, white: neutral, blue: negative). Fecal samples from 14 patients were cultured anaerobically for 3 days in BHI broth containing 1 mM progesterone, with daily sampling. Asterisks (*) denote genera with members isolated and characterized through culturomics.

Figure 6. Correlation analysis between gut bacterial genera and neurosteroid production in progesterone-supplemented DCB-1 broth. Fecal samples from 14 patients were cultured anaerobically in DCB-1 broth containing 1 mM progesterone for 6 days, with sampling every 2 days. Correlations between bacterial abundance and microbial products were calculated using SparCC with 1000 bootstrap replicates. Asterisks (*) indicate genera with characterized representatives. Genera in bold were also identified under BHI growth conditions (**Figure 5**).

Figure 7. Distribution of progesterone metabolism genes in Enterobacteriaceae and Veillonellaceae. (**A**) Metabolic pathway showing eukaryotic and microbial conversion of progesterone to pregnanolone. (B) Heatmap showing the presence of progesterone metabolism genes across selected Enterobacteriaceae and Veillonellaceae genomes, based on BLAST searches using biochemically characterized enzyme sequences or deduced amino acid sequences of putative genes identified through comparative genomics.

Figure 8. Gut bacterial community composition in healthy women (HW, n = 19) and women with recurrent miscarriage (MC, n = 41). (**A**) NMDS ordination based on Bray-Curtis dissimilarity showing bacterial community relationships between groups. (**B**) Relative abundance (%) of Enterobacteriaceae and Veillonellaceae families across groups (mean values shown). Detailed abundance data for individual genera (mean ± SE) are provided in **Figure S2**. Statistical significance was determined using unpaired nonparametric *t*-test. *$p < 0.05$.

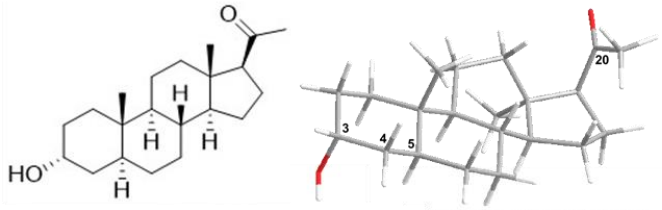
Figure 1



Progesterone (reproductive hormone)

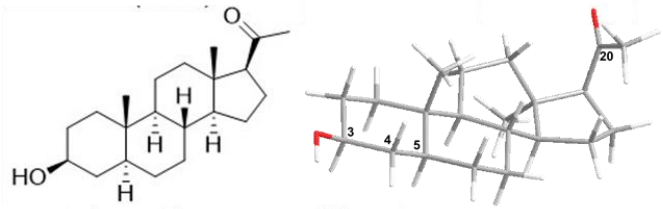
neurosteroids

5α-progestogens (positive modulators of the GABA receptor)



3α-hydroxy-5α-pregnan-20-one (allopregnanolone)

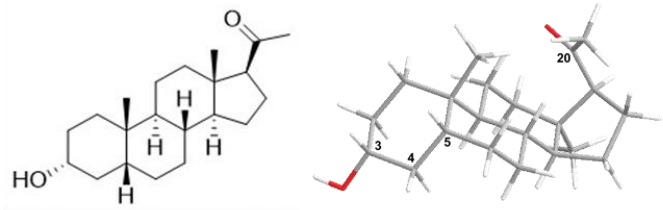
- a neuroprotective agent
- a drug for postpartum depression
- approved by FDA



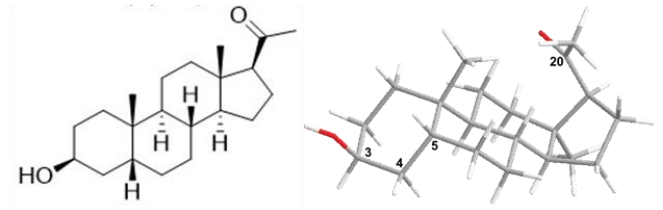
3β-hydroxy-5α-pregnan-20-one (isopregnanolone)

- treat Tourette's syndrome (in clinical trials)

5β-progestogens (negative modulators of the GABA receptor)



3α-hydroxy-5β-pregnan-20-one (pregnanolone)



3β-hydroxy-5β-pregnan-20-one (epipregnanolone)

Figure 2

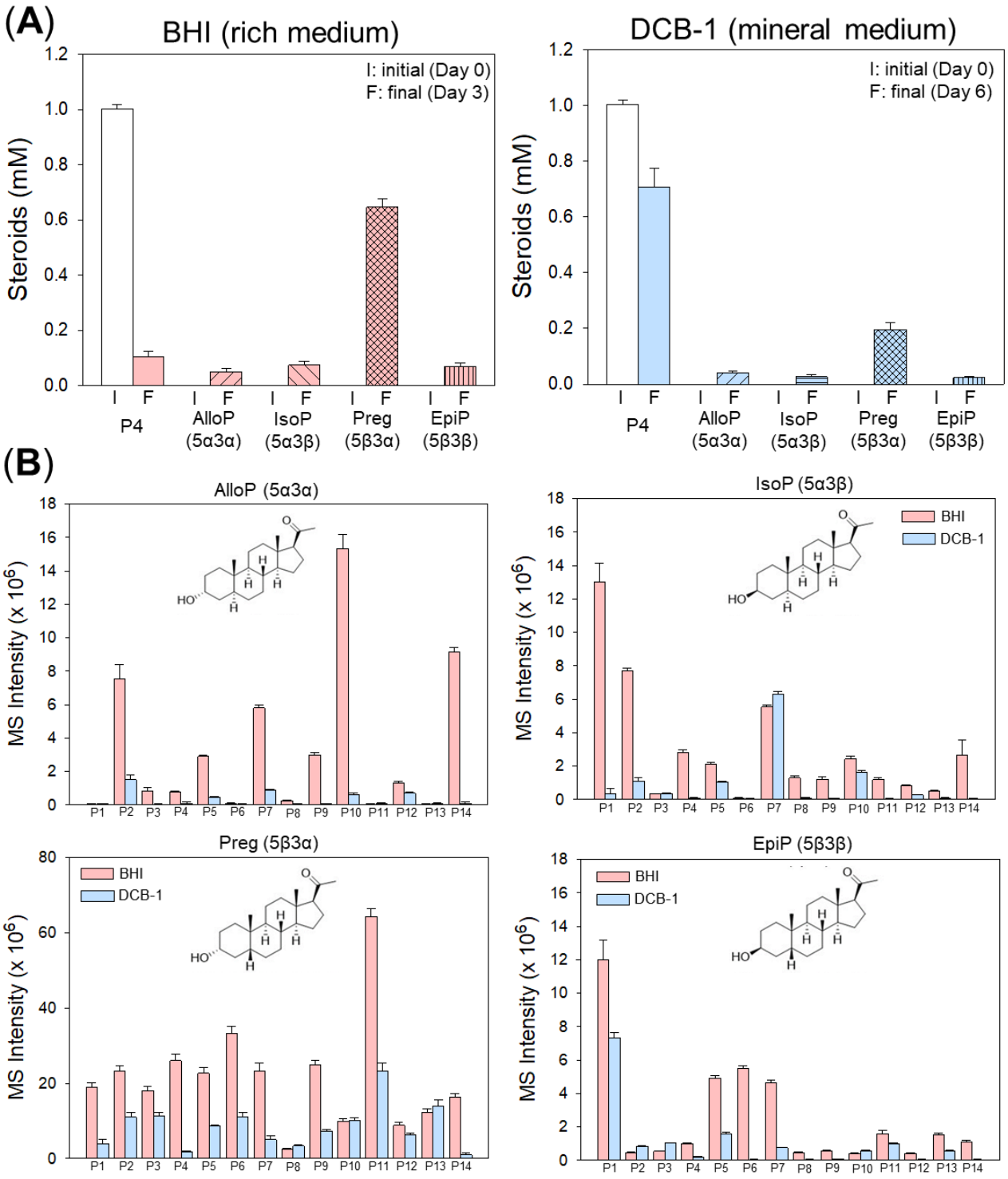

(A)
BHI (rich medium)
I: initial (Day 0)
F: final (Day 3)
Steroids (mM)
P4
AlloP (5α3α)
IsoP (5α3β)
Preg (5β3α)
EpiP (5β3β)
DCB-1 (mineral medium)
I: initial (Day 0)
F: final (Day 6)
(B)
AlloP (5α3α)
IsoP (5α3β)
Preg (5β3α)
EpiP (5β3β)
BHI
DCB-1
MS Intensity (x 10^6)
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13 P14

Figure 3

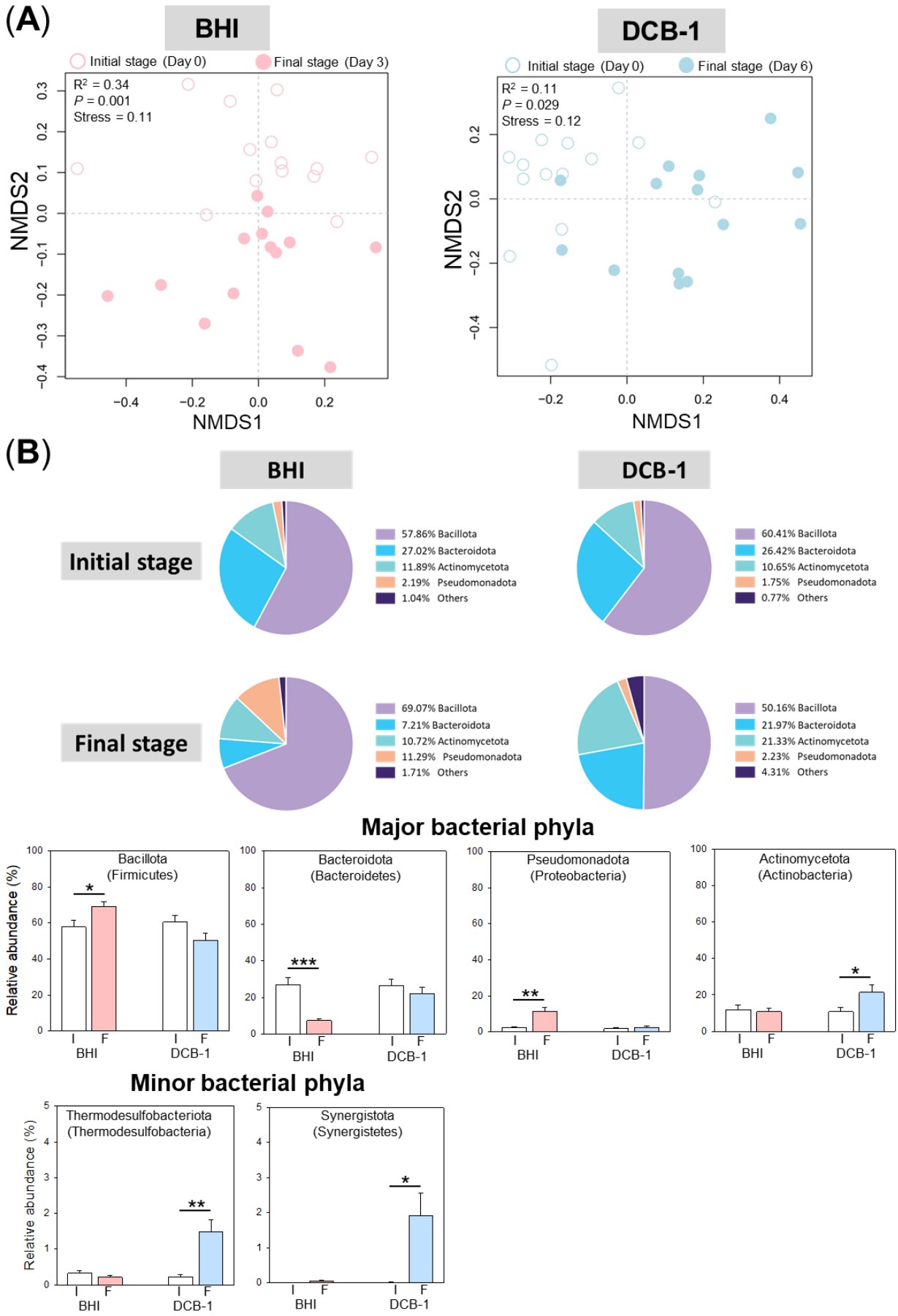

(A)
BHI
Initial stage (Day 0)
Final stage (Day 3)
R² = 0.34
P = 0.001
Stress = 0.11
NMDS2
NMDS1
DCB-1
Initial stage (Day 0)
Final stage (Day 6)
R² = 0.11
P = 0.029
Stress = 0.12
(B)
BHI
DCB-1
Initial stage
57.86% Bacillota
27.02% Bacteroidota
11.89% Actinomycetota
2.19% Pseudomonadota
1.04% Others
60.41% Bacillota
26.42% Bacteroidota
10.65% Actinomycetota
1.75% Pseudomonadota
0.77% Others
Final stage
69.07% Bacillota
7.21% Bacteroidota
10.72% Actinomycetota
11.29% Pseudomonadota
1.71% Others
50.16% Bacillota
21.97% Bacteroidota
21.33% Actinomycetota
2.23% Pseudomonadota
4.31% Others
Major bacterial phyla
Relative abundance (%)
Bacillota (Firmicutes)
Bacteroidota (Bacteroidetes)
Pseudomonadota (Proteobacteria)
Actinomycetota (Actinobacteria)
I F
BHI
DCB-1
Minor bacterial phyla
Thermodesulfobacteriota (Thermodesulfobacteria)
Synergistota (Synergistetes)

Figure 4

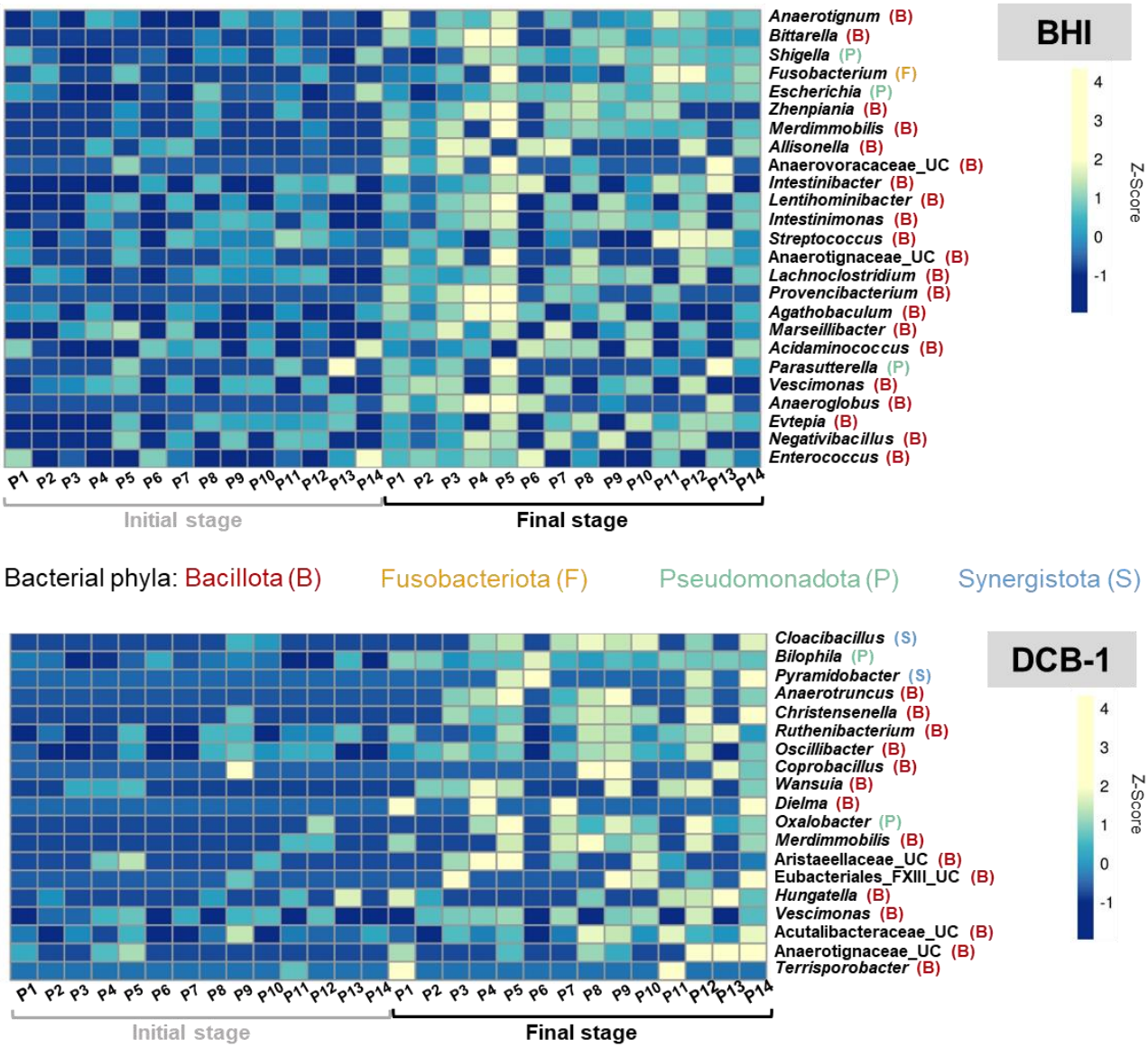

BHI
Anaerotignum (B)
Bittarella (B)
Shigella (P)
Fusobacterium (F)
Escherichia (P)
Zhenpiania (B)
Merdimmobilis (B)
Allisonella (B)
Anaerovoracaceae_UC (B)
Intestinibacter (B)
Lentihominibacter (B)
Intestinimonas (B)
Streptococcus (B)
Anaerotignaceae_UC (B)
Lachnoclostridium (B)
Provencibacterium (B)
Agathobaculum (B)
Marseillibacter (B)
Acidaminococcus (B)
Parasutterella (P)
Vescimonas (B)
Anaeroglobus (B)
Evtepia (B)
Negativibacillus (B)
Enterococcus (B)
Z-Score
4
3
2
1
0
-1
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13 P14 P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13 P14
Initial stage
Final stage
Bacterial phyla: Bacillota (B) Fusobacteriota (F) Pseudomonadota (P) Synergistota (S)
DCB-1
Cloacibacillus (S)
Bilophila (P)
Pyramidobacter (S)
Anaerotruncus (B)
Christensenella (B)
Ruthenibacterium (B)
Oscillibacter (B)
Coprobacillus (B)
Wansuia (B)
Dielma (B)
Oxalobacter (P)
Merdimmobilis (B)
Aristaeellaceae_UC (B)
Eubacteriales_FXIII_UC (B)
Hungatella (B)
Vescimonas (B)
Acutalibacteraceae_UC (B)
Anaerotignaceae_UC (B)
Terrisporobacter (B)
Z-Score
4
3
2
1
0
-1
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13 P14 P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13 P14
Initial stage
Final stage

Figure 5

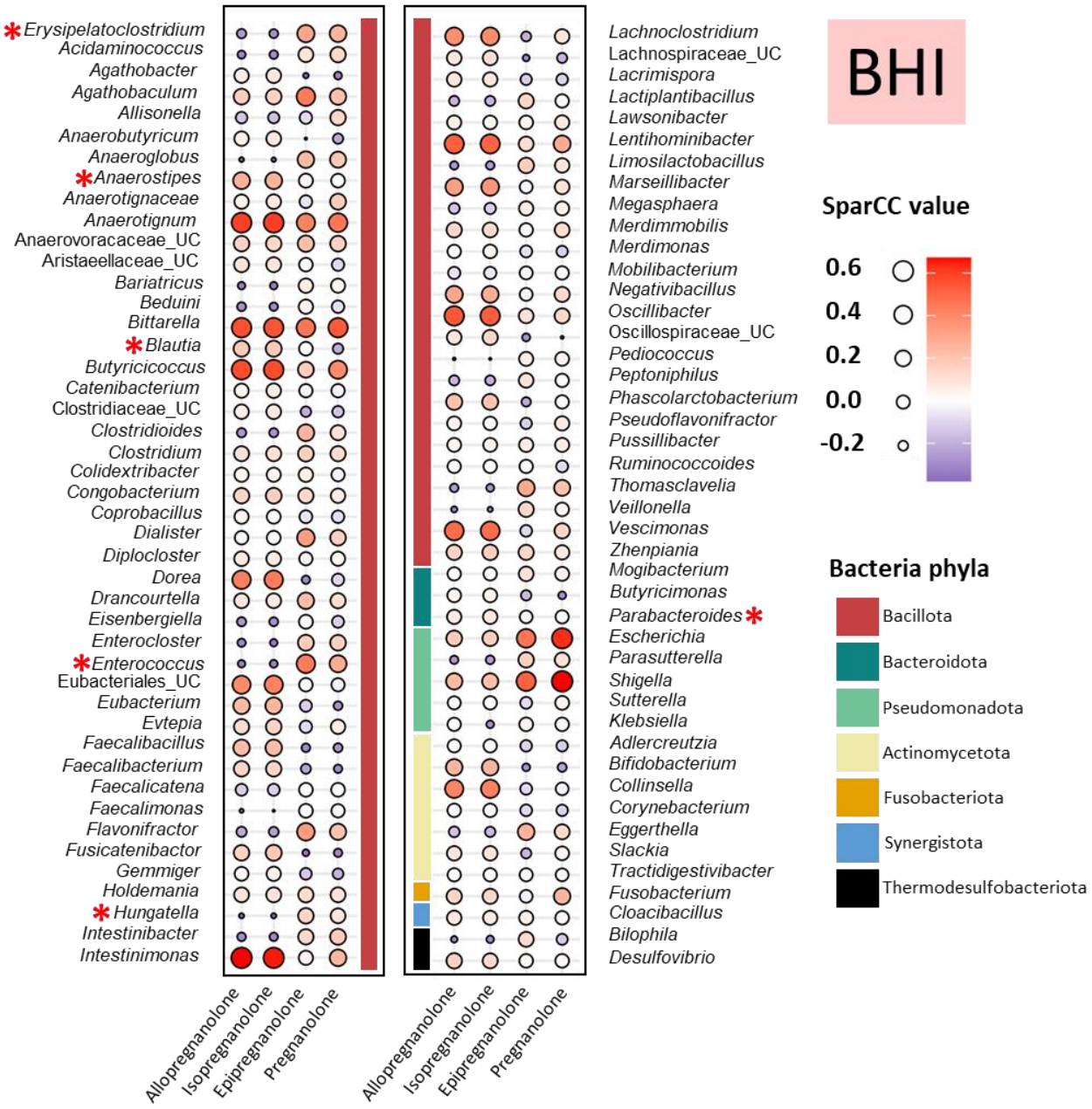

BHI
SparCC value
0.6
0.4
0.2
0.0
-0.2
Bacteria phyla
Bacillota
Bacteroidota
Pseudomonadota
Actinomycetota
Fusobacteriota
Synergistota
Thermodesulfobacteriota
Erysipelatoclostridium
Acidaminococcus
Agathobacter
Agathobaculum
Allisonella
Anaerobutyricum
Anaeroglobus
Anaerostipes
Anaerotignaceae
Anaerotignum
Anaerovoracaceae_UC
Aristaeellaceae_UC
Bariatricus
Beduini
Bittarella
Blautia
Butyricicoccus
Catenibacterium
Clostridiaceae_UC
Clostridioides
Clostridium
Colidextribacter
Congobacterium
Coprobacillus
Dialister
Diplocloster
Dorea
Drancourtella
Eisenbergiella
Enterocloster
Enterococcus
Eubacteriales_UC
Eubacterium
Evtepia
Faecalibacillus
Faecalibacterium
Faecalicatena
Faecalimonas
Flavonifractor
Fusicatenibactor
Gemmiger
Holdemania
Hungatella
Intestinibacter
Intestinimonas
Allopregnanolone
Isopregnanolone
Epipregnanolone
Pregnanolone
Lachnoclostridium
Lachnospiraceae_UC
Lacrimispora
Lactiplantibacillus
Lawsonibacter
Lentihominibacter
Limosilactobacillus
Marseillibacter
Megasphaera
Merdimmobilis
Merdimonas
Mobilibacterium
Negativibacillus
Oscillibacter
Oscillospiraceae_UC
Pediococcus
Peptoniphilus
Phascolarctobacterium
Pseudoflavonifractor
Pussillibacter
Ruminococcoides
Thomasclavelia
Veillonella
Vescimonas
Zhenpiania
Mogibacterium
Butyricimonas
Parabacteroides
Escherichia
Parasutterella
Shigella
Sutterella
Klebsiella
Adlercreutzia
Bifidobacterium
Collinsella
Corynebacterium
Eggerthella
Slackia
Tractidigestivibacter
Fusobacterium
Cloacibacillus
Bilophila
Desulfovibrio
Allopregnanolone
Isopregnanolone
Epipregnanolone
Pregnanolone

Figure 6

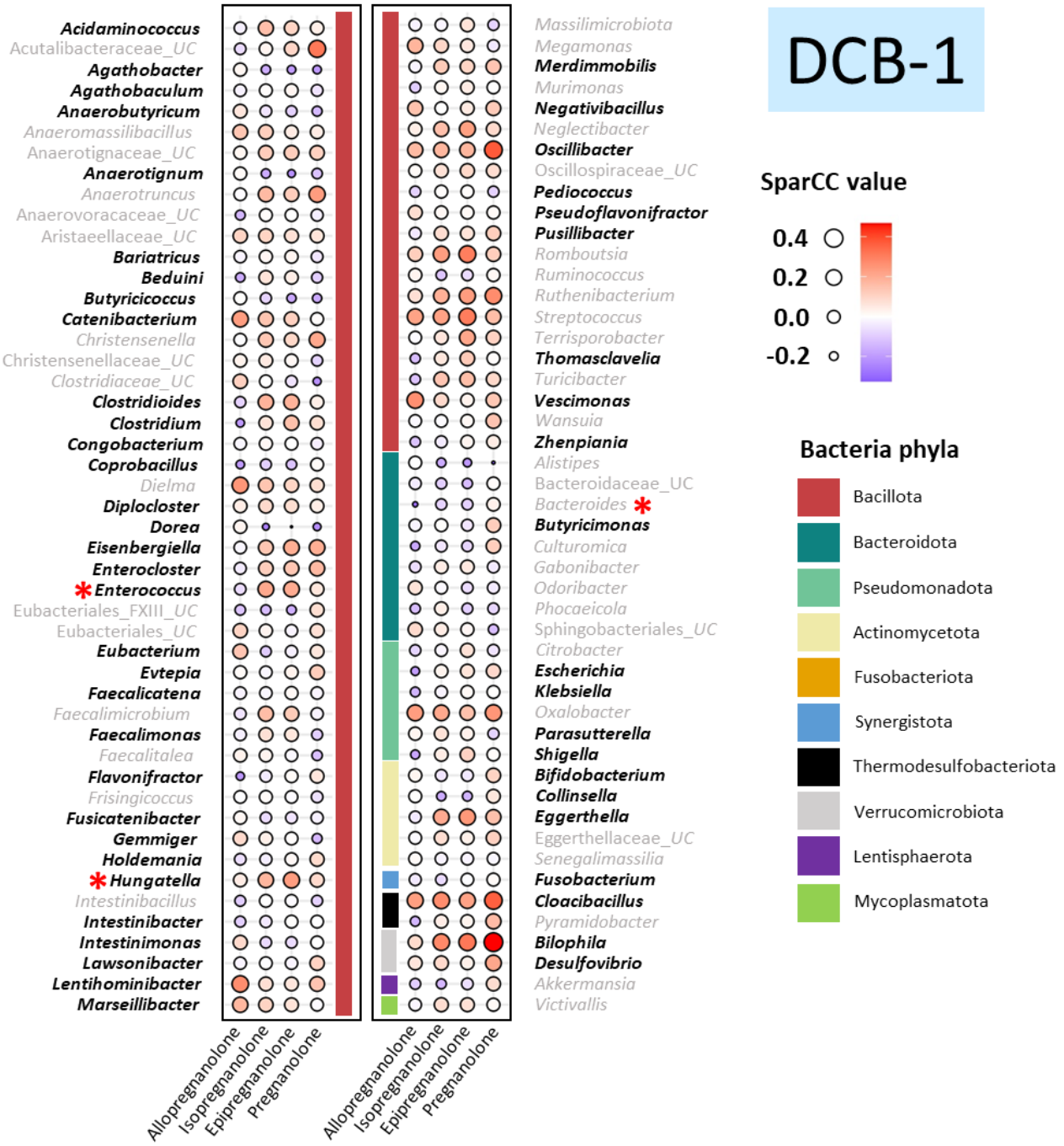

DCB-1
SparCC value
0.4
0.2
0.0
-0.2
Bacteria phyla
Bacillota
Bacteroidota
Pseudomonadota
Actinomycetota
Fusobacteriota
Synergistota
Thermodesulfobacteriota
Verrucomicrobiota
Lentisphaerota
Mycoplasmatota
Acidaminococcus
Acutalibacteraceae_UC
Agathobacter
Agathobaculum
Anaerobutyricum
Anaeromassilibacillus
Anaerotignaceae_UC
Anaerotignum
Anaerotruncus
Anaerovoracaceae_UC
Aristaeellaceae_UC
Bariatricus
Beduini
Butyricicoccus
Catenibacterium
Christensenella
Christensenellaceae_UC
Clostridiaceae_UC
Clostridioides
Clostridium
Congobacterium
Coprobacillus
Dielma
Diplocloster
Dorea
Eisenbergiella
Enterocloster
*Enterococcus
Eubacteriales_FXIII_UC
Eubacteriales_UC
Eubacterium
Evtepia
Faecalicatena
Faecalimicrobium
Faecalimonas
Faecalitalea
Flavonifractor
Frisingicoccus
Fusicatenibacter
Gemmiger
Holdemania
*Hungatella
Intestinibacillus
Intestinibacter
Intestinimonas
Lawsonibacter
Lentihominibacter
Marseillibacter
Massilimicrobiota
Megamonas
Merdimmobilis
Murimonas
Negativibacillus
Neglectibacter
Oscillibacter
Oscillospiraceae_UC
Pediococcus
Pseudoflavonifractor
Pusillibacter
Romboutsia
Ruminococcus
Ruthenibacterium
Streptococcus
Terrisporobacter
Thomasclavelia
Turicibacter
Vescimonas
Wansuia
Zhenpiania
Alistipes
Bacteroidaceae_UC
Bacteroides *
Butyricimonas
Culturomica
Gabonibacter
Odoribacter
Phocaeicola
Sphingobacteriales_UC
Citrobacter
Escherichia
Klebsiella
Oxalobacter
Parasutterella
Shigella
Bifidobacterium
Collinsella
Eggerthella
Eggerthellaceae_UC
Senegalimassilia
Fusobacterium
Cloacibacillus
Pyramidobacter
Bilophila
Desulfovibrio
Akkermansia
Victivallis
Allopregnanolone
Isopregnanolone
Epipregnanolone
Pregnanolone
Allopregnanolone
Isopregnanolone
Epipregnanolone
Pregnanolone

Figure 7

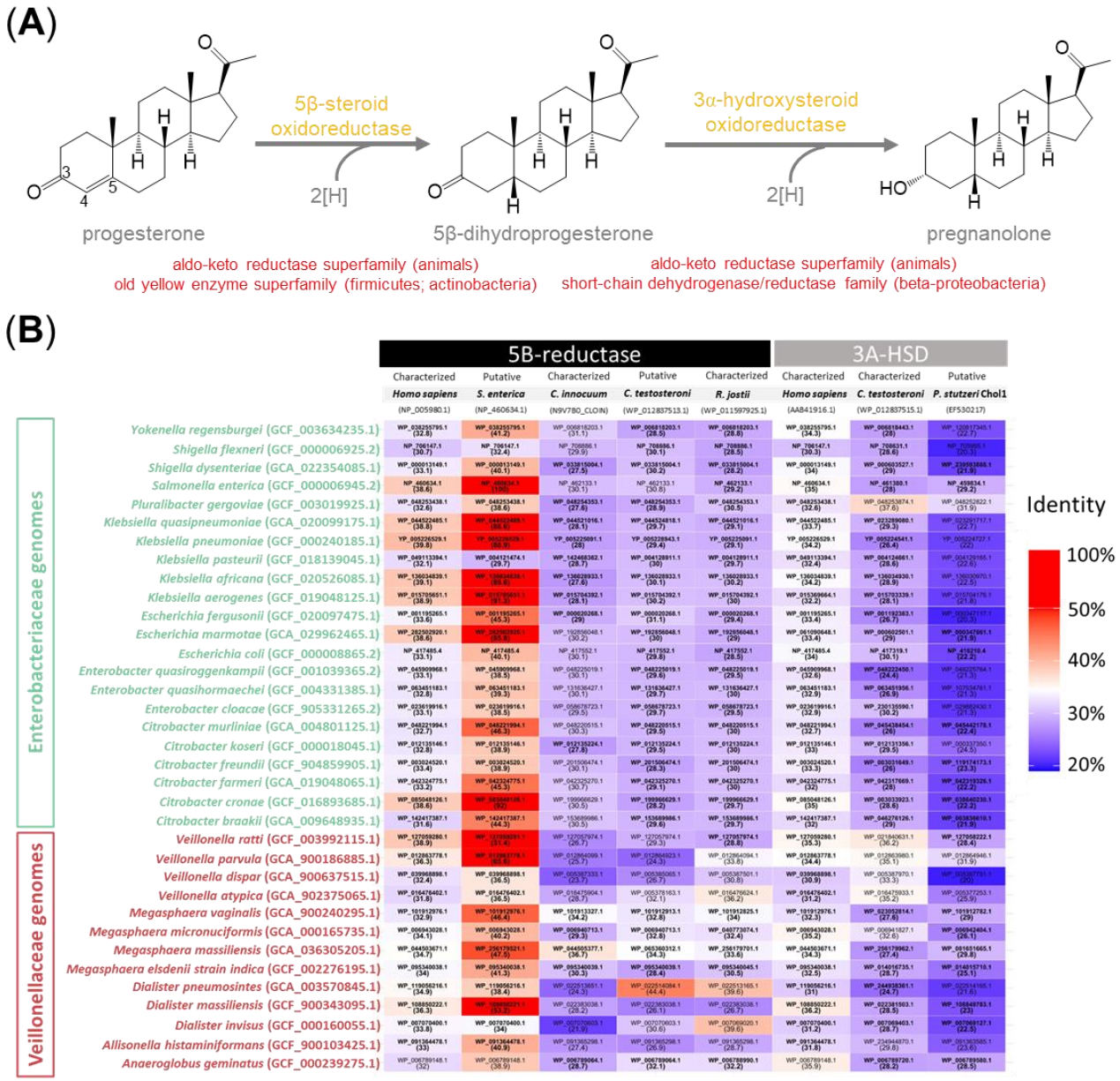
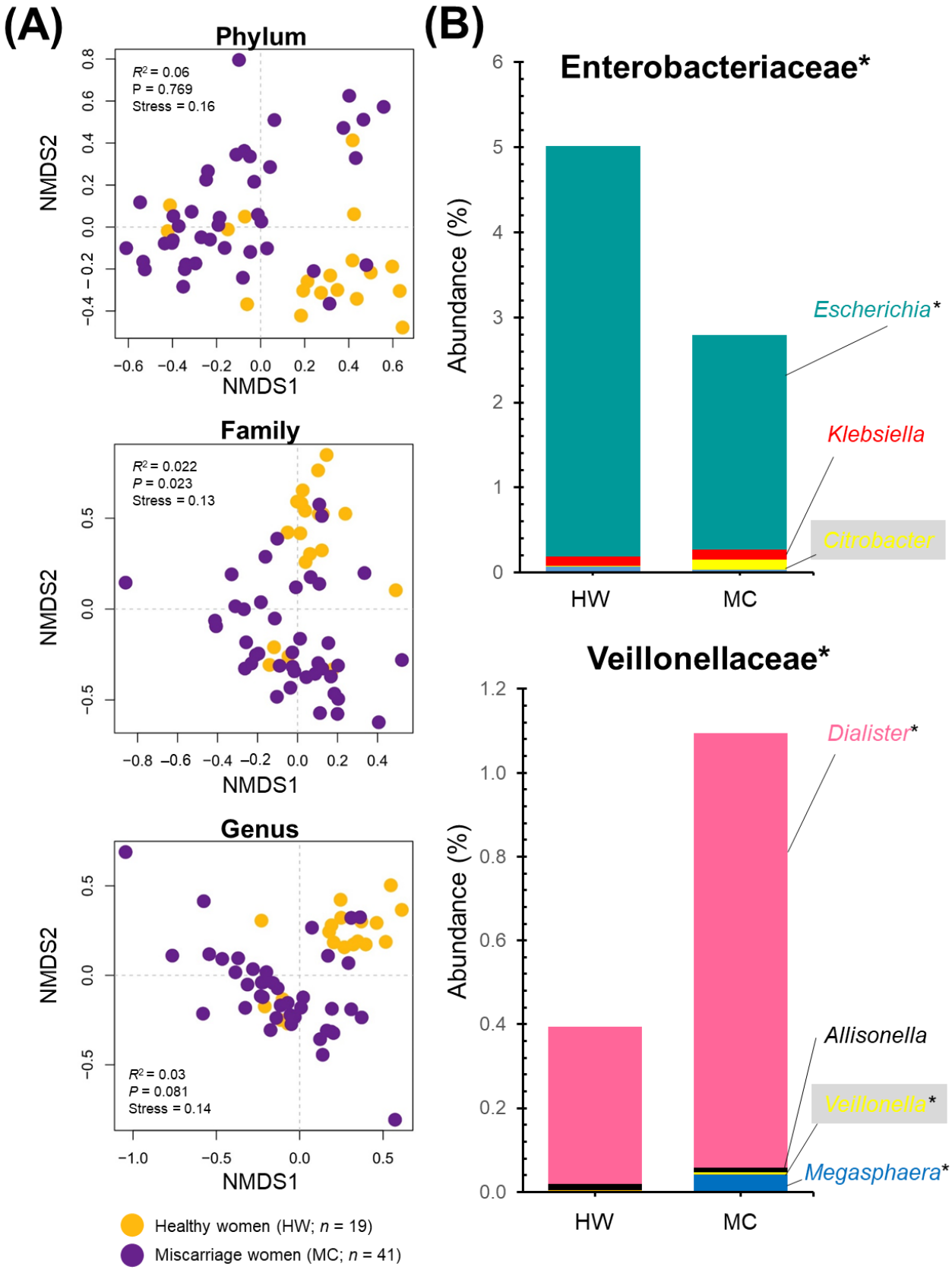

(A)
5β-steroid oxidoreductase
2[H]
3α-hydroxysteroid oxidoreductase
2[H]
progesterone
5β-dihydroprogesterone
pregnanolone
aldo-keto reductase superfamily (animals)
old yellow enzyme superfamily (firmicutes; actinobacteria)
aldo-keto reductase superfamily (animals)
short-chain dehydrogenase/reductase family (beta-proteobacteria)
(B)
5B-reductase
3A-HSD
Characterized
Putative
Characterized
Putative
Characterized
Characterized
Characterized
Putative
Homo sapiens
S. enterica
C. innocuum
C. testosteroni
R. jostii
Homo sapiens
C. testosteroni
P. stutzeri Chol1
(NP_005980.1)
(NP_460634.1)
(N9V7B0_CLOIN)
(WP_012837513.1)
(WP_011597925.1)
(AAB41916.1)
(WP_012837515.1)
(EF530217)
Enterobacteriaceae genomes
Veillonellaceae genomes
Yokenella regensburgei (GCF_003634235.1)
Shigella flexneri (GCF_000006925.2)
Shigella dysenteriae (GCA_022354085.1)
Salmonella enterica (GCF_000006945.2)
Pluralibacter gergoviae (GCF_003019925.1)
Klebsiella quasipneumoniae (GCA_020099175.1)
Klebsiella pneumoniae (GCF_000240185.1)
Klebsiella pasteurii (GCF_018139045.1)
Klebsiella africana (GCF_020526085.1)
Klebsiella aerogenes (GCF_019048125.1)
Escherichia fergusonii (GCF_020097475.1)
Escherichia marmotae (GCA_029962465.1)
Escherichia coli (GCF_000008865.2)
Enterobacter quasiroggenkampii (GCF_001039365.2)
Enterobacter quasihormaechei (GCF_004331385.1)
Enterobacter cloacae (GCF_905331265.2)
Citrobacter murliniae (GCA_004801125.1)
Citrobacter koseri (GCF_000018045.1)
Citrobacter freundii (GCF_904859905.1)
Citrobacter farmeri (GCA_019048065.1)
Citrobacter cronae (GCF_016893685.1)
Citrobacter braakii (GCA_009648935.1)
Veillonella ratti (GCF_003992115.1)
Veillonella parvula (GCA_900186885.1)
Veillonella dispar (GCA_900637515.1)
Veillonella atypica (GCA_902375065.1)
Megasphaera vaginalis (GCA_900240295.1)
Megasphaera micronuciformis (GCF_000165735.1)
Megasphaera massiliensis (GCF_036305205.1)
Megasphaera elsdenii strain indica (GCF_002276195.1)
Dialister pneumosintes (GCA_003570845.1)
Dialister massiliensis (GCF_900343095.1)
Dialister invisus (GCF_000160055.1)
Allisonella histaminiformans (GCF_900103425.1)
Anaeroglobus geminatus (GCF_000239275.1)
Identity
100%
50%
40%
30%
20%

Figure 8


(A)
Phylum
R^2 = 0.06
P = 0.769
Stress = 0.16
NMDS2
NMDS1
Family
R^2 = 0.022
P = 0.023
Stress = 0.13
NMDS2
NMDS1
Genus
R^2 = 0.03
P = 0.081
Stress = 0.14
NMDS2
NMDS1
Healthy women (HW; n = 19)
Miscarriage women (MC; n = 41)
(B)
Enterobacteriaceae*
Abundance (%)
Escherichia*
Klebsiella
Citrobacter
HW
MC
Veillonellaceae*
Abundance (%)
Dialister*
Allisonella
Veillonella*
Megasphaera*
HW
MC