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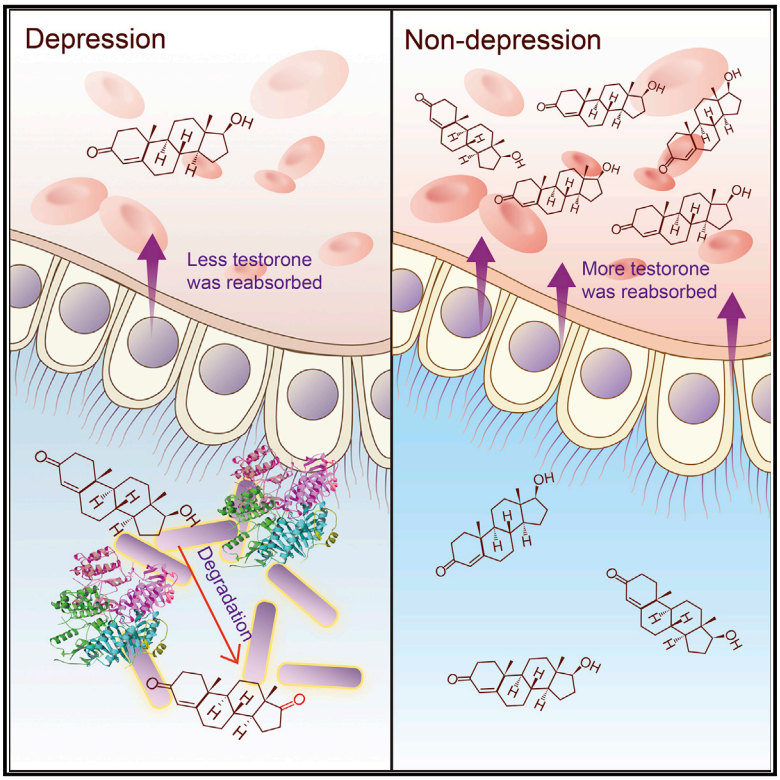
3β-Hydroxysteroid dehydrogenase expressed by gut microbes degrades testosterone and is linked to depression in males

Graphical abstract



Highlights

- *Mycobacterium neoaurum* from patients with depression can degrade testosterone
- The 3β-HSD enzyme in *M. neoaurum* degrades testosterone into androstenedione
- Gavaging rats with 3β-HSD-producing bacteria lowers testosterone and induces depression
- The prevalence of 3β-HSD is higher in patients with depression

Authors

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In brief

Li et al. isolated *Mycobacterium neoaurum* from the fecal samples of testosterone-deficient patients with depression and showed that this strain degrades testosterone. Gavaging rats with 3β-HSD-producing bacteria led to decreased testosterone levels and depression-like behavior in them, suggesting a potential causal role for gut microbes in certain types of depression.

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3β-Hydroxysteroid dehydrogenase expressed by gut microbes degrades testosterone and is linked to depression in males

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SUMMARY

Testosterone deficiency can lead to depressive symptoms in humans; however, the causes of this deficiency are incompletely understood. Here, we isolated *Mycobacterium neoaurum* from the fecal samples of testosterone-deficient patients with depression and showed that this strain could degrade testosterone *in vitro*. Furthermore, gavaging rats with *M. neoaurum* reduced their serum and brain testosterone levels and induced depression-like behaviors. We identified the gene encoding 3β-hydroxysteroid dehydrogenase (3β-HSD) as the enzyme causing testosterone degradation. Introducing 3β-HSD into *Escherichia coli* enhanced its ability to degrade testosterone. Gavaging rats with 3β-HSD-producing *E. coli* reduced their serum and brain testosterone levels and caused depression-like behaviors. Finally, compared with 16.67% of participants without depression, 42.99% (46/107) of the fecal samples of patients with depression harbored *3β-HSD*, and 60.87% (28/46) of these fecal samples expressed 3β-HSD. These results suggest that 3β-HSD expressed by gut microbes may be associated with depressive symptoms due to testosterone degradation.

INTRODUCTION

Depressive disorders are a major public health concern. In 2017, the World Health Organization (WHO) announced that depressive disorders became the leading cause of disability worldwide (W.H.O., 2017). The incidence of depression in men increases with age-related decline in testosterone levels (Bhasin and Seidman, 2019; Wu et al., 2010). However, testosterone deficiency also occurs in young men and is associated with depressive symptoms (Bremner, 2010). In the United States of America, testosterone deficiency occurs in approximately 6% of the general male population (aged 30 to 79 years) (Araujo et al., 2007). Sometimes, testosterone deficiency can be a primary manifestation (e.g., mumps orchitis and Klinefelter's syndrome), and in others, it may be a secondary manifestation (e.g., pituitary injury and excessive hunger) (Basaria, 2014). However, the causes of testosterone deficiency in patients with depression have not been clarified.

Testosterone is a neuroactive steroid hormone produced by the testis, adrenal glands, and adipose tissue (Baulieu, 1991). After being metabolized by the liver, testosterone is excreted through the bile into the gut, and then 83% of it is reabsorbed into the blood (Sandberg and Slaunwhite, 1956). Recently, it was reported that gut microbes influence the levels of some steroid hormones by shifting the ratio of active and inactive forms of the steroid hormones (Needham et al., 2020). Therefore, steroid hormone metabolism by gut microbes may influence the onset or treatment of certain diseases. For example, the gut microbiota contribute to endocrine resistance in castration-resistant prostate cancer by providing an alternative source of androgens (Pernigoni et al., 2021). The gut microbiota of patients with depressive disorders differ significantly from those of healthy controls and are characterized by increased abundances of *Proteobacteria* (Jiang et al., 2015), *Actinobacteria* (Sharon et al., 2016), and *Bacteroides* (Zheng et al., 2016). In diverse natural environments, *Proteobacteria* and *Actinobacteria* have been identified as steroid degraders (Holert et al., 2018). Thus, the gut microbiota in patients with depression are likely to have a stronger ability to degrade steroids than those in normal individuals.

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Given that testosterone is a steroid hormone, we hypothesized that some microbes in the gut of patients with depression may degrade testosterone, resulting in decreased serum testosterone levels and subsequent depression. Accordingly, in this study, we evaluated the relationships among testosterone deficiency, depression, and the gut microbiota.

RESULTS

Testosterone deficiency in participants with and without depression

To explore the incidence of testosterone deficiency in patients with depression, psychiatric in patients who met the diagnostic criteria for major depressive disorder (MDD) or dysthymic disorder from the *Diagnostic and Statistical Manual for Psychiatric Diseases* (DSM-V) were included in the depression group (n = 77). Healthy participants who did not meet the DSM-V diagnostic criteria for MDD or dysthymic disorder were included in the non-depression group (n = 102).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was conducted to quantify the serum testosterone levels in the 2 groups. The average serum testosterone level was significantly higher in the non-depression group (549.24 ± 269.16 ng/dL) than in the depression group (312.97 ± 173.18 ng/dL; $p < 0.001$; Figure S1A). According to the European Association of Urology, European Academy of Andrology, and International Society of Andrology, testosterone thresholds for the diagnosis of testosterone deficiency are less than 350 ng/dL (Aversa and Morgentaler, 2015). The incidence rates of testosterone deficiency in the depression and non-depression groups were 54.55% (42/77) and 11.76% (12/102), respectively. Antidepressants affect serum testosterone levels because fluoxetine, venlafaxine, and ketamine decrease the testosterone levels after treatment in men whereas amitriptyline and imipramine could increase testosterone levels (Pavlidi et al., 2021). Therefore, we excluded 47 patients from the depression group who used antidepressants and found that the incidence of testosterone deficiency was 30.00% (9/30) in untreated patients with depression.

Fecal transplantation from patients with depression induced depression-like behavior in rats

To investigate whether the gut microbiota from patients with depression could degrade testosterone, we cultured the gut microbiota with testosterone (2 g/L). A thin-layer chromatography (TLC) analysis showed that in 83% (5/6) of the depressed patients, the feces can degrade testosterone into androstenedione (AD), and in 67% (4/6) of depressed patients, feces can further degrade AD. All (6/6) feces samples from the non-depressed group showed no obvious testosterone degradation (Figure S2).

Furthermore, to determine whether the gut microbiota from patients with depression could decrease testosterone levels and cause depression-like behavior, we performed fecal transplantation by gavage. The rats were divided into the following 3 groups: (1) control group, administered 1 mL of saline by gavage once daily for 3 successive days; (2) non-depression microbiota group, transplantation with 1 mL of the gut microbiota from a non-depressive participant once daily for 3 successive days; and (3) depression microbiota group, transplantation with 1 mL of the gut microbiota from a depressive patient once daily for 3 successive days (Figure 1A).

Because all the rats were treated with an antibiotic cocktail (ABX) in their drinking water for 1 week to deplete the gut microbiota before gavage, which may affect the level of serum testosterone, we detected the level of serum testosterone between untreated rats and ABX-treated rats. Four weeks after treatment, there was no obvious difference between the 2 groups (Figure S1B), which further shows that ABX treatment had no influence on the level of serum testosterone in this condition. Then, we gavaged the ABX-treated rats with the gut microbiota from participants; the quantification of serum testosterone using LC-MS/MS showed that the serum testosterone levels in the depression microbiota group were significantly lower than those in the control group (118 ± 31 ng/dL versus 232 ± 90 ng/dL; $p = 0.018$; Figure 1B) and non-depression microbiota group (118 ± 31 ng/dL versus 225 ± 75 ng/dL; $p = 0.033$; Figure 1B). Next, we quantified the level of brain testosterone using LC-MS/MS and showed that the brain testosterone levels in the depression microbiota group were statistically significantly lower than those in the control group (5.04 ± 2.37 ng/g versus 12.61 ± 3.78 ng/g; $p = 0.042$; Figure 1C) and non-depression microbiota group (5.04 ± 2.37 ng/g versus 14.39 ± 7.16 ng/g; $p = 0.012$; Figure 1C).

Behavioral experiments showed that in the tail suspension test (TST) and forced swimming test (FST), the immobility time of the rats in the depression microbiota group was longer than that of the rats in the control and non-depression microbiota groups, and in the novelty-suppressed feeding (NSF) and splash tests, the latent time of the rats in the depression microbiota group was longer than that of the rats in the control and non-depression microbiota groups (Figures 1D and S3A–S3C).

These results suggested that the microbiota from patients with depression can decrease the levels of serum and brain testosterone and induce depression-like behavior in rats.

Isolation of testosterone-degrading bacteria from testosterone-deficient patients with depression

Because the degradation of testosterone by gut microbes can reduce serum testosterone, we used testosterone as the sole carbon source (Yang et al., 2011) to culture the feces of an untreated patient with depression; we isolated several bacteria, but only one could degrade testosterone (Figure 2A). Then, we performed sequencing analysis for its identification. In the phylogenetic tree, the 16S rRNA sequence of the strain displayed similarities of 99.0%–99.8% to *Mycobacterium neoaurum* and 98.6%–99.7% to other species of *Mycobacterium*. We named this strain *M. neoaurum* MN2019. Although *M. neoaurum* was isolated using testosterone as the sole carbon source, this strain can also grow in a medium with a general carbon source, such as in Luria-Bertani (LB) or blood agar plates. Drug sensitivity tests showed that *M. neoaurum* MN2019 was sensitive to erythromycin and amikacin; the minimum inhibitory concentration (MIC) of erythromycin in *M. neoaurum* was 0.25 mg/L and that of amikacin in *M. neoaurum* was 0.75 mg/L (Figure S4A). *M. neoaurum* was originally isolated from soil in 1972 (Tsukamura, 1972) and has also been identified in clinical infections, such as bloodstream infections (Washer et al., 2007), prosthetic valve endocarditis (Kumar et al., 2014), vascular catheter infections (George and Schlesinger, 1999), and pulmonary infections

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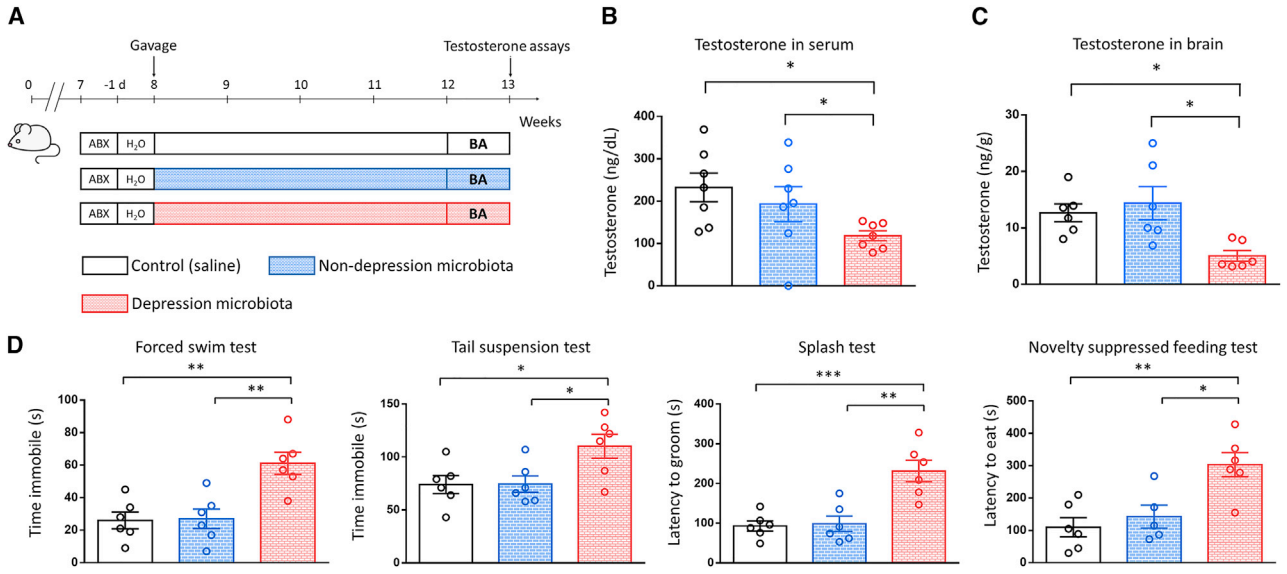

Figure 1. Rats that received fecal transplantation from patients with depression exhibited depression-like behaviors

(A) Experimental timeline for the control rats, non-depression-microbiota-recipient rats, and depression-microbiota-recipient rats. All rats (aged 7 weeks) were treated with 7 days of antibiotics followed by 1-day autoclaved water before being gavaged. The control rats were gavaged with saline (1 mL) once daily for 3 successive days. The non-depression-microbiota group rats were gavaged with 1 mL of gut microbiota from a non-depression participant once daily for 3 successive days. Depression microbiota group rats were gavaged with 1 mL of gut microbiota from a patient with depression once daily for 3 successive days. Four weeks after the first day of gavage, behavioral observation of rats was performed within a week, followed by testosterone levels measurement. BA, behavior assays.

(B) Concentration of testosterone in the serum of the control rats (n = 6), non-depression-microbiota-recipient rats (n = 6), and depression-microbiota-recipient rats (n = 6), as determined using targeted LC-MS/MS.

(C) Concentration of testosterone in the brain of the control rats (n = 6), non-depression-microbiota recipient rats (n = 6), and depression-microbiota-recipient rats (n = 6), as determined using targeted LC-MS/MS.

(D) Time spent immobile in the FST, time spent immobile in the TST, latency to groom in the splash test, and latency to eat in the NSF for the control rats (n = 6), non-depression-microbiota-recipient rats (n = 6), and depression-microbiota-recipient rats (n = 6). Data are presented as means ± standard errors of the means (± SEM), and one-way analysis of variance with Tukey's multiple comparisons tests was performed to calculate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

See also Figures S1–S3.

(Morimoto et al., 2007). Although *M. neoaurum* can transform several sterols (Wei et al., 2010), its ability to degrade testosterone has not been reported.

We used a fermentation medium for sterol degradation and added testosterone (2 g/L) as the carbon source for assisted growth to verify the degradation ability of *M. neoaurum*, in accordance with a previous study (Sun et al., 2019). TLC results showed that compared with those of the standards, the testosterone levels gradually decreased and the levels of AD, the main product, gradually increased (Figure S4B). High-performance liquid chromatography (HPLC) quantitative analysis showed that the testosterone levels decreased. After 96 h of culture, the testosterone level decreased by 91.78%. Metabolites included AD, androsta-1,4-diene-3,17-dione (ADD), and 9α-hydroxyandrosta-1,4-diene-3,17-dione (Figures 2B and 2C). The main metabolite was AD, accounting for 91.85% of all metabolites (Figure 2C). These results indicated that testosterone could be degraded by *M. neoaurum*.

Gavage of *M. neoaurum* induced depression-like behavior in rats

To explore whether *M. neoaurum* in the gut could cause depression-like behavior, we administered *M. neoaurum* by gavage to rats. The rats were divided into the following 3 groups: (1) control group, administered 1 mL of saline by gavage once daily for 3 successive days; (2) *M. neoaurum*-recipient group, administered *M. neoaurum* (approximately 1×10^8 colony-forming units [cfu] once daily for 3 successive days); and (3) erythromycin treatment group, treated with erythromycin (10 mg/kg once daily for 7 successive days) after the oral administration of *M. neoaurum* (Figure 3A).

The quantification of the serum testosterone levels using LC-MS/MS showed that the serum testosterone levels in *M. neoaurum*-recipient rats were significantly lower than those in the control group (93 ± 75 ng/dL versus 243 ± 104 ng/dL; $p = 0.002$; Figure 3B). Next, we quantified the level of brain testosterone using LC-MS/MS and showed that brain testosterone levels in *M. neoaurum*-recipient rats were statistically significantly lower than those in the control group (4.56 ± 3.07 ng/g versus 12.41 ± 4.67 ng/g; $p < 0.001$; Figure 3C).

Behavioral experiments showed that in the TST and FST, the immobility time of the *M. neoaurum*-recipient rats was longer than that of the control rats, and in the NSF and splash tests, the latent time of the *M. neoaurum*-recipient rats was longer than that of the control rats (Figures 3D and S3D–S3F). To evaluate the survival of *M. neoaurum* following oral gavage, we

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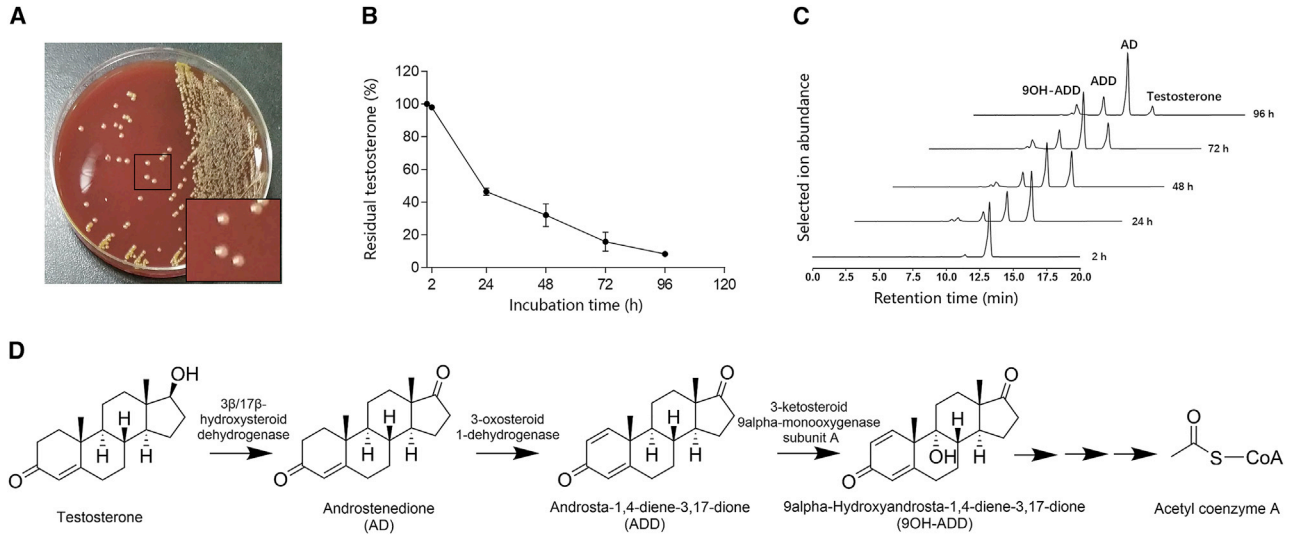

Figure 2. Isolation and validation of testosterone-degrading bacteria

(A) Images of typical colonies of *M. neoaurum*.

(B) Time course of testosterone degradation by *M. neoaurum*. Data points indicate mean testosterone levels ± standard deviations (3 biological replicates).

(C) Combined extracted ion chromatograms (EICs) showing time-dependent degradation of testosterone by *M. neoaurum*. The strain was grown with 2 g/L testosterone, and aliquots from the indicated time points were analyzed using HPLC.

(D) Testosterone degradation pathway.

See also Figure S4.

cultured the feces of the *M. neoaurum*-recipient rats after the behavioral test, and colonies looking like *M. neoaurum* were isolated and further identified using 16S rRNA sequencing for confirmation. However, no *M. neoaurum* was isolated from the feces of the control rats. Next, we also performed real-time reverse transcription-polymerase chain reaction (qRT-PCR) on total RNA extracted from the feces of the *M. neoaurum*-recipient rats. We selected specific marker genes for *M. neoaurum* (Data S1) to design specific primers for *M. neoaurum* and showed that the gene transcript was in the gut of the *M. neoaurum*-recipient rats (n = 12) and not in the gut of the control rats (n = 12) (Figures S5A and S5B). These results demonstrate that *M. neoaurum* survived in the gut of the *M. neoaurum*-recipient rats, from gavage to the behavioral test.

To determine whether antibiotic treatment could alleviate the serum testosterone deficiency and depression-like behaviors of the *M. neoaurum* recipients, we administered erythromycin for 1 week (10 mg/kg daily) by gavage (Figure 3A). Two weeks after erythromycin gavage, LC-MS/MS analysis showed that the level of serum testosterone in the *M. neoaurum* recipients treated with erythromycin (238 ± 115 ng/dL) recovered to the level of that in the control group (243 ± 104 ng/dL; p = 0.994; Figure 3B). We also quantified the level of brain testosterone using LC-MS/MS and showed that the level of brain testosterone in the *M. neoaurum* recipients treated with erythromycin (11.45 ± 4.78 ng/g) recovered to the level of that in the control group (12.41 ± 4.67 ng/g; p = 0.844; Figure 3C).

In behavioral experiments, the immobility time in the FST and TST and the latent time in the NSF and splash tests were similar in the erythromycin-treated *M. neoaurum* rats and control rats. These results indicated that the depression-like behavior of the *M. neoaurum* recipients was alleviated after erythromycin treatment (Figures 3D and S3D–S3F). To determine whether erythromycin treatment affects the survival of *M. neoaurum*, we cultured the feces of the erythromycin-treated *M. neoaurum* rats after the behavioral test, and no *M. neoaurum* were isolated. Next, we performed qRT-PCR on the total RNA content extracted from the feces of the erythromycin-treated *M. neoaurum* rats and showed that the gene was not transcript in the gut of the erythromycin-treated *M. neoaurum* rats (n = 12) (Figure S5C). These results demonstrate that *M. neoaurum* were killed by erythromycin.

These data showed that gavage with *M. neoaurum* decreased the serum and brain testosterone levels and induced depression-like behaviors in rats. Moreover, supplementation with erythromycin normalized the testosterone levels and behavior.

Identification of the key enzyme mediating testosterone degradation in *M. neoaurum*

We reasoned that the effects of *M. neoaurum* gavage on depression-like behaviors could be attributed to its capacity to degrade testosterone; therefore, we hypothesized that the construction of a testosterone-degrading strain by introducing a testosterone-degrading enzyme gene could induce depression-like behaviors after its administration by gavage in rats. To construct recombinant *Escherichia coli*, we attempted to find the gene sequence of a testosterone-degrading enzyme in *M. neoaurum*.

Information on steroid degradation was processed using BLAST from the Kyoto Encyclopedia of Genes and Genomes database (https://www.kegg.jp/) (Moriya et al., 2007). Unfortunately, the testosterone-degrading enzyme 3β/17β-hydroxysteroid dehydrogenase (EC 1.1.1.51) has not been identified in *M. neoaurum* VKM Ac-1815D. Therefore, we next sequenced the genome of *M. neoaurum* MN2019. In the *M. neoaurum* MN2019 chromosome (66.26% GC content in nucleobase,

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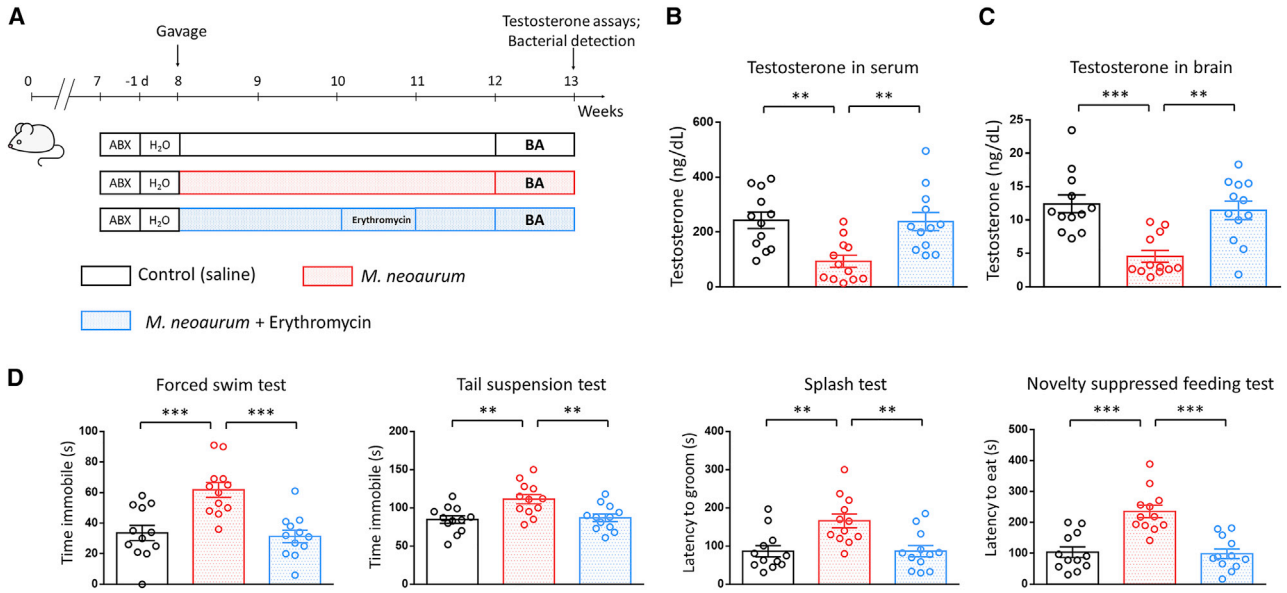

Figure 3. *M. neoaurum*-recipient rats exhibited depression-like behaviors

(A) Experimental timeline for control rats, *M. neoaurum*-recipient rats, and *M. neoaurum*-recipient rats treated with erythromycin. All rats (aged 7 weeks) were treated with 7-day antibiotics followed by 1-day autoclaved water before being gavaged. The control rats were gavaged with saline (1 mL) once daily for 3 successive days. The *M. neoaurum*-recipient rats were gavaged with *M. neoaurum* (approximately 1×10^8 cfu) once daily for 3 successive days. The *M. neoaurum*-recipient rats treated with erythromycin (*M. neoaurum* + erythromycin) were gavaged with *M. neoaurum* (approximately 1×10^8 cfu) once daily for 3 successive days; then, erythromycin treatment (10 mg/kg by gavage) started when rats aged 10 weeks and lasted for 1 week. Four weeks after the first day of gavage, behavioral observation of the rats was performed within a week, followed by testosterone levels measurement and bacterial detection. BA, behavior assays.

(B) Concentration of testosterone in the serum of the control rats (n = 12), *M. neoaurum*-recipient rats (n = 12), and *M. neoaurum*-recipient rats treated with erythromycin (n = 12), as determined using targeted LC-MS/MS.

(C) Concentration of testosterone in the brain of the control rats (n = 12), *M. neoaurum*-recipient rats (n = 12), and *M. neoaurum*-recipient rats treated with erythromycin (n = 12), as determined using targeted LC-MS/MS.

(D) Time spent immobile in the FST, time spent immobile in the TST, latency to groom in the splash test, and latency to eat in the NSF for the control rats (n = 12), *M. neoaurum*-recipient rats (n = 12), and *M. neoaurum*-recipient rats treated with erythromycin (n = 12). Data are presented as means ± standard errors of the means (± SEM), and one-way analysis of variance with Tukey's multiple comparisons tests was performed to calculate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

See also Figures S3 and S5 and Data S1.

accession number: CP074376), the gene MN2019_09885, encoding 3β-hydroxysteroid dehydrogenase (3β-HSD), was identified via annotation (Figure 4A; Data S2). To construct a testosterone-degrading strain, the pET28a containing MN2019_09885 gene was transformed into *E. coli* BL21 (DE3) cells and *E. coli* BL21 (DE3)/pDL01 was obtained (Figure S6A). The vector pET28a was introduced into *E. coli* BL21 (DE3) to construct *E. coli* BL21 (DE3)/pET28a as a control (Figure S6B). Using PCR, we verified that the MN2019_09885 gene was found in *E. coli* BL21 (DE3)/pDL01 but not in *E. coli* BL21 (DE3)/pET28a (Figure S6C).

Subsequently, we incubated *E. coli* BL21 (DE3)/pDL01 or *E. coli* BL21 (DE3)/pET28a with testosterone (2 g/L) in LB medium. TLC analysis showed that, compared with the standards, *E. coli* BL21 (DE3)/pDL01 transformed testosterone into AD in a time-dependent manner (Figure S6D). In contrast, testosterone was not transformed by *E. coli* BL21 (DE3)/pET28a (Figure S6E). The metabolites were identified using LC-MS/MS, which confirmed that the product of testosterone degradation by *E. coli* BL21 (DE3)/pDL01 was AD (Figure 4B). Testosterone and AD levels were quantified using HPLC, and *E. coli* BL21 (DE3)/pDL01 could convert 71.20% of testosterone into AD within 24 h and 89.41% of testosterone into AD within 48 h (Figure 4C).

To explore the activity of the 3β-HSD protein encoded by the MN2019_ 09885 gene, we overexpressed 3β-HSD with His-Tag in *E. coli* BL21 (DE3) and purified the enzyme using Ni-NTA column. The purified-enzyme (Figure S6F) kinetic constants k_{cat} and K_M for testosterone hydroxysteroid dehydrogenase reactions were determined (Figure 4D). The enzyme 3β-HSD efficiently catalyzed the β-hydroxy dehydrogenation of testosterone with k_{cat}, K_M, and k_{cat}/K_M values of 0.222 ± 0.017 min^{-1}, 101.9 ± 25.46 μM, and 0.002 min^{-1} μM^{-1}, respectively, for 3β oxidation. In a previous study, the k_{cat}, K_M, and k_{cat}/K_M values of the 17β-HSD from *Comamonas testosteroni* were 0.0012 ± 0.0002 min^{-1}, 11.8 ± 2.0 μM, and 0.0001 min^{-1} μM^{-1}, respectively (Benach et al., 2002), indicating that 3β-HSD in *M. neoaurum* had a higher testosterone degradation activity. These data showed that the MN2019_09885 gene in *M. neoaurum* would express 3β-HSD in *E. coli*.

Administration of *E. coli* BL21 (DE3)/pDL01 induced depression-like behaviors in rats

We next assessed whether the rats administered with *E. coli* BL21 (DE3)/pDL01 by gavage experienced depression-like

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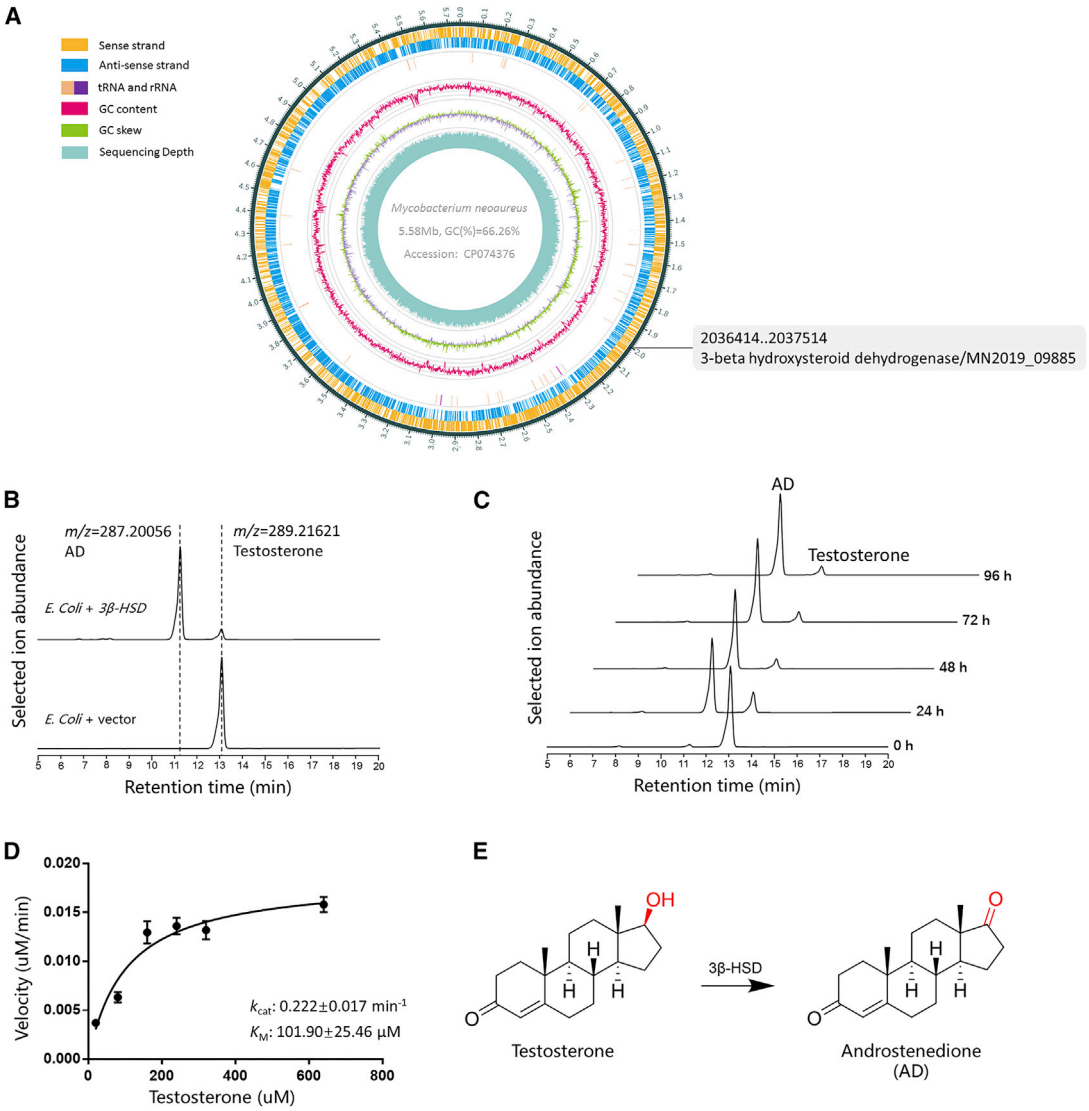

Figure 4. Assessment of the 3β-dehydrogenation pathway *in vitro*

(A) Structure and distribution of *3β-HSD* for testosterone degradation on the chromosome of *M. neoaurum* MN2019. The proposed gene products are enclosed in gray boxes.

(B) Combined EICs showing the conversion of testosterone to AD by *E. coli* BL21 (DE3)/pDL01 harboring *3β-HSD* versus a control strain of *E. coli* BL21 (DE3)/pET28a harboring the vector plasmid pET28a. The strains were grown with 2 g/L testosterone for 96 h, extracted with ethyl acetate, and analyzed using LC-MS. The experiment was independently repeated three times.

(C) Combined EICs showing time-dependent conversion of testosterone to AD by engineered *E. coli*. The strain was grown with 2 g/L testosterone, and aliquots from the indicated time points were quantified using HPLC. The experiment was repeated twice, independently, and showed similar results.

(D) Nonlinear regression for testosterone reduction catalyzed by 3β-HSD. The reaction mixture contained 200 μM NAD^+, 25 mM Tris-HCl (pH 7.6), 5 μM 3β-HSD, and testosterone at 37°C. Enzyme kinetic constants $k_{cat} = 0.222 \pm 0.017\ min^{-1}$ and $K_M = 101.9 \pm 25.46$ μM. Data points indicate mean levels ± SEM (3 biological replicates).

(E) Proposed pathway for the 3β-dehydrogenation of testosterone to AD.

See also Figure S6.

behaviors. The rats were divided into 3 groups: (1) control group, administered 1 mL of saline by gavage once daily for 3 successive days; (2) *E. coli* BL21 (DE3)/pDL01-recipient group, administered *E. coli* BL21 (DE3)/pDL01 (approximately 1×10^8 cfu once daily for 3 successive days) by gavage; and (3) *E. coli* BL21 (DE3)/pET28a-recipient group, administered *E. coli* BL21 (DE3)/pET28a by gavage (approximately 1×10^8 cfu once daily for 3 successive days; Figure 5A).

The quantification of the serum testosterone levels using LC-MS/MS demonstrated that the serum testosterone levels in the *E. coli* BL21 (DE3)/pDL01 recipients was significantly lower than those in the control group (94 ± 60 ng/dL versus 246 ± 134 ng/dL; p = 0.007), with no significant changes observed in the serum testosterone levels of the *E. coli* BL21 (DE3)/pET28a recipients compared with those of the control rats (221 ± 93 ng/dL versus 246 ± 134 ng/dL; p = 0.975; Figure 5B).

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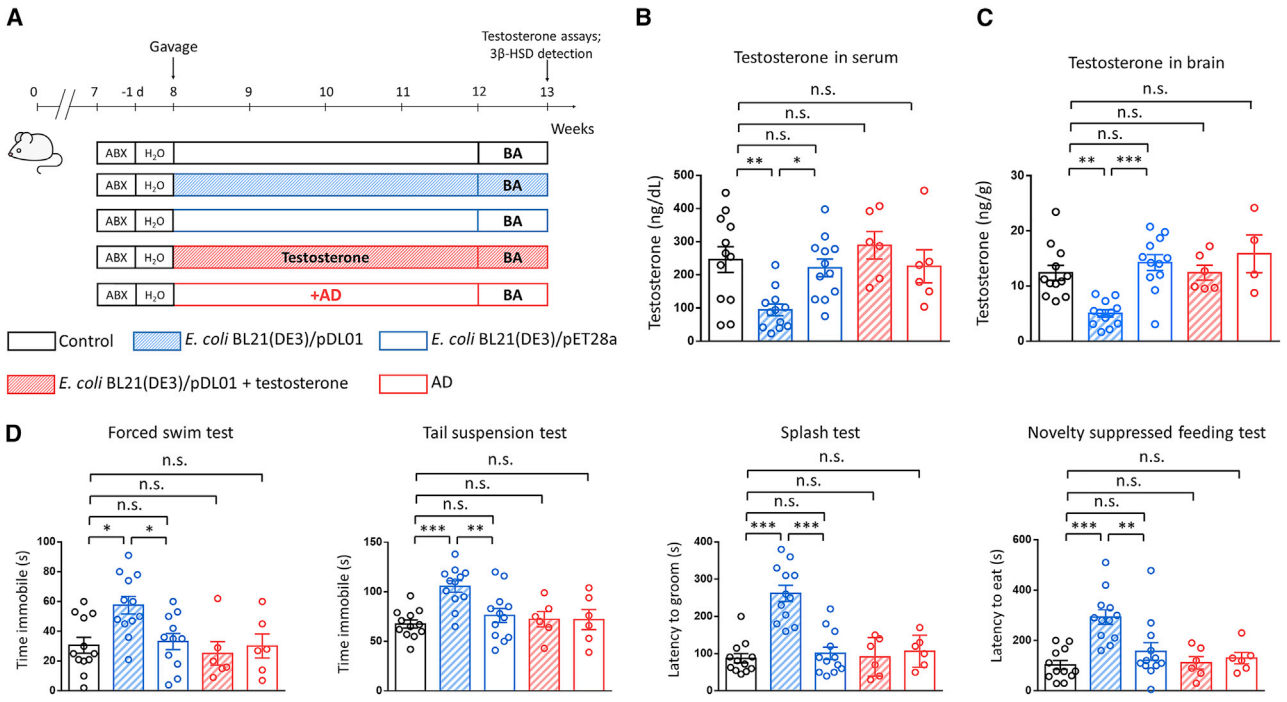

Figure 5. Administration of *E. coli* BL21 (DE3)/pDL01 by gavage induced depression-like behaviors in rats

(A) Experimental timeline for the control rats, *E. coli* BL21 (DE3)/pDL01 recipients, *E. coli* BL21 (DE3)/pET28a recipients, *E. coli* BL21 (DE3)/pDL01 recipients treated with testosterone, and the AD-treated rats. All rats (aged 7 weeks) were treated with 7-day antibiotics followed by 1-day autoclaved water before being gavaged. The control rats were gavaged with saline (1 mL) for 3 successive days. The *E. coli* BL21 (DE3)/pDL01 recipients and *E. coli* BL21 (DE3)/pET28a recipients were gavaged with *E. coli* BL21 (DE3)/pDL01 or *E. coli* BL21 (DE3)/pET28a (approximately 1×10^8 cfu) for 3 successive days. The *E. coli* BL21 (DE3)/pDL01 recipients treated with testosterone (*E. coli* BL21 (DE3)/pDL01+testosterone) were treated with 0.5 mg testosterone daily for 28 days by gavage at the beginning of the day, after *E. coli* BL21 (DE3)/pDL01 gavage. The AD-treated rats (AD) were treated with AD (0.5 mg/day for 28 days) by gavage, without any bacterial gavage. Four weeks after the first day of gavage, behavioral observation of rats was performed within a week, followed by testosterone levels measurement and 3β-HSD detection. BA, behavior assays.

(B) Concentration of testosterone in the serum of control rats (n = 12), *E. coli* BL21 (DE3)/pDL01 recipients (n = 12), *E. coli* BL21 (DE3)/pET28a recipients (n = 12), *E. coli* BL21 (DE3)/pDL01 recipients treated with testosterone (n = 6), and the AD-treated rats (n = 6), as detected by using LC-MS/MS.

(C) Concentration of testosterone in the brain of the control rats (n = 12), *E. coli* BL21 (DE3)/pDL01 recipients (n = 12), *E. coli* BL21 (DE3)/pET28a recipients (n = 12), *E. coli* BL21 (DE3)/pDL01 recipients treated with testosterone (n = 6), and the AD-treated rats (n = 6), as detected by using LC-MS/MS.

(D) Time spent immobile in the FST, time spent immobile in the TST, latency to groom in the splash test, and latency to eat in the NSF for the control rats (n = 12), *E. coli* BL21 (DE3)/pDL01 recipients (n = 12), *E. coli* BL21 (DE3)/pET28a (n = 12), *E. coli* BL21 (DE3)/pDL01 recipients treated with testosterone (n = 6), and the AD-treated rats (n = 6). *E. coli* BL21 (DE3)/pDL01: plasmid pDL01, which contained *3β-HSD*, was introduced into *E. coli* BL21 (DE3). *E. coli* BL21 (DE3)/pET28a: vector plasmid pET28a was introduced into *E. coli* BL21 (DE3). Data are presented as mean levels ± SEM, and one-way analysis of variance with Tukey's multiple comparisons tests was performed to calculate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

See also Figure S3 and Data S2.

Next, we quantified the level of brain testosterone using LC-MS/MS and showed that the brain testosterone levels in the *E. coli* BL21 (DE3)/pDL01 recipients were significantly lower than those in the control group (5.04 ± 2.28 ng/g versus 14.03 ± 5.01 ng/g; $p < 0.001$; Figure 5C) and *E. coli* BL21 (DE3)/pET28a recipients (5.04 ± 2.28 ng/g versus 14.27 ± 4.91 ng/g; $p < 0.001$; Figure 5C).

Behavioral experiments showed that in the FST and TST, the immobility time of the *E. coli* BL21 (DE3)/pDL01 recipients was longer than that of the *E. coli* BL21 (DE3)/pET28a recipients and the control rats. In the NSF and splash tests, the latent time was longer in the *E. coli* BL21 (DE3)/pDL01 recipients than in the *E. coli* BL21 (DE3)/pET28a recipients and control rats (Figures 5D and S3G–S3I). No significant depression-like behaviors were observed in the *E. coli* BL21 (DE3)/pET28a recipients compared with the control rats (Figures 5D and S3G–S3I). Next, to verify whether 3β-HSD is expressed during the experiment, we conducted a western blot using the protein obtained from feces samples after behavioral assays. No obvious bands in the samples from the rats gavaged with *E. coli* BL21(DE3)/pET28a recipients were observed, whereas bands were observed in the samples from the *E. coli* BL21 (DE3)/pDL01 recipients (Figures S6G and S6H), suggesting that the *E. coli* BL21 (DE3)/pDL01 recipients expressed 3β-HSD during the experiment.

Taken together, these results indicated that expression of 3β-HSD in the gut could degrade serum and brain testosterone to induce depression-like behaviors in rats.

Testosterone supplementation alleviated depression-like behaviors in *E. coli* BL21 (DE3)/pDL01 recipients

To further evaluate the roles of testosterone deficiency in the depression-like behaviors of the *E. coli* BL21 (DE3)/pDL01

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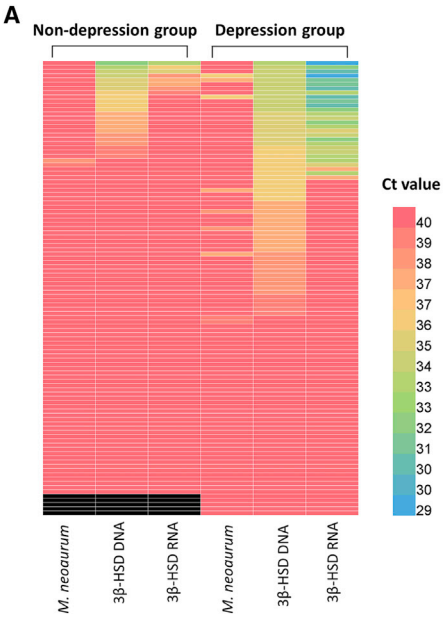

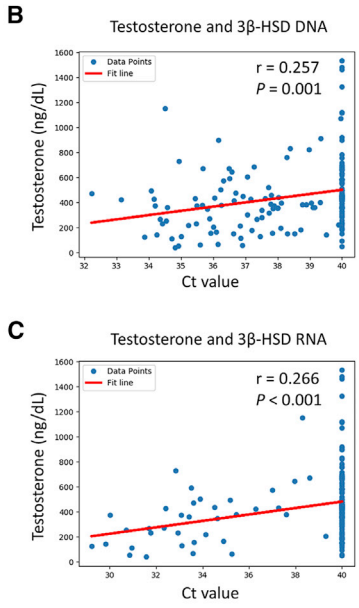

Figure 6. Metagenomic prevalence of *M. neoaurum* and *3β-HSD*

(A) The metagenomic prevalence of *M. neoaurum* and *3β-HSD* was examined in non-depression individuals (n = 102) and patients with depression (n = 107). A Ct value of less than 38 was considered positive.

(B) Correlation analysis between the Ct value of *3β-HSD* DNA and serum testosterone (n = 184).

(C) Correlation analysis between the Ct value of *3β-HSD* RNA and serum testosterone (n = 184).

recipients, we determined whether testosterone supplementation could alleviate these phenotypes. The *E. coli* BL21 (DE3)/pDL01 recipients were treated with 0.5 mg testosterone daily for 28 days by gavage, beginning from the day after the *E. coli* BL21 (DE3)/pDL01 gavage (Spritzer et al., 2011; Figure 5A).

The quantification of serum testosterone using LC-MS/MS demonstrated that the testosterone levels in *E. coli* BL21 (DE3)/pDL01 recipients (289 ± 101 ng/dL) were restored to the level of those in the control rats (246 ± 134 ng/dL) after testosterone supplementation (Figure 5B). The brain testosterone levels in the *E. coli* BL21 (DE3)/pDL01 recipients (12.44 ± 3.32 ng/g) were restored to the level of those in the control rats (14.03 ± 5.01 ng/g, p = 0.989) (Figure 5C). Results from the behavioral experiments showed that the immobility time in the FST and TST and the latent time in the NSF and splash tests in the *E. coli* BL21 (DE3)/pDL01 recipients with testosterone were similar to those of the control rats (Figures 5D and S3G–S3I), which suggested that testosterone supplementation could alleviate depression-like behaviors in the *E. coli* BL21 (DE3)/pDL01 recipients. Next, to verify whether 3β-HSD is expressed during the experiment, we conducted western blotting using the protein obtained from feces samples after behavioral assays. Bands were observed in the samples from the *E. coli* BL21 (DE3)/pDL01 recipients treated with testosterone (Figure S6I), suggesting that 3β-HSD was expressed in the gut of rats during the experiment.

Because 3β-HSD converts testosterone to AD, there are at least 3 situations that cause depression-like behaviors in the *E. coli* BL21 (DE3)/pDL01 recipients: (1) testosterone deficiency induces depression-like behaviors, (2) elevated serum AD levels induce depression-like behaviors, and (3) both testosterone deficiency and elevated serum AD levels induce depression-like behaviors. The relationship between testosterone deficiency and depression-like behaviors has been reported in a previous study (Carrier and Kabbaj, 2012). Therefore, based on this study, we supplemented ABX-treated rats with AD (0.5 mg/day for 28 days) by gavage to further elucidate the associations between 3β-HSD and depression-like behaviors.

In behavioral experiments, the immobility time in the FST and TST and the latent time in the NSF and splash tests for the AD-treated rats were similar to those of the control rats (Figures 5D and S3G–S3I). This suggested that AD supplementation did not lead to depression-like behaviors in rats. Therefore, the expression of 3β-HSD in the gut may cause depression-like behavior in rats by reducing their testosterone levels.

M. neoaurum and *3β-HSD* were prevalent in patients with depression

To assess the prevalence of *M. neoaurum* and *3β-HSD* in the human gut, we examined the metagenomic prevalence of *M. neoaurum* and *3β-HSD* in the fecal DNA of the participants without (n = 102) and with depression (n = 107) using real-time polymerase chain reaction (qPCR). The genes of *M. neoaurum* and *3β-HSD* were amplified using specific and degenerate primers, respectively. For the degenerate primer of the enzyme, MN2019_09885 sequences were compared using BLAST, and all homologous genes were selected to design primers for the conserved regions.

The results showed that *M. neoaurum* was present in 4.67% (5/107) of the patients in the depression group and in none of the patients in the non-depression group. Moreover, *3β-HSD* was present in 42.99% (46/107) of the patients in the depression group and 16.67% (17/102) of the patients in the non-depression group (Figure 6A). These data suggested that *M. neoaurum* mainly existed in the patients with depression and that *3β-HSD* was distributed more widely in the patients with depression than in healthy individuals. Furthermore, the distribution of *3β-HSD* was more extensive than that of *M. neoaurum*, indicating that *M. neoaurum* may not be the only bacterium expressing 3β-HSD in the gut.

Next, to investigate the expression level of *3β-HSD* in the participants without (n = 102) and with depression (n = 107), we used qRT-PCR to test the fecal RNA. The result showed that 3β-HSD was transcribed in 26.17% (28/107) of the patients in the depression group and 4.90% (5/102) of the participants in the non-depression group (Figure 6A). Upon comparing these results with the qPCR data, we observed that 60.87% (28/46) of the patients with depression showed the transcription of *3β-HSD* in

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their gut, whereas this percentage was 29.41% (5/17) in the non-depression group, which suggests that the testosterone-degrading bacteria in patients with depression are more active in expressing 3β-HSD.

To explore the relationship between *3β-HSD* and serum testosterone, correlation analyses were conducted. The result shows that both Ct values of *3β-HSD* DNA ($r = 0.257$; $p = 0.001$; Figure 6B) and *3β-HSD* RNA ($r = 0.266$; $p < 0.001$; Figure 6C) were positively associated with serum testosterone, which suggests that serum testosterone could be affected by gut *3β-HSD*.

DISCUSSION

The idea that testosterone deficiency can lead to depressive disorders was proposed over 50 years ago; however, relatively few studies have investigated the causes of testosterone deficiency. Diczfalusy et al. (1962) found for the first time that steroid hormones can be converted into other products in the intestine, resulting in less reabsorption of steroid hormones into the blood (Diczfalusy et al., 1962). Moreover, recent studies suggest that gut microbes can change the ratio of active and inactive forms of steroid hormones through the degradation pathway, thus affecting the levels of some steroid hormones (Needham et al., 2020). However, the role of gut microbes in the metabolism of testosterone in the pathogenesis of depression has not yet been studied. In this study, we found that gut microbes could reduce the serum and brain testosterone levels by degrading testosterone and lead to depressive symptoms.

Our results provided evidence for a previously unknown mechanism, i.e., the relationship between the gut microbiota and testosterone levels of serum and brain. It has been reported that the serum testosterone level of female mice administered with adult male cecal contents by gavage increased significantly (Markle et al., 2013). This indicates that gut microbes can change the testosterone level. In this study, *M. neoaurum*, which can degrade testosterone, reduced the testosterone levels of serum and brain, possibly because testosterone is degraded by 3β-HSD produced by *M. neoaurum* before it is reabsorbed into circulation. The presence of *M. neoaurum* MN2019 was not surprising because *Mycobacteria* have been reported to use natural sterols or some other steroidal compounds as sole energy and carbon sources (Yao et al., 2014). *M. neoaurum* is a rapidly growing *Mycobacterium* and can degrade natural steroids (Voĭshvillo et al., 2003). In addition, *M. neoaurum* NwIB-01 was isolated from soil using steroids as the sole carbon source (Wei et al., 2010) and was found to convert phytosterols into AD, ADD, and 9α-hydroxy-4-androstene-3,17-dione (Yao et al., 2014). Notably, *M. neoaurum* is currently used in the pharmaceutical industry to produce AD or ADD by phytosterol degradation (Rodríguez-García et al., 2016). Because the engineered *E. coli* that expressed 3β-HSD in the gut resulted in depression-like behaviors in rats, we speculate that the bacteria expressing 3β-HSD in the gut may have the common feature of reducing serum testosterone and resulting in host depression. These data demonstrated a role for the gut-microbiota degradation of testosterone in depression-like behaviors and provided a genetic basis for understanding testosterone-deficiency-related depressive disorders. AD is a weakly active form of testosterone and has a lower affinity for androgen receptor (Young et al., 2013). Therefore, when testosterone is converted into a weakly active form by 3β-HSD in the gut, the testosterone levels in the serum and brain decrease. These results are consistent with the previous results demonstrating that the gut microbiota can influence the levels of some steroid hormones by shifting the ratio of active and inactive steroid levels through degradation pathways (Needham et al., 2020).

Our study identified a type of depression caused by testosterone-degrading bacteria in the gut, causing a decrease in testosterone levels and depressive symptoms secondary to clinical hypogonadism. Notably, our results show only a particular niche of depression linked to low testosterone levels, rather than all those meeting the clinical cutoff for hypogonadism. A previous review indicates that for depressive symptoms secondary to clinical hypogonadism, there are strong evidences for the efficacy of testosterone replacement therapy; for subthreshold depressive symptoms in men without clinical hypogonadism, there is preliminary evidence for the efficacy of testosterone augmentation; and for treatment-resistant MDD in men without clinical hypogonadism, there are strong evidences for there being no benefit to testosterone augmentation (Dwyer et al., 2020). Our result demonstrated that testosterone supplementation is effective in alleviating depression-like behaviors in rats, which reinforced the previous conclusion. Future studies can compare the effect of testosterone supplementation in patients with testosterone-degrading bacterial infection with other patients exhibiting depressive symptoms secondary to clinical hypogonadism. Determining the effect of using antibiotics to clear bacteria in this patient population will provide further insights.

Many studies have shown that *Actinomycetes* are more abundant in the gut of the patients with depression than in that of the control subjects (Jiang et al., 2015; Naseribafrouei et al., 2014; Sharon et al., 2016; Zheng et al., 2016). However, it is not clear how *Actinomycetes* are associated with depression. In fact, *Actinomycetes* can metabolize steroids (Holert et al., 2018). Therefore, many bacteria in the *Actinomycetes* family may degrade testosterone. We speculated that *Actinomycetes* may reduce the testosterone levels of serum and brain through the degradation of testosterone, resulting in depression.

However, there remain several unaddressed questions. First, our results showed that the distribution of 3β-HSD was more common than that of *M. neoaurum* in the depression group, indicating that *M. neoaurum* was not the only bacterium in the gut harboring *3β-HSD*. Other testosterone-degrading bacteria were not evaluated in this study. Second, *3β-HSD* was also detected in the gut of the non-depressed group, indicating that the investigation on whether *3β-HSD* in the gut causes depression should consider other factors, such as enzyme activity, enzyme expression, enzyme inhibitors in the intestinal environment, and the production and regulation of serum testosterone in the host. The relationship between enzyme activity and the testosterone levels of serum and brain should be investigated in further studies.

The discovery of relationships among testosterone deficiency, gut microbial metabolism, and depression pathogenesis has extensive health-related implications. Our study revealed a mechanism potentially associating the degradation of testosterone by

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gut microbes harboring 3β-HSD with elevated depression risk. The role of the gut microbe in this mechanism suggests modifiable risk factors for preventing testosterone deficiency and/or depressive disorders. Further studies should focus on randomized, double-blind controlled clinical trials to explore effective interventions, such as antibiotic treatment, testosterone supplementation, or 3β-HSD inhibitors, to prevent testosterone deficiency or depressive disorders.

Limitations of the study

In this study, all rats were given short-term antibiotics before further experiment to deplete their gut microbiota. However, in the subsequent feeding for 4 weeks, the gut microbiota of rats may have slowly recovered to a different composition (Ni et al., 2017); so the background gut microbiota (gut microbiota other than gavage bacteria) may be different, and we have not conducted an in-depth evaluation of the background gut-microbiota composition. However, we set up a control group in each batch of experiments to exclude the influence of the background gut microbiota on the experimental conclusions.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.chom.2022.01.001.

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AUTHOR CONTRIBUTIONS

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

DECLARATION OF INTERESTS

The authors applied patents based on this research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-His Tag Mouse Monoclonal Antibody	Abbkine	Cat#A02050
Bacterial and virus strains		
M. neoaurum MN2019	This paper	N/A
E. coli BL21 (DE3)	AngyuBio	Cat#G6030
Biological samples		
Human fecal and plasma samples	Renmin Hospital of Wuhan University	N/A
Chemicals, peptides, and recombinant proteins		
Neomycin	Solarbio	Cat#N8090
Metronidazole	Solarbio	Cat#M8060
Vancomycin	BioFroxx	Cat#1161GR001
Ampicillin	Aladdin	Cat#A102048
Erythromycin	Aladdin	Cat#E105345
Testosterone	Aladdin	Cat#1915399
Androstenedione	Aladdin	Cat#A101164
Critical commercial assays		
QIAamp PowerFecal Pro DNA Kit	Qiagen	Cat#51804
RevertAid First Strand cDNA Synthesis Kit	Thermo Fisher Scientific	Cat#K1622
Fecal RNA Rapid Extraction Kit	BioTeke	Cat#RP8001
Deposited data		
Genome for *M. neoaurum* MN2019	GenBank	Accession: CP074376
Experimental models: Organisms/strains		
SD rats	Hunan STJ Laboratory	N/A
Oligonucleotides		
Specific primers and probe for *M. neoaurum*	This paper	N/A
M. neoaurum forward primer (5′-CTGACCAATCACTACTACC-3′)		
M. neoaurum reverse primer (5′-CCATGTACTGGTTCTTGG-3′)		
M. neoaurum probe (5′ROX-AGTT GATCGACAGCACCTCCG-3′MGB)		
Specific primers and probe for 3β-HSD	This paper	N/A
3β-HSD forward primer (5′-CGGC CSAGYGGNATCTGGGG-3′)		
reverse primer (5′-CGTCRTTG AYGAARTASGCCTG-3′)		
Probe (5′VIC-TSGAYAAYT CCTACGT-3′MGB)		
Primers for constructing pDL01	This paper	N/A
pDL01 forward primer (5′-AATTCGG ATCCTCAGCTCTTCGGTGCCGCG-3′)		
pDL01 reverse primer (5′-CAGCCATA TGGGTGACCCAACTTTGCGTAC-3′)		
Recombinant DNA		
Plasmid: pDL01	This paper	N/A

(*Continued on next page*)

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
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, Tiangang Liu (liutg@whu.edu.cn).

Materials availability

No unique reagents were generated in the study. Strains, plasmids, or other materials in this study can be got by contact with lead contact with reasonable request.

Data and code availability

- The genome of *M. neoaurum* MN2019 is deposited at GenBank and is publicly available as of publication. Accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Rats

Animal studies were conducted in strict accordance with protocols approved by the Animal Experiment Center Renmin Hospital of Wuhan University (approval no. S0271902190A). Sprague-Dawley rats (male, 6 weeks of age, n = 6–12 per group) were purchased from Hunan SJA Laboratory Animal Corporation (Hunan, China) and reared in the specific pathogen-free (SPF) sector of the Animal Experiment Center of Renmin Hospital of Wuhan University and acclimatized for 1 week before being used for experiments. Rats were maintained under SPF conditions on a 12-h light/dark schedule in a humidity- and temperature-controlled (22–25 °C) environment with autoclaved standard chow and water.

Human subjects

All protocols were approved by the Renmin Hospital of Wuhan University Institutional Review Board (approval no. WDRY2021-K041). Informed consent was waived by the committee. Participants were recruited from Renmin Hospital of Wuhan University. For the depression group, male volunteers (aged 18–65 years) meeting the Diagnostic Criteria for Depression from the DSM-V published by the American Psychiatric Association in 2013 for major depressive disorders (such as MDD and dysthymic disorder) were eligible. Exclusion criteria included patients with active psychosis or dementia, those with both clear intent and suicidal ideation, those meeting criteria for substance dependence, and those with comorbidities that could affect testosterone level, including endocrine disorders and kidney diseases (Egede et al., 2015). Finally, 107 patients with depression were included in our study. Serum samples were obtained from 77 of the 107 patients with depression.

Age-matched non-depression controls (n = 102) were also included in the current study, recruited from volunteers who visited Renmin Hospital of Wuhan University for their annual physical examination and who did not meet the DSM-V criteria for MDD. As controls, all clinical indices were within normal ranges, including chest X-ray, urine and feces evaluations, routine examination of blood, physical examination, and preoperative serological tests (kidney and liver function tests). Exclusion criteria included endocrine disorders, kidney diseases, cancer, celiac disease, inflammatory bowel disease, metabolic syndrome, diabetes, hypertension, and all types of viral infections, including human immunodeficiency virus and hepatitis virus.

METHOD DETAILS

Detection of serum and brain testosterone

Blood samples of rats were collected from the inferior vena cava after inhaling anesthesia. Blood samples of human were collected via elbow venous blood. For serum (100 μL) from humans or rats, samples were added into 1.5 mL centrifuge tubes spiked with the testosterone internal standard (2 μL). The whole brain tissue was mashed, and 0.25 g of the brain homogenate was collected in 1.5 mL centrifuge tubes spiked with the testosterone internal standard (2 μL). Next, methyl tert-butyl ether (500 μL) was added for extraction. Then the upper organic phase was transferred into a new tube and was evaporated using nitrogen (60 °C, 5 min), and

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redissolved using 50% methanol. The testosterone was analyzed using an Ekspert ultraLC 100-XL system (AB SCIEX, Framingham, MA, USA) coupled to an AB SCIEX 4500 QTRAP mass spectrometer with a 1.7 μm, 2.1 × 50 mm ACQUITY UPLC BEH Shield RP18 column (Waters, Milford, MA, USA). Methanol with 0.2% formic acid (A) and 0.2% formic acid aqueous solution (B) were used as the mobile phase at a flow rate of 0.3 mL/min. Data were collected in positive-ion mode.

Fecal sample collection

Each participant provided a fresh fecal pellet sample between 06:00 and 09:00 AM during normal defecation. The samples were immediately stored in liquid nitrogen and used in the experiment or stored at -80 °C immediately.

Testosterone degradation by fecal microbiota

Feces pellets (0.8–1.2 g) were collected from random six participants in depression group or non-depression group and suspended using 10 mL of normal saline in sterile tubes. After centrifugation at 2,000 × *g* at 4 °C for 1 min, bacteria-enriched supernatants were collected in sterile tubes and centrifuged at 15,000 × *g* for 5 min. Then, the supernatant was discarded and the fecal microbiota of bacterial pellets was resuspended using 10 mL normal saline following addition of testosterone (2 g/L) in the sterile tubes. Then, 0.5 mL of the suspension was harvested every 24 h.

Gut microbiota collection

According to a published fecal microbiota transplant method (Yuan et al., 2019), fresh feces pellets (0.20–0.30 g) from participants were collected and suspended using 10 mL normal saline in sterile tubes. After centrifugation at 2,000 × *g* at 4 °C for 1 min, bacteria-enriched supernatants were collected in sterile tubes and centrifuged at 15,000 × *g* for 5 min. Then, the supernatant was discarded and the fecal microbiota of bacterial pellets was resuspended using 10 mL normal saline. This microbial preparation was used for fecal microbiota transplant or stored at -80 °C. According to the recommendations from transplantation protocols (Secombe et al., 2021), the details for fecal microbiota transplant are listed in Table S1.

Gavage with bacteria in rats

Rats were treated with short-term antibiotic before gavage (Chevalier et al., 2020). Briefly, rats were given a cocktail comprising 0.5 mg/mL vancomycin, 1 mg/mL neomycin, 1 mg/mL metronidazole, and 1 mg/mL ampicillin in their drinking water for 1 week to deplete intestinal microorganisms, followed by one-day autoclaved water (Miyauchi et al., 2020). All antibiotics were obtained from Solarbio (Beijing, China). Rats receiving the bacteria were orally gavaged with 1 mL of microbiota solution or approximately 1 mL overnight cultures of bacteria (approximately 1 × 10^8 cfu once daily for three successive days) (Funabashi et al., 2020). *E. coli* BL21 (DE3)/pDL01 and *E. coli* BL21 (DE3)/pET28a were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.25 mM) before gavage.

Gavage with testosterone or AD in rats

For testosterone treatment, *E. coli* BL21 (DE3)/pDL01 recipients were treated with 0.5 mg testosterone daily for 28 days by gavage, beginning the day after *E. coli* BL21 (DE3)/pDL01 gavage. For AD treatment, ABX-treated rats (without any bacterial gavage) were supplemented with AD (0.5 mg/day for 28 days) by gavage.

Behavioral assays

Rats were subjected to splash tests, NSF tests, TSTs, and FSTs, in this order (Chevalier et al., 2020). Behavioral assays were conducted within one week, with at least 24 h between each behavioral assay. All analyses were conducted with the experimenter blinded to the experimental conditions.

Splash test

Sucrose solution (10%) was squirted on the dorsal coat of the rat in its home cage. The sucrose solution soils the rat fur because of its viscosity, and rats initiate grooming behavior. After splashing sucrose solution, the time spent grooming, the frequency of grooming, and latency to grooming were measured for 6 min as an index of motivational and self-care behavior.

NSF

After depriving food for 24 h, the rats were transferred into a novel white methacrylate box (100 cm × 100 cm × 40 cm) with the floor covered in wooden bedding. A single food pellet was positioned on a piece of white filter paper (10 cm in diameter), placed in the center of the container. The rat was positioned in one corner of the container, and the latent time to feed was recorded for 10 min. Feeding was defined as biting food, not touching or sniffing. After the assay, the rats were placed immediately into their home cage, and the consumption of food during the subsequent 5-min period was recorded as a control of the feeding drive.

TST

By holding tails wrapped in an adhesive tape, rats were suspended approximately 40 cm above the floor under dim light environment (~40 lx). The total immobility time was recorded during a 5-min period.

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FST

Rats were transferred individually in glass cylinders (20 cm diameter) that were filled to a depth of 40 cm with water (23–27 °C) under dim light conditions (~40 lx). The total immobility time was recorded during a 5-min period.

Isolation of testosterone-degrading strains

Fresh fecal samples (0.5 g) from the patient with depression, who provide feces for former fecal microbiota transplant (Table S1), were added into 250 mL Erlenmeyer flasks containing 50 mL of a selective medium comprising basic medium (pH 7.0) and 3 mg/L testosterone. The basic medium composed of 19 mM NH_4Cl, 9 mM NaCl, 22 mM KH_2PO_4, 0.1 mM $CaCl_2 \cdot 2H_2O$, 48 mM Na_2HPO_4 and 2 mM $MgSO_4 \cdot 7H_2O$ (Yang et al., 2011). Incubation was performed on a rotary shaker at 250 rpm at 37 °C in the dark. Three days after incubation, 1 mL cell suspension was added into new 250 mL Erlenmeyer flasks containing 49 mL of the fresh selective medium. This process was repeated nine times over a 27-day period. The final culture suspension was inoculated onto nutrient-rich Columbia blood agar plates to obtain bacterial colonies. The strains were tested for their ability to degrade testosterone, and those that degraded testosterone were identified by 16S rRNA sequencing and whole genome sequencing.

Testosterone degradation by *M. neoaurum*

Before testosterone degradation, testosterone (100 g/L) was dissolved in methanol. For testosterone fermentation (Sun et al., 2019), *M. neoaurum* strains were incubated in 5 mL of the LB medium (10 g/L NaCl, 10 g/L tryptone and 5 g/L yeast extract, pH 7.0) and cultivated at 37 °C to an OD_{600} of 2.0–3.0. Then, 3 mL of this bacterial culture was added into 30 mL of a seed medium (2 g/L citric acid, 20 g/L glycerol, 1.65 g/L $(NH_4)_2HPO_4$, 0.05 g/L ferric ammonium citrate, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 2.52 g/L KNO_3, pH 7.5) and cultured at 37 °C to an OD_{600} of 3.0–4.0. Next, 3 mL cell suspension from the seed medium was added into 30 mL of the fermentation medium (3.5 g/L $(NH_4)_2HPO_4$, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L ferric ammonium citrate, 10 g/L glucose, 2 g/L citric acid, pH 7.5) with a final testosterone concentration of 2 g/L. Then, 1 mL of the culture was harvested every 24 h and stored at -80 °C until further analysis.

TLC for steroids

Stored samples (500 μL) were thawed in a 37 °C water bath and were extracted with 500 μL ethyl acetate. After centrifugation, 2 μL supernatant were added to TLC plates (Qingdao Marine Chemical Factory, Qingdao, China). Ethyl acetate/petroleum ether (4:6, v/v) was used to observe the formation of products. Testosterone and its products were visualized using 20% sulfuric acid solution before baking (80 °C, 30 min).

Quantification of steroids using HPLC

Stored samples (500 μL) were thawed in a 37 °C water bath and were extracted with 500 μL ethyl acetate. After centrifugation, supernatant was quantified on a 3.9*150mm, 5 μm, 30 °C Xterra RP-C18 column (Waters, Milford, MA, USA) using methanol/water (80:20, v/v) as the mobile phase on UtiMate 3000 (Thermo Fisher Scientific, Waltham, MA, USA). The flow rate was 0.8 mL/min. Testosterone and its products were detected with ultraviolet light at 254 nm.

E-test

The E-test was conducted using a kit (Autobio, Zhengzhou, China), according to the manufacturer's instructions. For anti-*M. neoaurum* susceptibility testing, Columbia blood agar plates were inoculated with a sterile cotton swab dipped into the *M. neoaurum* inoculum suspension. After 37 °C incubation for 48 h, MIC was the lowest drug concentration on the scale of the elliptical edge contact test paper.

Whole genome sequencing of *M. neoaurum*

The genomic DNA of *M. neoaurum* was extracted using Bacterial Genomic DNA Extraction Kit (Tiangen, Beijing, China), following the manufacturer's instructions. The whole genome sequencing was performed and assembled by GrandOmics (Wuhan, China) using T7 (MGI, shenzhen, China) and PromethION (Oxford nanopore, oxford, UK) platforms.

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

Plasmid pET28a was used as an overexpression vector to express 3β-HSD from *M. neoaurum* in *E. coli*. Genomic DNA of *M. neoaurum* was used as a template to amplify a fragment of *3β-HSD* via PCR (forward primer: 5′-AATTCGGATCCTCAGCTCT TCGGTGCCGCG-3′, reverse primer: 5′-CAGCCATATGGGTGACCCAACTTTGCGTAC-3′), and the amplified fragment was digested by *Bam*HI and *Nde*I and cloned into the pET28a plasmid to construct the plasmid pDL01. The sequence of pDL01 was confirmed via DNA sequencing. The correct plasmid pDL01 was introduced into *E. coli* BL21 (DE3) to construct *E. coli* BL21 (DE3)/pDL01, and the vector pET28a was introduced into *E. coli* BL21 (DE3) to construct *E. coli* BL21 (DE3)/pET28a as a control. *E. coli* BL21 (DE3)/pDL01 or *E. coli* BL21 (DE3)/pET28a were incubated in 30 mL of the LB medium at 37 °C on a rotary shaker at 220 rpm. When the OD_{600} reached 0.8–1.2, 0.5 mM IPTG was added into the medium to induce 3β-HSD expression.

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Analysis of the kinetic parameters of 3β-HSD

Based on a previous method (Liu et al., 2020), recombinant 3β-HSD protein was purified from intracellular proteins through sonication and Ni-affinity chromatography. Briefly, after overexpression cells were harvested by centrifugation at 5000 × *g* for 20 min at 4 °C and resuspended in buffer A (20 mmol L^{-1} Tris, pH 7.6, 500 mmol L^{-1} NaCl, 10% glycerol). The cell suspension was lysed by sonication and centrifuged at 25000 × *g* for 1 h at 4 °C. The Ni-NTA column (GE Healthcare, Marlborough, MA, USA) was charged and washed with two column volumes (CV) of buffer A, and the filtered supernatant was applied to the column. The resin was washed with two CV each of buffers containing 0, 25, 50, 100, 200, and 500 mmol L^{-1} imidazole. The purified protein was concentrated using Amicon Ultra-15 centrifugal filter devices (Millipore, Billerica, MA, USA) and the buffer was replaced with storage buffer (100 mmol L^{-1} phosphate buffer, 10% glycerol, pH 7.6) via the PD-10 column (GE Healthcare). Then, the purified protein was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Kinetic parameters for 3β-HSD from *M. neoaurum* were determined in assays containing 200 μM NAD^+, 25 mM Tris-HCl (pH 7.6), and 5 μM 3β-HSD. The reaction mixture was incubated for 20 min at 37 °C. Substrate testosterone concentrations varied from 1 to 50 μM, and a 20 μL sample was pipetted and mixed with an equal volume of acetonitrile to stop the reaction. Product formation was determined using HPLC, as described above. Reactions were performed in triplicate, and data were fitted to the Michaelis-Menten equation using the least-squares method.

Western blot

Stored feces samples (0.20–0.30 g) were thawed in a 37 °C water bath then suspended in 10 mL normal saline in sterile tubes. After centrifugation at 2,000 × *g* at 4 °C for 1 min, bacteria-enriched supernatants were collected in sterile tubes and centrifuged at 15,000 × *g* for 5 min. After discarding the supernatant, fecal microbiota was treated with 250 μL RIPA lysis buffer (Servicebio, Wuhan, China) followed by sonication and centrifugation at 12,000 × *g* for 5 min. Then, 30 μg of protein was loaded in 8% separating gel and 5% concentrated gel for electrophoresis and blotted onto a PVDF membrane (Millipore, Shanghai, China). Membranes were blocked in 5% skimmed milk and incubated with primary antibodies for His-Tag (Abbkine Scientific, Wuhan, China). Finally, membranes were developed using ECL kit (Bio-Rad, Shanghai, China) and captured using QuickChemi Imager (QuickChemi 5100, Monna, Jiangsu, China).

Fecal sample DNA/RNA extraction

DNA was extracted from 0.25 g feces samples using a QIAamp PowerFecal DNA Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. RNA was extracted from 0.25 g feces samples using fecal RNA extraction kit (Bioteke, Beijing, China) following the manufacturer's instructions. The extracted RNA was transcripted into DNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, Shanghai, China) for qPCR.

qPCR assay for prevalence of *3β-HSD* and *M. neoaurum*

The *3β-HSD*-related gene sequences in the GenBank database were searched using protein-protein BLAST (http://www.ncbi.nlm.nih.gov/blast/) with *M. neoaurum* 3β-HSD as a query. Multiple alignment of the *3β-HSD*-related gene sequences was conducted using BioEdit (version 7.2.5). Conserved regions were used to deduce a degenerate primer pair (forward primer: 5′-CGGCCSAGYGGN ATCTGGGG-3′, reverse primer: 5′-CGTCRTTGAYGAARTASGCCTG-3′, and probe: 5′VIC-TSGAYAAYTCCTACGT-3′MGB).

For *M. neoaurum*-specific primers, we compared the complete genome of *M. neoaurum* in the NCBI database and our sequencing data *of M. neoaurum* MN2019 with all *Mycobacterium* and *Mycolicibacterium* sequences in the NCBI database and selected conserved and specific genes for *M. neoaurum*. The obtained sequences were compared with the existing species marker genes in metaphlan2 (Truong et al., 2015), and we ensured the markers were not selected as marker genes for other species. In total, 129 marker genes were selected for analysis. Based on NCBI BLAST, the gene with the best specificity was selected for designing specific primers. Finally, *M. neoaurum* fragments were amplified using specific primers and probes (forward primer: 5′-CTGACCAAT CACTACTACC-3′, reverse primer: 5′-CCATGTACTGGTTCTTGG-3′, and probe 5′ROX-AGTTGATCGACAGCACCTCCG-3′MGB).

The gene *3β-HSD* and *M. neoaurum* fragments were amplified using qPCR with specific primers and probes on a ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction system included 10 μL Premix Ex Taq (Takara, Shiga, Japan; cat. no. RR390Q), 1 μL forward and reverse primers (10 μM), 1 μL probe (10 μM), 2 μL fecal DNA, and 5 μL H_2O. The PCR was performed at 95 °C for 30 s; 40 cycles: 95 °C for 20 s, 57 °C for 20 s, and 72 °C for 20 s.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were presented in the figures as mean ± SEM. Differences among more than two groups were assessed using one-way ANOVA with Tukey's multiple comparisons tests. *P values* < 0.05 were considered significant. Significant differences emerging from the above tests are stated in the figures by $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Notable non-significant differences are stated in the figures by NS. Correlation between serum testosterone and the Ct value of 3β-HSD RNA or DNA were assessed using correlation analysis. Statistical analyses were performed in SPSS.