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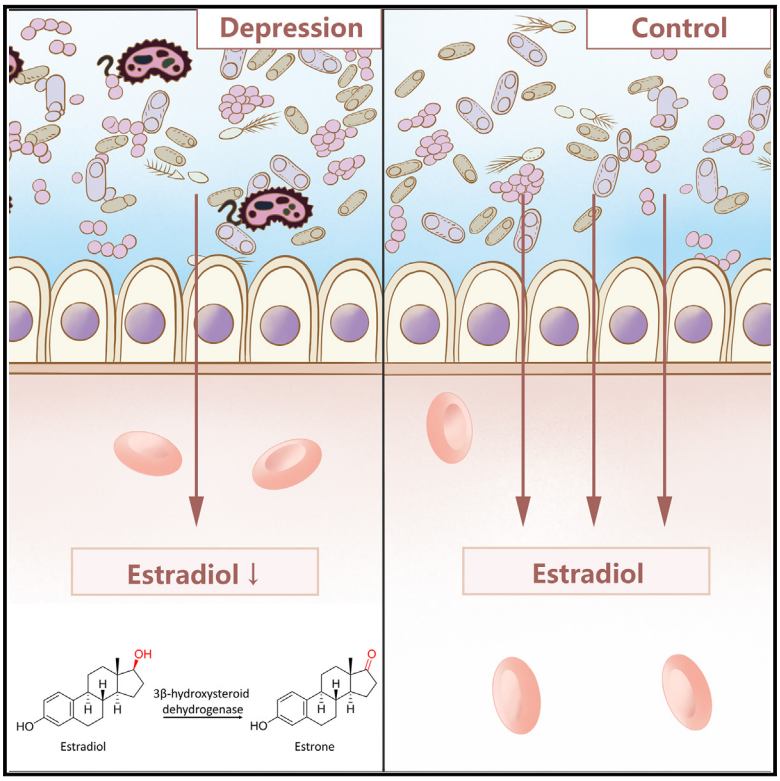
Article

Gut-microbiome-expressed 3β-hydroxysteroid dehydrogenase degrades estradiol and is linked to depression in premenopausal females

Graphical abstract



Highlights

- Depression is the leading cause of disease burden and disability
- Women are about twice as likely as men to develop depression
- Estradiol decline can lead to depressive symptoms in women
- Estradiol-degrading bacteria and enzymes are intervention targets for depression

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In brief

Our research demonstrates that the degradation of estradiol by gut microbes containing 3β-HSD leads to a decrease in serum estradiol levels, causing depression in female mice. Our study reveals both a novel mechanism for depression in females linked to gut microbes and possible intervention targets.

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Gut-microbiome-expressed 3β-hydroxysteroid dehydrogenase degrades estradiol and is linked to depression in premenopausal females

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SUMMARY

Estradiol decline can result in depressive disorders in females; nevertheless, the causes of this decline are unclear. In this study, we isolated estradiol-degrading *Klebsiella aerogenes* from the feces of premenopausal females with depression. In mice, gavaging with this strain led to estradiol decline and depression-like behaviors. The gene encoding the estradiol-degrading enzyme in *K. aerogenes* was identified as 3β-hydroxysteroid dehydrogenase (3β-HSD). Heterologously expressing *3β-HSD* resulted in *Escherichia coli* obtaining the ability to degrade estradiol. Gavaging mice with 3β-HSD-expressing *E. coli* decreased their serum estradiol levels, causing depression-like behaviors. The prevalence of *K. aerogene* and 3β-HSD was higher in premenopausal women with depression than in those without depression. These results suggest that the estradiol-degrading bacteria and 3β-HSD enzymes are potential intervention targets for depression treatment in premenopausal women.

INTRODUCTION

Depression, a global problem, is a leading cause of disease burden and disability.[1] It is about twice as common in women as in men.[2] This sex difference is linked to changes in estradiol levels.[3] Estradiol is important for supporting a healthy mood.[4] In rodents, low serum estradiol levels could induce depression-like behaviors.[5] Some studies have observed a decline in estradiol levels in premenopausal female patients with depression.[6] However, the causes of this decline are still not fully understood.

In humans and animals, estrogens are synthesized mainly in the ovary. Estrogen hormones derive from cholesterol and pregnenolone that are transformed into androstenedione and testosterone, which are the direct precursors of estradiol. Estradiol is a C18 steroid, constituted by 18 carbon atoms distributed in one pentagonal and three hexagonal rings.[7] Following liver metabolism, estradiol is excreted via the biliary tract into the gut and partly reabsorbed back into the blood.[8] Gut microbes could increase serum steroid hormone levels by shifting their active-to-inactive form ratio.[9] Estradiol degradation pathways in environmental bacteria have been reported. The major degradation products of estradiol were estrone and 4,16-OH-estrone, which can be further degraded through five possible pathways.[10] 17β-Hydroxysteroid dehydrogenase or 3-oxoacyl-(acyl-carrier-protein) reductase is the initial step reaction enzyme.[11] However, the relationship between gut bacteria metabolism and estradiol decline remains unclear.

Therefore, this study aims to explore whether there are estradiol-degrading microbes in the guts of female patients and whether these microbes are linked to depression in premenopausal women.

RESULTS

Serum estradiol decreased in premenopausal women with depression

To verify if the serum estradiol levels in premenopausal women with depression were lower than those in non-depressed premenopausal women, we included 91 premenopausal women with depression (27.59 ± 6.86 years, depression group) and 98 premenopausal women without depression (26.85 ± 9.30 years, non-depression group).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) showed that, compared with the non-depression group

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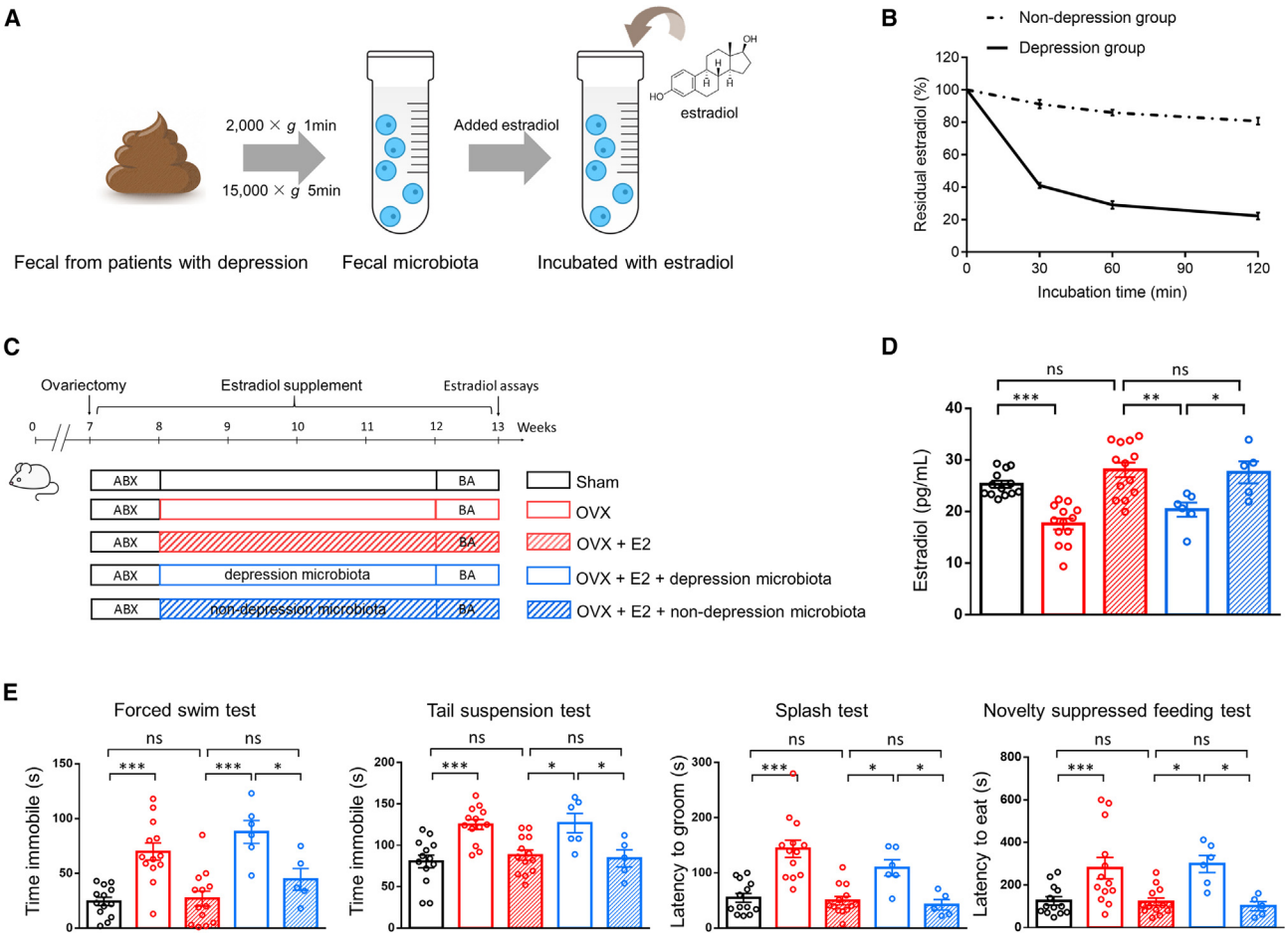

Figure 1. Gut microbiota from depressed premenopausal women induces depression-like behaviors

(A) Flow chart of fecal microbiota extraction.

(B) Incubation of gut microbiota from the non-depression (n = 5) and depression group (n = 5) with estradiol (100 mg/L) tested *in vitro*. Data are presented as means ± SEM.

(C) Experimental timeline. All mice (aged 7 weeks) were treated with 7-day antibiotics. Mice in the sham group underwent sham operation. Mice in the OVX group underwent ovariectomy. The OVX + E2 group underwent ovariectomy followed by estradiol supplementation by gavage for 6 weeks. The OVX + E2 + depression microbiota group underwent ovariectomy followed by gavage estradiol for 6 weeks and depression microbiota for 4 weeks. The OVX + E2 + non-depression microbiota group underwent ovariectomy followed by gavage estradiol for 6 weeks and non-depression microbiota for 4 weeks. All mice (aged 12 weeks) underwent behavioral assessment. Serum estradiol was detected after behavioral assessment.

(D) Serum concentration of estradiol in the sham group (n = 12), OVX group (n = 12), OVX + E2 group (n = 12), OVX + E2 + depression microbiota group (n = 6), and OVX + E2 + non-depression microbiota group (n = 5).

(E) Immobility time in FST and TST, latency to groom in the splash test, and latency to eat in NSF in the sham group (n = 12), OVX group (n = 12), OVX + E2 group (n = 12), OVX + E2 + depression microbiota group (n = 6), and OVX + E2 + non-depression microbiota group (n = 5).

ABX, antibiotic mixture consisting of 100 mg/kg neomycin, 100 mg/kg metronidazole, 50 mg/kg vancomycin, and 1 mg/mL ampicillin; OVX, ovariectomized mice; E2, estradiol; BA, behavior assays.

Data are presented as means ± SEM, and one-way ANOVA with Tukey's multiple comparisons tests was performed to calculate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

See also Figures S2 and S3.

(95.20 ± 28.17 pg/mL), the depression group had significantly lower serum estradiol levels (54.27 ± 25.17 pg/mL) ($p < 0.001$; Figure S1A).

Gut microbiota from depressed premenopausal women degrade estradiol

To investigate whether the gut microbiota of premenopausal women with depression can degrade estradiol, we incubated estradiol (100 mg/L) with gut microbiota from premenopausal women with depression and low serum estradiol (n = 5) (Figure 1A). Gut microbiota from age-matched premenopausal women without depression who had normal serum estradiol were included as controls (n = 5). LC-MS/MS showed that estradiol was degraded by 77.8% and 19.3% within 120 min in the depression and non-depression groups, respectively (Figure 1B). These results suggested that the gut microbiota in the depression group degraded estradiol more efficiently than that in the non-depression group.

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Mice receiving fecal transplants from depressed premenopausal women exhibit depression-like behaviors

To determine whether the gut microbiota in the premenopausal women with depression and low estradiol could reduce serum estradiol and thereby cause depression-like behaviors, we transplanted the above-mentioned gut microbiota that can efficiently degrade estradiol from five depressed women into recipient mice.

We removed the mice ovaries and supplemented them with estradiol before gavaging with the gut microbiota to exclude the menstrual cycle influence on estradiol detection. To verify whether the ovariectomy was successful and whether estradiol recovered after supplementation, the mice were divided into three groups: (1) sham, sham-operated mice; (2) OVX, ovariectomized mice; and (3) OVX + E2, ovariectomized mice supplemented with estradiol (0.2 mg/kg/day, for 6 consecutive weeks) (Figure 1C).

The results showed that the estradiol level in the OVX group (17.63 ± 3.86 pg/mL) was significantly lower than that in the sham group (25.30 ± 2.40 pg/mL, $p < 0.001$). After estradiol supplementation, the estradiol levels in the OVX + E2 group (28.09 ± 5.18 pg/mL, $p > 0.05$) recovered compared with those in the sham group (Figure 1D).

Behavioral experiments were performed to determine whether ovariectomy and estradiol supplementation affected mouse depression-like behaviors. The results showed that in the tail suspension test (TST) and forced swim test (FST), immobility time was not significantly different between the sham and OVX + E2 groups. In the splash test and novelty-suppressed feeding test (NSF), latency time was not significantly different between the sham and OVX + E2 groups. These results indicated that ovariectomy and estradiol supplementation did not affect depression-like behaviors in the mice in our study (Figures 1E and S2A).

Moreover, to investigate whether the gut microbiota in premenopausal women with depression could reduce serum estradiol levels and, thereby, cause depression-like behaviors, the mice were divided into three groups: (1) OVX + E2, 0.2 mg/kg/day for 28 days; (2) OVX + E2 + non-depression microbiota, ovariectomized mice gavaged with gut microbiota from the non-depression group (1×10^8 CFU for 3 successive days per week for 4 weeks) and supplemented with 0.2 mg/kg/day estradiol for 28 days; and (3) OVX + E2 + depression microbiota, OVX + E2, gavaged with gut microbiota from the depression group (1×10^8 CFU for 3 successive days per week for 4 weeks) and supplemented with 0.2 mg/kg/day estradiol for 28 days (Figure 1C).

The results showed that the serum estradiol level in the OVX + E2 + depression microbiota group was lower than that in the OVX + E2 group (20.38 ± 3.36 versus 27.81 ± 5.30 pg/mL, $p = 0.001$) and the OVX + E2 + non-depression microbiota group (20.38 ± 3.36 versus 26.41 ± 5.13 pg/mL, $p = 0.013$). The serum estradiol level in the OVX + E2 + non-depression microbiota group was not significantly different from that in the OVX + E2 group ($p > 0.05$) (Figure 1D).

Behavioral assessment showed that in TST and FST, the OVX + E2 + depression microbiota group had longer immobility time than the OVX + E2 and OVX + E2 + non-depression microbiota groups. In the splash test experiment, the OVX + E2 + depression microbiota group had longer latency than the OVX + E2 and OVX + E2 + non-depression microbiota groups. In NSF, the OVX + E2 + depression microbiota group had longer latency time than the OVX + E2 and OVX + E2 + non-depression microbiota groups (Figures 1E and S2A).

These results suggest that gut microbiota from premenopausal women with depression can reduce serum estradiol in mice and induce depression-like behaviors.

Estradiol-degrading *Klebsiella aerogenes* was isolated from female patients with depression

To isolate gut microbe that can degrade estradiol, we used estradiol (100 mg/L) as the single carbon source and incubated it with gut microbiota from premenopausal women with depression and low estradiol levels. Finally, a pale white colony with fuzzy edges and a smooth surface was isolated (Figure 2A). This strain was identified as *K. aerogenes* using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) (Bruker Daltonics, USA) (Figure S3A). We named this strain as *Klebsiella aerogenes* TS2020. The drug susceptibility testing showed that *K. aerogenes* TS2020 was sensitive to cefotaxime (Figure S3B).

We incubated *K. aerogenes* TS2020 with estradiol (100 mg/L) and collected culture medium samples every 24 h to verify the estradiol-degrading ability. The culture medium in the control group contained estradiol (100 mg/L) without any microbes. Thin-layer chromatography (TLC) showed that estradiol was not degraded in the control group (Figure S3C), whereas estradiol was degraded by *K. aerogenes* TS2020 (Figure S3D). High-performance liquid chromatography (HPLC) showed that the estradiol level gradually decreased and the level of the estrone metabolite increased. After incubating for 120 h, 61.8% of estradiol was converted into estrone (Figure 2B). These results showed that *K. aerogenes* TS2020 could degrade estradiol.

K. aerogenes TS2020 gavage induces depression-like behavior in mice

We gavaged mice with *K. aerogenes* TS2020 to determine whether *K. aerogenes* TS2020 can induce depression-like behaviors in female mice. Additionally, we administered *K. aerogenes*-recipient mice with cefotaxime to investigate whether serum estradiol reduction and depression-like behavior in the mice could be alleviated by antibiotic treatment. We divided the mice into three groups: (1) OVX + E2 group, gavaged with estradiol (0.2 mg/kg/day for 4 consecutive weeks); (2) OVX + E2 + KA group, gavaged with estradiol (0.2 mg/kg/day for 4 consecutive weeks) and *K. aerogenes* TS2020 (approximately 1×10^8 CFU, three times a week for 4 weeks); and (3) OVX + E2 + KA + cefotaxime group, gavaged with estradiol (0.2 mg/kg/day for 6 consecutive weeks) and *K. aerogenes* TS2020 (approximately 1×10^8 CFU, three times a week for 4 weeks). After *K. aerogenes* TS2020 gavage, the mice were treated with cefotaxime (200 mg/kg) for 1 week (Figure 2C).

The mouse serum estradiol levels in the OVX + E2 + KA group were significantly lower than those in the OVX + E2 group (17.70 ± 3.66 versus 28.41 ± 5.51 pg/mL, $p < 0.001$) and OVX + E2 + KA + cefotaxime group (17.70 ± 3.66 versus 26.98 ± 0.63 pg/mL, $p < 0.001$). The serum E2 levels of the

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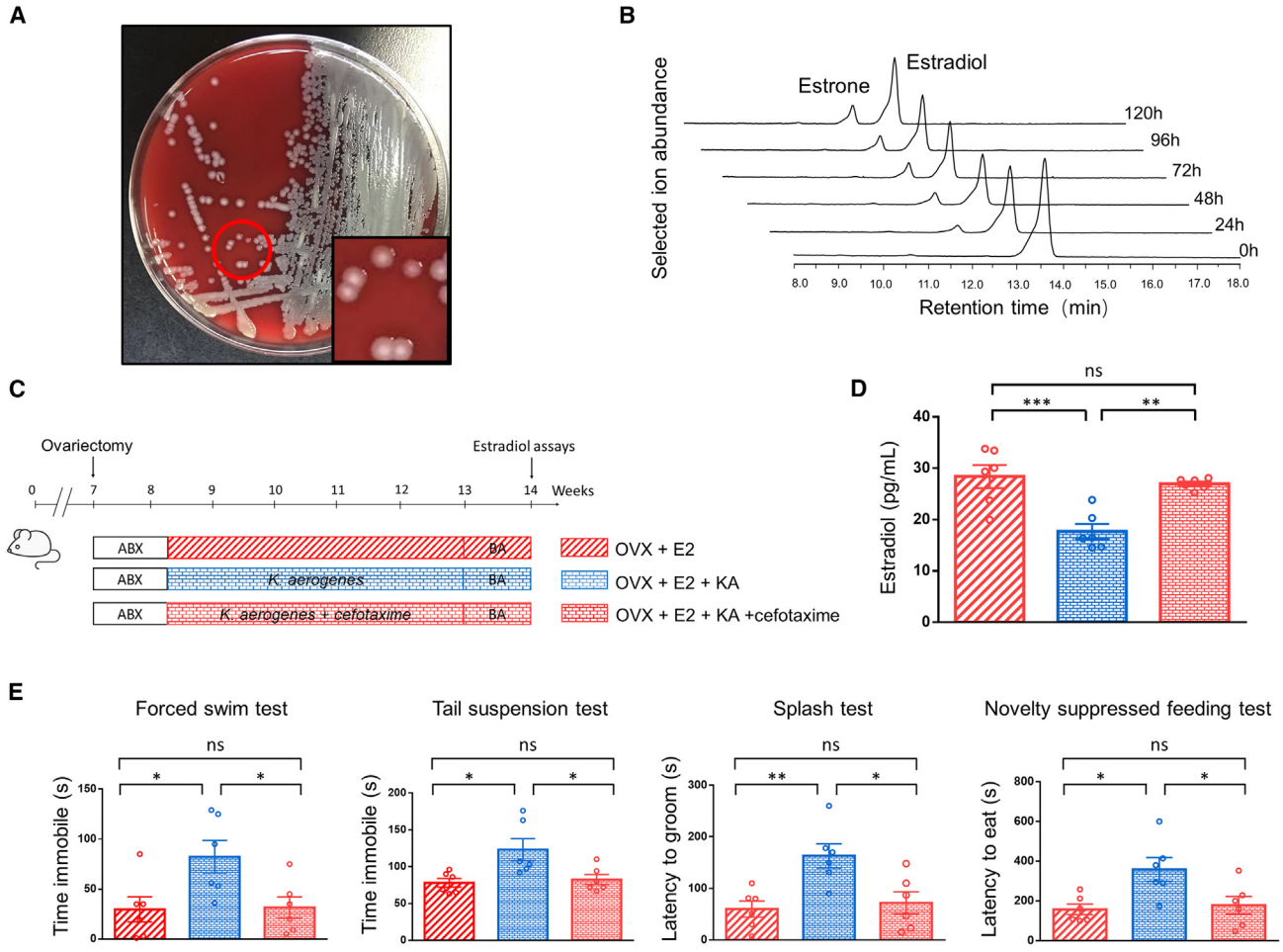

Figure 2. Colony morphology and ability of estradiol degradation of *Klebsiella aerogenes*

(A) *K. aerogenes* TS2020 typical colonies.

(B) Combined EICs showing that *K. aerogenes* converted estradiol into estrone time dependently. *K. aerogenes* was incubated with estradiol (100 mg/L) and hormones were detected using HPLC.

(C) Experimental timeline. All mice (aged 7 weeks) were treated with 7-day antibiotics. The OVX + E2 group underwent ovariectomy followed by estradiol supplementation by gavage for 6 weeks. The OVX + E2 + KA group underwent ovariectomy followed by gavage estradiol for 6 weeks and *K. aerogenes* TS2020 for 4 weeks. The mice in OVX + E2 + KA + cefotaxime group gavaged with estradiol for 7 weeks and *K. aerogenes* TS2020 for 4; after *K. aerogenes* TS2020 gavage, the mice were treated with cefotaxime for 1 week. The OVX + E2 and OVX + E2 + KA groups underwent behavior assays at age 12 weeks. The OVX + E2 + KA + cefotaxime group underwent behavior assays at age 13 weeks. Serum estradiol was detected after behavior assays finished.

(D) Estradiol concentration in the OVX + E2 (n = 6), OVX + E2 + KA (n = 6), and OVX + E2 + KA + cefotaxime groups (n = 6).

(E) Immobility time in FST and TST, latency to groom in the splash test, and latency to eat in the NSF in the OVX + E2 (n = 6), OVX + E2 + KA (n = 6), and OVX + E2 + KA + cefotaxime groups (n = 6).

OVX, ovariectomy; E2, estradiol; cefo, cefotaxime. Data are presented as means ± SEM, and a one-way ANOVA with Tukey's multiple comparisons tests was performed to calculate statistical significance. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

See also Figures S2 and S3.

OVX + E2 and OVX + E2 + KA + cefotaxime groups were not significantly different (28.41 ± 5.51 versus 26.98 ± 0.63 pg/mL, $p = 0.541$) (Figure 2D).

Behavioral assessments showed that in TST and FST, the OVX + E2 + KA group had longer resting times than the OVX + E2 and OVX + E2 + KA + cefotaxime groups, and the OVX + E2 + KA group had a longer latency time than the OVX + E2 and OVX + E2 + KA + cefotaxime groups. In NSF, the latency time was longer in the OVX + E2 + KA group than in the OVX + E2 and OVX + E2 + KA + cefotaxime groups. The OVX + E2 + KA + cefotaxime and OVX + E2 groups showed no significant difference in FST, TST, NSF, and splash experiment results (Figures 2E and S2B).

These results show that *K. aerogenes* TS2020 can reduce the serum estradiol level in mice and induce depressive-like behaviors. Additionally, cefotaxime administration can alleviate such depressive-like behaviors in mice.

Identification of key enzymes for degrading estradiol in *K. aerogenes* TS2020

The above results showed that estradiol was converted into estrone by *K. aerogenes* TS2020. Comparison using the

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KEGG pathway showed that the enzyme that converts estradiol into estrone is either 3β-HSD (EC: 1.1.1.51) or 17β-estradiol 17-dehydrogenase (EC:1.1.1.62) (https://www.genome.jp/pathway/map00140+C00280). We assembled and annotated *K. aerogenes* TS2020 using whole-genome sequencing to determine the enzyme-encoding gene in *K. aerogenes* TS2020. Finally, in *K. aerogenes* TS2020 chromosome (54.67% GC, GenBank: CP102479), the gene TS2020_3397 encoding 3β-HSD was found (Figure 3A).

To verify whether the gene TS2020_3397 can express an enzyme that can convert estradiol into estrone, we transformed pET28a, which contains the TS2020_3397 gene, into *E. coli* BL21(DE3) cells to obtain *E. coli*-pTS07 (Figure 3B). Similarly, we introduced the empty vector pET28a to obtain *E. coli*-pET28a as a control. Through PCR, we confirmed the presence of the gene TS2020_3397 in *E. coli*-pTS07 (Figure 3C). After incubation with estradiol (100 mg/L) in lysogeny broth (LB) medium, TLC showed that *E. coli*-pET28a did not degrade estradiol (Figure S4A), whereas *E. coli*-pTS07 degraded estradiol time dependently (Figure S4B). Moreover, HPLC showed that *E. coli*-pTS07 could degrade 89.52% of estradiol to estrone within 24 h (Figures 3D and 3E).

These results showed that the *K. aerogenes* TS2020 gene, TS2020_3397, could express 3β-HSD with the ability to convert estradiol into estrone.

Gavaging with *E. coli*-pTS07 induced depression-like behavior in female mice

We supplemented *E. coli*-pTS07 into mice to explore whether *E. coli*-pTS07 could induce depression-like behavior in mice. The experimental mice were divided into three groups: (1) OVX + E2; (2) OVX + E2 + pTS07, gavaged with estradiol (0.2 mg/kg/day for 6 consecutive weeks) and *E. coli*-pTS07 (approximately 1×10^8 CFU, three times a week for 4 weeks); and (3) OVX + E2 + pET28a, gavaged with estradiol (0.2 mg/kg/day for 6 consecutive weeks) and *E. coli*-pET28a (approximately 1×10^8 CFU, three times a week for 4 weeks) (Figure 4A).

The results showed that the mouse serum estradiol levels in the OVX + E2 + pTS07 group were significantly lower than those in the OVX + E2 (15.74 ± 1.85 versus 28.41 ± 5.51 pg/mL, $p < 0.001$) and OVX + E2 + pET28a groups (15.74 ± 1.85 versus 25.57 ± 2.45 pg/mL, $p < 0.001$). There was no significant difference in serum estradiol levels between the OVX + E2 and OVX + E2 + pET28a groups (28.41 ± 5.51 versus 25.57 ± 2.45 pg/mL, $p = 0.184$) (Figure 4B).

The behavioral assessment results showed that in FST and TST, the OVX + E2 + pTS07 group had a longer resting time than the OVX + E2 and OVX + E2 + pET28a groups. In the splash experiment, the OVX + E2 + pTS07 group had longer latency time than the OVX + E2 and OVX + E2 + pET28a groups. In NSF, the OVX + E2 + pTS07 group had longer latency than the OVX + E2 and OVX + E2 + pET28a groups. There were no significant differences in any of the behavioral experiments between the OVX + E2 + pET28a and OVX + E2 groups (Figures 4C and S2C). We obtained mouse fecal proteins for western blotting after behavioral assessments to further investigate whether 3β-HSD is produced during the experiment. The results showed that there were obvious bands in the mouse fecal samples from the OVX + E2 + pTS07 group. Conversely, none were observed in the mouse fecal samples from the OVX + E2 + pET28a group (Figure S4C), indicating that the *E. coli*-pTS07 receptor continued to express 3β-HSD during the experiment.

To investigate whether 3β-HSD-producing bacteria-induced decline in circulating estradiol levels also affected the central nervous system (CNS), we measured estradiol levels in the whole brain. As the hippocampus has been extensively documented as a critical site involved in depression in the brain,[12] estradiol levels in the hippocampus were also assayed. The results showed that mice in the OVX + E2 + pTS07 group had lower brain and hippocampus estradiol levels than mice in the OVX + E2 + pET28a group and OVX + E2 (Figures S4D and S4E).

The close relationship between estradiol and the serotonin (5-HT) system provides insight as to why some women experience increased susceptibility to mood symptoms during periods of estradiol alteration.[13] To investigate whether the 3β-HSD producing-bacteria-induced decline in circulating estradiol levels was associated with molecular alterations affecting the behavioral phenotype, 5-HT levels in mouse brain and hippocampus were measured. The results showed that mice in the OVX + E2 + pTS07 group had lower brain and hippocampus 5-HT levels than mice in the OVX + E2 + pET28a group and OVX + E2 (Figures S4F and S4G).

The above results suggest that 3β-HSD produced by the gut microbiota can degrade estradiol, inducing depression-like behavior in mice.

Estrone supplementation did not affect depression-like behavior in mice

Because 3β-HSD can convert estradiol to estrone, there are at least three scenarios in the *E. coli*-pTS07 recipient mice that lead to depression-like behaviors: (1) estradiol deficiency, (2) elevated estrone levels, and (3) estradiol deficiency and elevated estrone levels. As low estradiol induced depression-like behavior in mice,[5] we supplemented the sham group with estrone (0.2 mg/kg/day) by gavage to explore the estrone effect on depression-like behavior.

The behavioral experiment results showed that there was no statistical difference in any of the behavioral experiments between the sham + E1 and sham groups (Figures 4C and S2C). These results indicated that estrone supplementation did not affect depression-like behaviors in mice. Therefore, 3β-HSD produced by gut microbiota may induce depression-like behaviors in mice by reducing estradiol levels.

Prevalence of *K. aerogenes* TS2020 and 3β-HSD in premenopausal women with depression

We used real-time qPCR to detect the relative abundance of *K. aerogenes* TS2020 and 3β-HSD in fecal DNA of 98 non-depressed and 91 depressed premenopausal women. The results showed that the relative abundance of *K. aerogenes* TS2020 ($p = 0.007$) and 3β-HSD ($p = 0.009$) was higher in the fecal DNA of the depressed premenopausal women than in the non-depressed women (Figure 5). These data indicated that *K. aerogenes* TS2020 and 3β-HSD are more widely distributed in the premenopausal women with depression.

Next, we used metagenomic sequencing to investigate whether there were other bacteria present in these patients that

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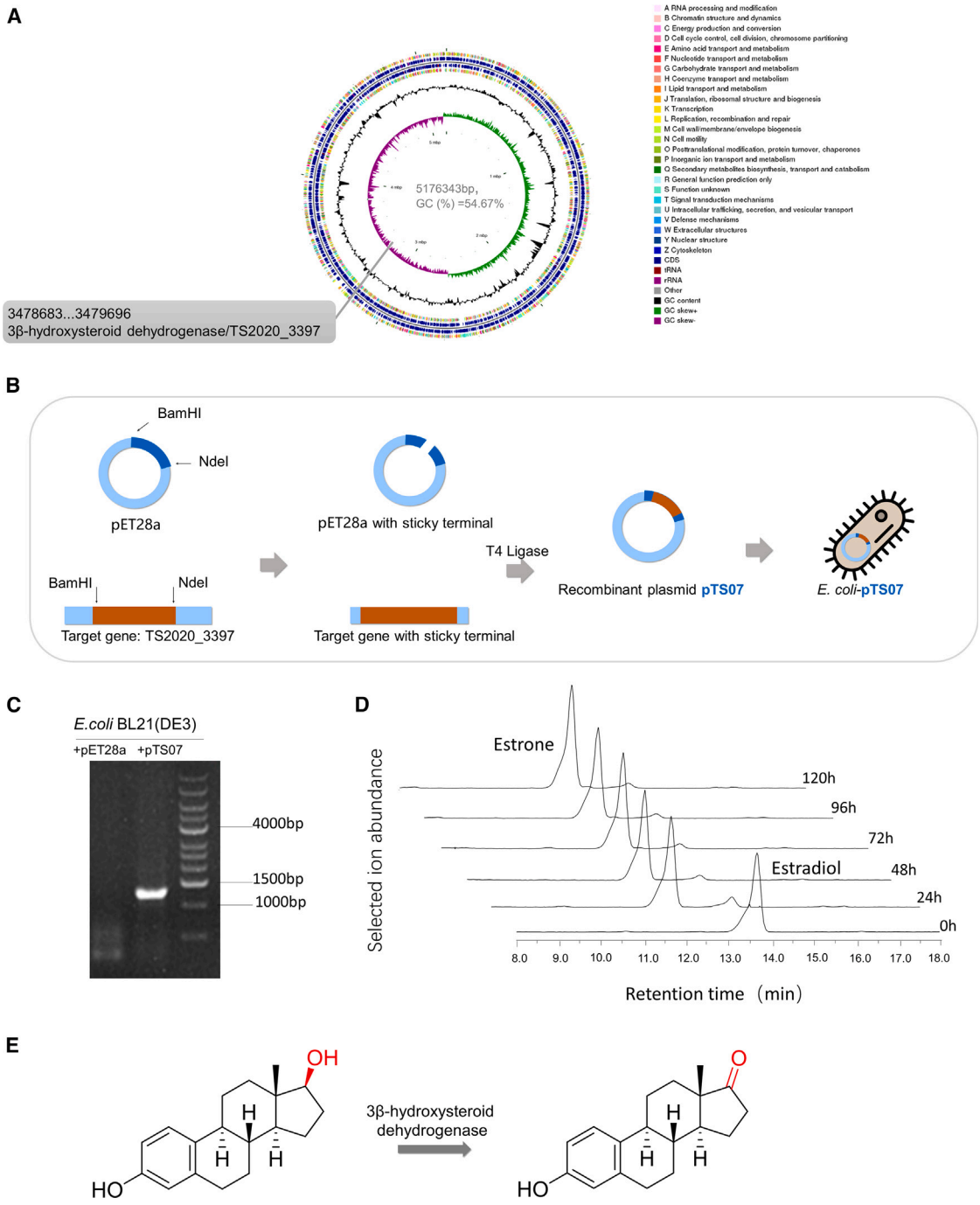

Figure 3. Identification of key enzymes for degrading estradiol in *Klebsiella aerogenes*

(A) The gene TS2020_3397 encodes 3β-hydroxysteroid dehydrogenase (3β-HSD) in *K. aerogenes* TS2020.

(B) The target gene TS2020_3397 was digested by NdeI and BamHI endonucleases, ligated by T4 DNA ligase, and cloned into the pET28a vector to obtain plasmid pTS07; the plasmid pTS07 was introduced into *E. coli* BL21(DE3) to obtain *E. coli*-pTS07.

(C) PCR confirmed the presence of the gene TS2020_3397 in *E. coli*-pTS07 and not in *E. coli*-pET28a.

(D) Combined EICs showing that *E. coli*-pTS07 containing the TS2020_3397 gene converted estradiol into estrone time dependently. *E. coli*-pTS07 was incubated with estradiol (100 mg/L) and hormones were detected using HPLC.

(E) 3β-HSD converted estradiol into estrone.

See also Figure S4.

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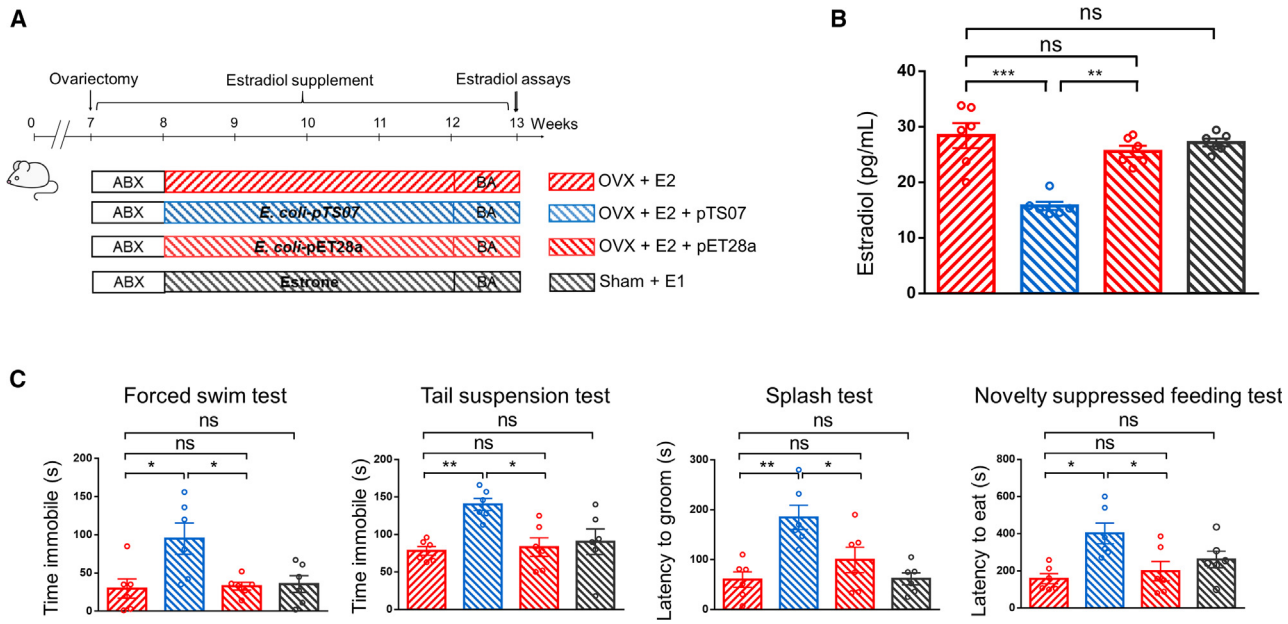

Figure 4. Gavage of *Escherichia coli*-pTS07 induces depression-like behavior in female mice

(A) Experimental timeline. All mice (aged 7 weeks) were treated with 7-day antibiotic courses. The OVX + E2 group underwent ovariectomy followed by supplemented estradiol by gavage for 6 weeks. The OVX + E2 + pTS07 group underwent ovariectomy followed by gavage estradiol for 6 weeks and *E. coli*-pTS07 for 4 weeks. The OVX + E2 + pET28a group underwent ovariectomy followed by gavage estradiol for 6 weeks and *E. coli*-pET28a for 4 weeks. The sham + E1 group received estrone by gavage for 6 weeks. All mice (aged 12 weeks) underwent behavior assessments. Serum estradiol was detected after the behavior assessments.

(B) Estradiol concentration in the OVX + E2 (n = 6), OVX + E2 + pTS07 (n = 6), OVX + E2 + pET28a (n = 6), and E1 groups (n = 6).

(C) Immobility time in FST and TST, latency to groom in the splash test, and latency to eat in the NSF in the OVX + E2 (n = 6), OVX + E2 + pTS07 (n = 6), OVX + E2 + pET28a groups (n = 6), and E1 groups (n = 6).

OVX, ovariectomy; E2, estradiol; E1, estrone.

Data are presented as means ± SEM, and one-way ANOVA with Tukey's multiple comparisons tests was performed to calculate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

See also Figure S4.

possessed 3β-HSD. The results showed that the *Bacteroides thetaiotaomicron* and *Clostridia bacterium* possess 3β-HSD.

Additionally, because the microbial factors determining serum estradiol concentrations are likely not to be solely due to this 3β-HSD enzyme, given the potential for microbial action on the deconjugation of estradiol glucuronide, fecal β-glucuronidase activity was also assessed. However, the results showed that there were no significant differences between the groups (Figure S1B).

DISCUSSION

The idea that estradiol was related to depression in women was proposed over 100 years ago.[14] Normal serum estrogen levels are beneficial for maintaining a healthy mood. Contrastingly, estradiol decline occurs during physiological conditions, including postpartum[15] and menopause,[16] predisposing women to depression. Approximately 3%–4% of women experience estradiol decline not due to menopause, lactation, or pregnancy.[17,18] The most common cause of estradiol decline is primary ovarian insufficiency affecting approximately 1 in every 100 women.[19] Other causes of estradiol decline include congenital adrenal hyperplasia, hyperprolactinemia, and polycystic ovary syndrome. However, the causes of this decline are still not fully understood.

In 1962, scholars discovered that estradiol in the gut is converted into other substances that reduce the amount of estrogen reabsorbed into the blood.[20] However, how estradiol in the gut is converted remains unknown. In the present study, we isolated *K. aerogenes*, an estrogen-degrading strain, in the gut of premenopausal women with low estrogen levels. It has been reported that this bacterium can perform some bioconversion[21,22]; however, whether it could degrade estradiol remained unknown. In the present study, we proved that *K. aerogenes* could degrade estradiol using in vitro experiments. Subsequently, animal experiments showed that *K. aerogenes* gavage decreased estrogen levels and depression-like behavior in mice. *K. aerogenes* is a facultative anaerobe. This *Klebsiella* can carry out metabolism under anaerobic conditions.[23,24] The abundance of *K. aerogenes* is increased in the gut of patients with atherosclerosis and decreased in patients with celiac disease, ulcerative colitis, pancreatic neoplasms, Crohn disease, colorectal neoplasms, and constipation, according to the Peryton database (https://dianalab.e-ce.uth.gr/peryton/#/associations).

The gut microbiota of patients with depressive disorders is characterized by increased abundance of *Actinobacteria*[25] and *Proteobacteria*.[26] Interestingly, *Actinobacteria* and *Proteobacteria* are mainly steroid degraders in nature.[27] In our previous study, we isolated a testosterone-degrading strain (*Mycobacterium*

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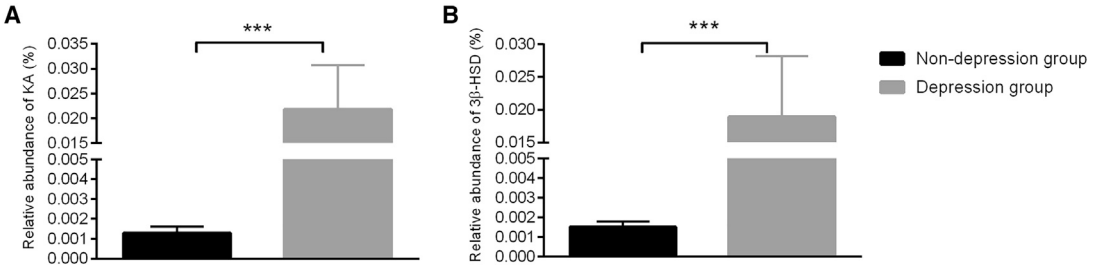

Figure 5. Prevalence of *Klebsiella aerogenes* and 3β-HSD in premenopausal women with depression

(A) *K. aerogenes* and (B) 3β-HSD are more widely distributed in the depression group (n = 91) than in the non-depression group (n = 98). KA, *K. aerogenes*. Data are presented as mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001).

See also Figure S1.

neoaurum, belonging to *Actinobacteria*) from the feces of depressed testosterone-deficient males.[28] This strain can induce depression-like behaviors in male rats. This study is a continuation and development of our previous research. In this study, we isolated an estradiol-degrading strain, *K. aerogenes*, belonging to *Proteobacteria*. *K. aerogenes* gavage induced depression-like behavior in female mice. The estradiol-degrading enzyme that we identified in the present study is 3β-HSD, which has the same name as the testosterone-degrading enzyme we found in the previous study. Moreover, we found that the 3β-HSD enzyme we previously identified in male patients with depression degrades testosterone and estradiol. Similarly, the 3β-HSD enzyme identified in this study in females with depression degrades testosterone and estradiol (data not shown). Taking these two studies together, we speculate that the 3β-HSD enzyme is involved in depression development and that this relationship is sex independent.

This study uncovered a previously unknown mechanism linking gut bacteria, estradiol, and depression in women. The relationship between intestinal bacteria and estradiol has been studied previously. Plottel and Blaser[29] found a bacterial collection called "estrobolome" in the gut, which produces β-glucuronidase that changes estrogen's bound form into a free form, resulting in increased estradiol reabsorption into the blood. This estrobolome mechanism may cause abnormally elevated serum estradiol levels, leading to the development of certain diseases, including breast cancer.[30] However, the mechanism by which intestinal bacteria lead to low estradiol has not been reported. In this study, through heterologous expression experiments, we found the genes encoding the 3β-HSD enzymes that degrade estradiol and proved that 3β-HSD expressed by gut bacteria could induce depression-like behaviors in mice. Although the abundance of *K. aerogenes* shown in our results seems very low, we believe that *K. aerogenes* in feces is not the only gut bacteria that can produce 3β-HSD. Our metagenomic sequencing data showed that the *Bacteroides thetaiotaomicron* and *Clostridia bacterium* possess 3β-HSD. However, there may be other 3β-HSD-producing gut bacteria below the detection limit of metagenomic sequencing. Which bacteria can produce 3β-HSD needs further study.

Estrogen replacement therapy is one of the interventions scholars are constantly exploring to improve depression in women.[31] As presented in our results, estradiol supplementation in mice ameliorated gavage-induced depression-like behavior. However, it is worth noting that, even though estradiol supplementation improved depression-like behaviors in mice in this study, active bacteria in the mouse gut expressing the 3β-HSD enzyme could cause depression relapse. Therefore, estradiol-degrading bacteria in the gut, particularly the enzymes expressed by these bacteria, may be better targets for intervention.

This study identified potential mechanisms between gut bacteria and estradiol in depression pathogenesis in women and revealed a mechanism by which gut bacteria degrade estradiol to cause depression. We also suggested that the estradiol-degrading bacteria and 3β-HSD enzymes may be potential intervention targets for depression in premenopausal women. Future studies should explore how to modulate the estradiol-degrading bacteria or the enzymes expressed by these bacteria in the gut.

Limitations of study

The antibiotic cocktail used to deplete the microbiota contained metronidazole, which can reach the CNS and may have potentially confounded the interpretation of the results.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cmet.2023.02.017.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.L., G.W., and Z.L.; methodology, D.L., T.S., Y.T., J.L., and H.L.; investigation, D.L., T.S., Y.T., J.L., J.T., Y.M., and J.C.; writing – original draft, D.L. and T.S.; writing – review & editing, D.L., T.S., J.L., W.J., Q.Y., and Y.T.; resources, D.L. and T.S.; funding acquisition, G.W. and Z.L.; supervision, Y.L. and G.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-His Tag Mouse Monoclonal Antibody	Abbkine	#A02050
Bacterial and virus strains		
K. aerogenes TS2020	This paper	N/A
E. coli BL21 (DE3)	Angyu	G6030G-10
Biological samples		
Human fecal and plasma samples	Renmin Hospital of Wuhan University	N/A
Chemicals, peptides, and recombinant proteins		
Neomycin	Solarbio	N8090
Metronidazole	Solarbio	M8060
Vancomycin	BioFroxx	1161GR001
Ampicillin	Aladdin	A102048
Erythromycin	Aladdin	E105345
Estradiol	Aladdin	E110145
Estrone	Aladdin	E105515
Critical commercial assays		
QIAamp PowerFecal Pro DNA Kit	Qiagen	51804
Bacterial Genomic DNA Extraction Kit	Tiangen	DP302
Mice 17β-E2 ELISA kit	Jianglai	JL50852
Mice 5-HT ELISA kit	Jianglai	JL12087
Deposited data		
Genome for *K. aerogenes* TS2020	GenBank	CP102479
Metagenomic sequencing data	Dryad	https://doi.org/10.5061/dryad.qfttdz0n4
Data S1 - Source Data	Dryad	https://doi.org/10.5061/dryad.31zcrjdqt
Experimental models: Organisms/strains		
C57BL/6J mice	Zhejiang Weitong Lihua Laboratory	N/A
Oligonucleotides		
Specific primers and probe for *K. aerogenes*	This paper	N/A
K. aerogenes forward primer (5′-GATCAATA AAATCAGGACCTCAGGC-3′)		N/A
K. aerogenes reverse primer (5′-CGTTTCTGT ATCTCAGCGATATTATCAA-3′)		N/A
Specific primers and probe for 3β-HSD	This paper	N/A
3β-HSD forward primer (5′-CCTGCCGATCT CACCGAACT -3′)		N/A
3β-HSD reverse primer (5′-GAAGGCGATGA AATATGGACGA -3′)		N/A
Universal primer EUB	Chen et al.[32]	N/A
EUB forward primer (5′-ACTCCTACGGG AGGCAGCAGT-3′)		N/A
EUB reverse primer (5′-ATTACCGCGGC TGCTGGC-3′)		N/A
Primers for constructing pTS07	This paper	N/A
pTS07 forward primer (5′- CGCGGCAGC CATATGAAGGTACTGGTTACCGGTG-3′)		N/A

(*Continued on next page*)

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pTS07 reverse primer (5′- TTCGGATCATC GTGTATTTACCGTGGTC -3′)		N/A
Recombinant DNA		
Plasmid: pTS07	This paper	N/A
Software and algorithms		
GraphPad Prism 6.01	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
SPSS 20.0	IBM	https://www.ibm.com/analytics/spss-statistics-software

RESOURCE AVAILABILITY

Lead contact

Further information and requests for reagents and resources should be directed to and will be fulfilled by the lead contact, Yan Li (yanlitf1120@163.com).

Materials availability

No unique reagents were generated in the study. The lead contact can provide strains, plasmids, or other materials in this study upon reasonable request.

Data and code availability

- *K. aerogenes* TS2020 genome and "Data S1 - Source Data" are deposited at GenBank and are publicly available as of publication. Accession numbers are listed in the key resources table.
- This paper does not report the original code.
- Any additional information required to reanalyze the data reported in this paper can be obtained from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human participants

Premenopausal female patients with depression who visited the clinical psychology and psychiatric department of the Renmin Hospital of Wuhan University (Wuhan, China) from May 2020 to January 2022 were recruited. Inclusion criteria included age between 18 and 45 years, and meeting the diagnostic criteria for depression of the Diagnostic and Statistical Manual for Psychiatric Diseases (DSM-V). The exclusion criteria included organic brain and serious physical diseases, endocrine system diseases, use of antidepressants in the past 3 months, use of steroid hormones in the past 3 months, use of hormonal contraception in past 3 months, traumatic life events, family history of mental diseases, heart or kidney diseases, endocrine system diseases, digestive system diseases, hypertension, cancer, and diabetes. Finally, 91 premenopausal females with depression were included (Data S1). In total, 98 age-matched premenopausal females without depression were included in the non-depression group. Inclusion criteria included age between 18 and 45 years, and not meeting DSM-V criteria for depression. The exclusion criteria included heart or kidney disease, endocrine disease, digestive system disease, hypertension, cancer, and diabetes.

Mice

All C57BL/6J female mice (6 weeks old) for this experiment were purchased from Zhejiang Weitong Lihua Laboratory Animal Technology (Zhejiang, China). Mice were housed at a temperature of 22°C–25°C with alternating 12-h light-dark cycles in specific pathogen-free (SPF) barrier environment. The mice were acclimated to the SPF environment for 1 week before any manipulation. To exclude the influence of the menstrual cycle on the estradiol detection, we removed the mice's ovaries and supplemented estradiol (0.2 mg/kg/day) to make serum estradiol stable until the mice were euthanized. After ovariectomy, the mice were treated with an antibiotic mixture (ABX) of 100 mg/kg metronidazole, 100 mg/kg neomycin, 50 mg/kg vancomycin, and 1 mg/mL ampicillin for 1 week before the experiment to deplete gut microbes.[33] The Laboratory Animal Welfare Ethics Committee (IACUC) of the Renmin Hospital of Wuhan University approved this study (approval number S0272107092A).

To reduce the influence of the menstrual cycle on estradiol detection and facilitate the observation of estradiol decline, serum samples were collected for analysis from all participants on days 9–12 of the menstrual cycle (late follicular phase). The medical ethics review committee of the People's Hospital of Wuhan University, China, approved this study (WDRY2021-K041). All participants signed written informed consent before inclusion in the study.

METHOD DETAILS

Detection of serum estradiol levels

Human participants fasted after 7:00 p.m. the day before the examination; 5 mL of venous blood was collected from the elbow from 6:00 a.m. to 9:00 a.m. on the same day. Human serum estradiol level was detected using LC-MS/MS. A 100-μL human serum sample was added to a 2-μL estradiol internal standard for LC-MS/MS analysis. Serum estradiol was detected and analyzed using an AB SCIEX 4500 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) and Ekspert ultraLC 100-XL system. The chromatographic column used in this experiment was ACQUITY UPLC BEH Shield RP18 (1.7 μm, 2.1 × 50 mm), flow rate was 0.3 mL/min, and column temperature was 45°C. Methanol solution containing 0.2% formic acid was used as mobile phase A, and an aqueous solution containing 0.2% formic acid as mobile phase B. Serum estradiol in mice was measured using an ELISA kit (Kamaishu, Shanghai, China).

Collection of fecal samples and extraction of fecal DNA

Each participant provided fresh stool samples from 6:00 a.m. to 9:00 a.m. According to the QIAamp PowerFecal Pro fecal DNA Extraction Kit (QIAGEN, Germany) operating steps, the fecal DNA of each subject was extracted. The extracted fecal DNA samples were stored at -20°C until analysis.

Collection of fecal microbiota

The participants' fresh fecal samples (0.25 g) were added to 10 mL of sterile normal saline. After 2000 ×g centrifugation for 1 min at 4°C, the sediment at the bottom of the sterile EP tube was discarded; the suspension was then centrifuged at 15000 ×g for 5 min at 4 °C, and the supernatant was discarded. The microbiota was then resuspended in 10 mL of normal saline.[34] The gut microbiota was used in mouse fecal transplantation.

Degradation of estradiol by gut microbiota

The patient's fecal sample (1 g) was suspended in 10 mL sterile normal saline. After full mixing, it was centrifuged at 2,000 ×g for 1 min at 4°C; the sediment at the bottom of the sterile EP tube was discarded. The suspension was then centrifuged at 15,000 ×g for 5 min, and the supernatant was discarded. Finally, 10 mL normal saline was used to resuspend the feces microbiota, and estradiol with a final concentration of 100 mg/L was added to the sterile tube. After estradiol addition, the bacterial suspension was collected at 0, 24, 48, 72 and 96 h under anaerobic conditions. Estradiol in the culture medium was detected and analyzed using an AB SCIEX 4500 QTRAP mass spectrometer (Applied Biosystems) and Ekspert ultraLC 100-XL system. The chromatographic column used in this experiment was ACQUITY UPLC BEH Shield RP18 (1.7 μm, 2.1 × 50 mm) at a flow rate of 0.3 mL/min and column temperature of 45°C. Methanol solution containing 0.2% formic acid was used as mobile phase A and an aqueous solution containing 0.2% formic acid as mobile phase B.

Gavage of microbes in mice

Mice recipients were gavaged with microbiota solution or overnight cultured bacteria (1×10^8 CFU for 3 consecutive days weekly). *E. coli*-pET28a and *E. coli*-pTS07 were incubated with 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) before gavage. Details of fecal transplant see Data S1.

Behavioral assessment

The behavioral assessment included TST, FST, splash test, and NSF. All behavioral experiments were completed within a week, and the time interval between each behavioral experiment was at least a day.

TST

The mice were acclimatized to the dark (approximately 40lx light) for 1 h. The mouse tails were wrapped with tape (approximately 2–3 mm from the tail end), and the mice were suspended approximately 20–25 cm from the ground for 6 min. The immobility time of the mice was recorded during the last 4 min.

FST

The mice were placed in a transparent glass drum with a height of 25 cm and diameter of 10 cm. The drum was filled with autoclaved water (water depth 15 cm, water temperature 25°C). Under dark conditions (approximately 40lx of light), the mice were timed for 6 min. Immobility time was recorded during the last 4 min.

Splash test experiment

A 10% sucrose solution was sprayed onto the mouse back. The mice groomed because the sucrose solution smeared their fur due to its viscous properties. After spraying the sucrose solution, the latency, grooming time, and grooming frequency of mice were recorded for 6 min.

NSF

The mice were fasted for 24 h the day before NSF and placed in a new white plastic box (length 50 cm, height 40 cm). In a bright environment (light = approximately 500lx), a piece of common feed was placed on a white filter paper approximately 10 cm

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long; the filter paper was then placed at the center of the container. The latency of the mice to nibble (touch or smell) food within 10 min was recorded. Immediately after the recording, the animals were transferred to their cages, and food consumption was measured for 5 min.

Isolation of estradiol-degrading strains

Fresh fecal pellets (0.3–0.5 g) from patients with depression were placed in a selective medium (50 mL) under anaerobic conditions. The selective medium included 5 mg/L estradiol and a basal medium (0.4 g/L $MgCl_2 \cdot 6H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 1.0 g/L NaCl, 0.5 g/L KCl, 0.25 g/L NH_4Cl, 0.2 g/L KH_2PO4, 2.52 g/L $NaHCO_3$, 0.36 g/L $Na_2S \cdot 9H_2O$). After incubating the selective medium for 3 days in a constant-temperature shaker at 37°C and 250 ×*g*, 1 mL of bacterial suspension was added to 49 mL of a new selective medium, and the process was repeated nine times. The resulting bacterial suspension was inoculated on Columbia blood solid medium to isolate colonies of bacteria.

Verification of the ability of K. aerogenes to degrade estradiol

The *K. aerogenes* sample was inoculated into an LB medium and cultured at 30°C and 220 ×*g*. When the optical density (OD) value of the bacterial liquid was 0.7–1.0, 5 mL of the bacterial suspension in the above LB medium was added to 45 mL of the fermentation medium [2.0 g/L $NaHCO_3 \cdot 10H_2O$, 0.3 g/L $MnSO_4 \cdot 4H_2O$, 0.02 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.2 g/L $ZnSO_4 \cdot 7H_2O$, 0.5 g/L $FeSO_4 \cdot 7H_2O$, 0.5 g/L $CoCl_2 \cdot 6H_2O$, 0.1 g/L $CuSO_4 \cdot 5H_2O$, 0.05 g/L $CaCl_2 \cdot 2H_2O$](1 mL)+[1.5 g/L KH_2PO4, 3.5 g/L K_2HPO4, 0.5 g/L $(NH_4)_2SO_4$, 0.15 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L NaCl, 0.005% yeast extract]. Next, 250 μL (20 g/L) estradiol solution was added to the medium simultaneously. Bacterial suspension samples were collected at 0, 24, 48, 72, 96, and 120 h. Five hundred microliters of ethyl acetate were then added to the bacterial suspension sample and centrifuged at 1,200 ×*g* for 10 min; 400 μL of the supernatant was carefully aspirated, the precipitate was discarded, and the supernatant was dried; and 20 μL ethyl acetate was re-added to reconstitute. Five microliters of the reconstituted solution in ethyl acetate was taken and spotted on a TLC plate (MacLean, Shanghai, China); 1 μL estradiol and estrone standards (both 10 g/L) were added; the plate was then placed in a developing agent prepared from ethyl acetate and petroleum ether at a ratio of 1:1, dried, and developed using ultraviolet irradiation at 265 nm.

Heterologous expression of 3β-HSD in *E. coli*

The 3β-HSD gene TS2020_3397 was overexpressed using pET28a as a vector. PCR (forward primer: 5′-CGCGGCAGCCATAT GAAGGTACTGGTTACCGGTG-3′; reverse primer: 5′ -TTCGGATCATCGTGTATTTACCGTGGTC-3′) was used to amplify the 3β-HSD fragment (*K. aerogenes* genomic DNA as template). The target gene TS2020_3397 was digested by NdeI and BamHI endonucleases, ligated by T4 DNA ligase, and cloned into the pET28a vector to obtain plasmid pTS07. The pTS07 sequence was verified by sequencing. The correct pTS07 sequence was introduced into *E. coli* BL21(DE3) to obtain the *E. coli*-pTS07, and the vector pET28a was introduced into *E. coli* BL21(DE3) to construct *E. coli*-pet28a as the control. *E. coli*-pet28a or *E. coli*-pTS07 were placed in a 50 mL LB medium, which was placed on a 37°C constant-temperature shaking table (220 rpm). When OD600 was 0.8–1.0, IPTG (0.1 mM) was added to induce the expression.

Verification of the ability of *E. coli*-pTS07 to degrade estradiol

E. coli-pTS07 was incubated in an LB medium. When OD600 was 0.8–1.0, IPTG (0.25 mM) was added into the medium for 12 h to induce 3β-HSD expression. After that, estradiol (100 mg/L) was added to the medium; 500 μL of the above suspension was sampled every 24 h and stored at -80°C until analysis. Then, 500 μL ethyl acetate was added to the bacterial suspension and centrifuged at 1,200 ×*g* for 10 min; 400 μL of the supernatant was carefully collected. Five microliters of the supernatant were taken and placed on the TLC plate (McLean, Shanghai, China); 1 μL estradiol and estrone (both 10 g/L) were added as standards; the TLC plate was placed into the developing agent prepared using ethyl acetate and petroleum ether in a ratio of 1:1, dried, and developed using ultraviolet irradiation at 265 nm.

A sample of bacterial suspension with ethyl acetate was extracted, and the supernatant was added into UtiMate 3000 (Waltham, MA, USA) for quantitative detection. The supporting chromatographic column of the UtiMate 3000 was the Xterra RP-C18 chromatographic column (Milford, MA, USA). The mobile phase was methanol/water (80:20, v/v). The flow rate was 8 mL/min. Estradiol and its products were detected at 280 nm using UV spectrophotometry.

Drug sensitivity test of *K. aerogenes*

The drug sensitivity test of *K. aerogenes* TS2020 was conducted according to the Autobio company kit (Zhengzhou, China). The *K. aerogenes* TS2020 suspension was dipped with a sterile cotton swab and laid (0.5 Michaelis turbidity) on an MH agar plate (Dijing, Guangzhou, China). The drug-sensitive paper was then stuck to the MH agar plate and incubated at 37°C for 16–18 h. Finally, the inhibition zone size was measured.

Western blot

A 0.25 g fecal sample was placed into a 10 mL sterile EP tube, and 5 mL of normal saline was added. After centrifuging at 2,000 ×*g* for 1 min at 4°C, the sediment at the bottom of the sterile EP tube was discarded; 250 μL Ripa lysate (Biyuntian, Shanghai, China) was added and centrifuged at 12,000×*g* ultrasonic for 10 min. The qmni easy TM one-step gel rapid preparation kit (10%) (Yamei, China, Shanghai) was then used to prepare the gel and perform electrophoresis. Next, electrophoretic protein was transferred to the NC

membrane (Merck, Darmstadt, Germany). It was then incubated with 5% skim milk and His tag-specific antibody (Abbkine Scientific, Wuhan, China). Finally, the NC film was processed and imaged using the ECL Kit (Biyuntian, Jiangsu, China) and G:box imager (Syngene, USA).

Prevalence of *K. aerogenes* and 3β-HSD by qPCR

For *K. aerogenes*-specific primers, we compared our *K. aerogenes* TS2020 sequencing data and the complete genome of *K. aerogenes* in the NCBI database with *Klebsiella* sequences in the NCBI database, selecting specific and conserved genes for *K. aerogenes*. Primer Premier 5.0 (Primer, Canada) was used to design specific primers against conserved *K. aerogenes* sequences. For 3β-HSD primers, Primer Premier 5.0 (Primer, Canada) was used to design specific primers against the gene TS2020_3397. The universal primer EUB was also used as an internal reference (EUB_F: 5′-ACTCCTACGGGAGGCAGCAGT-3′; EUB_R: 5′-ATTACCGCGGCTGCTGGC-3′). The relative abundance of *K. aerogenes* and 3β-HSD was calculated using $2^{-\Delta ct}$.[32]

Metagenomic sequencing

Total genomic DNA was fragmented to an average size of about 400 bp using Covaris M220 (Gene Company Limited, China), followed by sequencing on an Illumina HiSeq4000 sequencing platform (Illumina, San Diego, CA, USA) at Majorbio Bio-Pharm Technology (Shanghai, China) according to the manufacturer's protocols. The raw reads were assembled by using MEGAHIT software to obtain contigs for the following annotation and prediction.

Detection of fecal β-glucuronidase activity

Fecal β-glucuronidase activity was detected using a β-glucuronidase activity kit (Lot#A053-2-1, Nanjing Jiancheng Bioengineering Institute, China).

Detection of estradiol and 5-HT in CNS

Estradiol in CNS was detected using a mice 17β-E2 ELISA kit (Lot#JL50852, ShanghaiJianglai, China). 5-HT in CNS was detected using a mice 5-HT ELISA kit (Lot#JL12087, ShanghaiJianglai, China).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are presented as mean ± SEM. Differences between the two groups were calculated using Mann–Whitney U test. Differences among more than two groups were calculated using ANOVA with Tukey's tests. P-values of <0.05 were considered statistically significant. Non-significant differences are presented in figures as ns. Significant differences are presented in figures by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Statistical analyses were performed in SPSS (IBM, Amonk, New York, USA) and GraphPad Prism (GraphPad Software, San Diego CA).