Chronic sleep deprivation causes delayed puberty onset in rats through activating proinflammatory cytokines and alternating the gut microbiome (#109757)

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Chronic sleep deprivation causes delayed puberty onset in rats through activating proinflammatory cytokines and alternating the gut microbiome

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Chronic sleep deprivation (CSD) in adolescents has become a secular trend with adverse health outcomes. Previous studies have demonstrated that sleep deprivation causes inflammation, altered puberty onset, and changes the composition of the gut microbiome; however, the relationship between these is still unknown. Therefore, we hypothesized that CSD affects the onset of puberty might via elevating proinflammatory cytokines and alteration of gut microbiome composition. Our results revealed that CSD in juvenile rats for 4 weeks were significantly reduced body weights, delayed onset of puberty, and elevated antioxidant enzyme activities in both sexes. In the sleep-deprivation female (SDF) rat, plasma levels of lipopolysaccharide binding protein (LBP), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) were significantly elevated; mRNA levels of *TNF-α* and *IL-1β* were also significantly elevated in the colon and reproductive organs, respectively. In the sleep-deprivation male (SDM) rat, only plasma levels of IL-6 were significantly elevated; in addition, mRNA levels of *IL-1β* and *TNF-α* were also significantly elevated in the colon and reproductive organs, respectively. Gut microbiome

analysis revealed that Predominant bacteria at the genus level were Muribaculaceae, Prevotellaceae UCG-001, and Ruminococcaceae UCG-005 in the SDF rat; Prevotellaceae NK3B31, Ruminococcaceae UCG-010, Eubacterium coprostanoligenes, and Shuttleworthia in the SDM rat. CSD rats with abundant genera were positively correlated with antioxidant enzyme activities and mRNA levels of proinflammatory cytokines. Overall, CSD causes delayed pubertal timing, possibly via an increase in the expression levels of proinflammatory cytokines and altering the gut microbiome composition, indicating proinflammatory cytokines and gut microbiome play an important role in pubertal timing change. These findings may guide the future studies to intervene sleep deprivation-related delays in the onset of puberty.

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ABSTRACT

Chronic sleep deprivation (CSD) in adolescents has become a secular trend with adverse health outcomes. Previous studies have demonstrated that sleep deprivation causes inflammation, altered puberty onset, and changes the composition of the gut microbiome; however, the relationship between these is still unknown. Therefore, we hypothesized that CSD affects the onset of puberty might via elevating proinflammatory cytokines and alteration of gut microbiome composition. Our results revealed that CSD in juvenile rats for 4 weeks were significantly reduced body weights, delayed onset of puberty, and elevated antioxidant enzyme activities in both sexes. In the sleep-deprivation female (SDF) rat, plasma levels of lipopolysaccharide binding protein (LBP), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) were significantly elevated; mRNA levels of *TNF-α* and *IL-1β* were also significantly elevated in the colon and reproductive organs, respectively. In the sleep-deprivation male (SDM) rat, only plasma levels of IL-6 were significantly elevated; in addition, Mrna levels of *IL-1β* and *TNF-α* were also significantly elevated in the colon and reproductive organs, respectively. Gut microbiome analysis revealed that Predominant bacteria at the genus level were Muribaculaceae, Prevotellaceae UCG-001, and Ruminococcaceae UCG-005 in the SDF rat; Prevotellaceae NK3B31, Ruminococcaceae UCG-010, Eubacterium coprostanoligenes, and Shuttleworthia in the SDM rat. CSD rats with abundant genera were positively correlated with antioxidant enzyme activities and Mrna levels of proinflammatory cytokines. Overall, CSD causes delayed pubertal timing, possibly via an increase in the expression levels of proinflammatory cytokines and altering the gut microbiome composition, indicating proinflammatory cytokines and gut microbiome play an important role in pubertal timing change. These findings may guide the future studies to intervene sleep deprivation-related delays in the onset of puberty.

Keywords: Chronic sleep deprivation, Proinflammatory cytokines, Puberty onset, Gut microbiome.

INTRODUCTION

Puberty is a developmental phase that determines the end of the growth phase and the beginning of the reproductive phase (Wood et al. 2019). The early onset of puberty has become a secular trend in adolescents worldwide (Hardy et al. 2006; Hui et al. 2012). Adolescents have been sleeping less over time (Matricciani et al. 2012; Shochat et al. 2014), and studies have revealed an association between sleep duration and the onset of puberty in adolescents (Hoyt et al. 2018; Sadeh et al. 2009; Wang et al. 2020). Chronic sleep deprivation (CSD) leads to the accumulation of reactive oxygen species (ROS), the presence of which activates antioxidant defense mechanisms to restore the balance of oxidants and antioxidants (Birben et al. 2012).

Major proinflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), are also associated with sleep deprivation (Garbarino et al. 2021; Mullington et al. 2010). Sleep deprivation leads to an inflammatory response, especially in the hypothalamus, inhibiting gonadotropin-releasing hormone (GnRH) expression, which reduces luteinizing hormone (LH) release and may alter the onset of puberty (Haziak et al. 2018).

In addition, some studies have shown that sleep deprivation alters the composition of the gut microbiome (Poroyko et al. 2016; Reynolds et al. 2017). Although the association between gut microbiome and puberty onset has not been well established; however, one study observed that the composition of the gut microbiome are different between pubertal and non-pubertal groups (Yuan et al. 2020). Another animal study also demonstrated that probiotics can reverse the early onset of puberty in rats (Cowan & Richardson 2019). Thus, alteration of gut microbiome composition might affect the onset of puberty.

We hypothesized that CSD affects the onset of puberty via elevating inflammation and alteration of gut microbiome composition. Here, we performed a sleep deprivation study by using animal models to establish the relationship between proinflammatory cytokines, gut microbiome and pubertal timing.

MATERILAS AND METHODS

Animal study

The study was conducted to investigate the impact of sleep deprivation on altering the puberty onset in juvenile Sprague-Dawley (SD) rats. Four pregnant SD rats were purchased from BioLASCO Taiwan Co. Ltd, and they were housed at the Laboratory Animal Center at Taipei Medical University in a controlled environment (12-hour light-dark cycle, 22–24°C, 40%–60% humidity). The juvenile SD rats were weaned and grouped at postnatal day 21 (PND 21), and they were divided into Control Female (CF) (n=6), Sleep-Deprivation Female (SDF) (n=6), Control Male (CM) (n=6), and Sleep-Deprivation Male (SDM) (n=6) groups, totally 24 rats. They were subjected to 15 hours of sleep deprivation per day for 4 weeks after weaning. Sleep deprivation is a highly stressful condition; therefore, the body weights of rats were monitored every other day, and all rats survived until euthanization. The rats were euthanized by using cardiac puncture after 4 weeks of sleep deprivation, and blood samples and tissues were collected and stored in the -80 ℃ refrigerator until used.

CSD model

The rats in the SDF and SDM groups were subjected to the sleep deprivation for 15 hours (from 08:30 to 23:30) per day for 4 weeks continuously based on the modified inverted flowerpot method (Machado et al. 2004). Sleep deprivation for 4 weeks was considered as CSD. The rats were placed inside a water tank containing 8 circular platforms, each with a diameter of 6 cm.

They were put in the tanks with the same sex and group-housed after they returned to the home cage. Water was added to within 1 cm of the upper surface of each platform. Each water tank had at most 6 rats. When the rats reached the rapid eye movement stage of sleep, muscle atonia caused them to fall into the water, at which point they had to climb up a platform to avoid being drowned.

Assessment of the onset of puberty

The onset of puberty in the female rats was determined by daily observation of the vaginal opening, which started at PND25. Vaginal cytology was observed from vaginal smears to determine the estrous cycle stage. Vaginal smears were performed daily until the end of the experiment by inserting a sterilized pipette tip filled with 10μL of normal saline into the vagina (Marcondes et al. 2002). The obtained vaginal fluid was placed on a glass slide, and unstained material was observed under a light microscope with 10× and 40× objective lenses. The onset of puberty in the male rats was determined according to the day of preputial separation, which is when the foreskin detaches from the glans penis (Korenbrot et al. 1977). The male rats were observed for preputial separation at PND 35.

Tissue preparation

Colon (cecum), ovary, and testes samples were dissected and washed immediately in 0.1 M phosphate buffer saline (PBS). They were homogenized on ice in PBS 1:2 (w/v; 1 g tissue with 3 mL PBS, pH 7.4) and centrifuged at 10,000 × *g* for 15 minutes at 4°C. The supernatants were collected for determining CAT, SOD, and glutathione GPx activity.

Protein determination

The protein levels of the colon, ovary, and testes homogenates were determined by using Bradford method (Bradford 1976).

Antioxidant enzyme activities

Antioxidant enzyme activities were determined by using CAT, SOD, and GPx activities. Antioxidant enzyme activities were analyzed by using assay kits which from Cayman Chemical. Analyzed procedures followed the manufacturer's protocols: CAT (item no. 707002), SOD (item no. 706002), and GPx (item no. 703102). Antioxidant enzyme activities were normalized to the total protein in the homogenates and expressed as units per mg of protein.

Determination of circulating levels of LBP and proinflammatory cytokines

Circulating levels of LBP and proinflammatory cytokines (including IL-1β, IL-6 and TNF-α) were determined in rat plasma. The levels of LBP in plasma was determined by using ELISA kit (Cusabio; CSB-E11184r); the levels of IL-1β, IL-6 and TNF-α were determined by using LEGENDp lex™Mul_-Analyte Flow Assay Kit (Biolegend; Cat. 741395 and Cat. 741396). Analyzed procedures followed the manufacturer's protocols.

RNA extraction and real-time quantitative reverse transcription-polymerase chain reaction

The RNA was extracted by using the RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. Following RNA isolation, 1 μg of RNA was used for reverse transcription to cDNA by using the MMLV Reverse Transcription Kit (Protech Technology Enterprise, Co., Ltd.). cDNA was used to quantify the transcript levels on the Smart Quant Green Master Mix system (Protech Technology Enterprise, Co., Ltd.). *GAPDH* is used as an internal control (Ct of target gene - Ct of *GAPDH* = ΔCt), and ΔCt of control is used as the calibrator (ΔCt of sample - ΔCt of calibrator = ΔΔCt). Relative mRNA levels of target genes = $2^{-\Delta\Delta CT}$ (fold change vs. control).

DNA sequences of rat-specific primers were summarized as follows:

Gene symbol	Forward primer 5' to 3' (F)	Reverse primer 5' to 3' (R)
GAPDH	5'-GTGCCAGCCTCGTCTCATAG-3'	5'-CGTTGATGGCAACAATGTCCA-3'
TNF-α	5'- CTCTTCTCATTCCTGCTCGT-3'	5'-GGGAGCCCATTTGGGAACTT-3'
IL-1B	5'-CACCTCTCAAGCAGAGCACA-3'	5'-TCCTGGGGAAGGCATTAGGA-3'
IL-6	5'-ACCCCAACTTCCAATGCTCT-3'	5'-AGCACACTAGGTTTGCCGAG-3'

Gut microbiome composition

Fecal samples were collected at vaginal opening days (PND 30~PND 40) from female rats and were collected at preputial separation days (PND 39~PND 49) from male rats during the experiment. In brief, collected fecal samples were transferred immediately to cold storage and remained stored at 80°C until processing near days of vaginal opening. Fecal genomic DNA was extracted using the QIAamp DNA Stool Mini Kit (cat. no. 51504, QIAGEN, Denmark) according to the manufacturer's instructions, stored at -80°C, and underwent processing including polymerase chain reaction (PCR) assays and 16S rRNA sequencing. The Pacbio sequencing for full-length 16S genes (V1-V9 regions) was performed. The full-length 16S genes was amplified using barcoded 16S gene-specific primers. Subsequently, the PCR reaction was carried out by KAPA HiFi HotStart ReadyMix (Roche), and its products were purified using the AMPure PB Beads for SMRTbell library construction and sequencing processes. Consequently, multiple sequence alignment was performed by QIIME2 alignment MAFFT against the NCBI database to analyze the sequence similarities among the amplicon sequence variants (ASVs).

Operational taxonomic unit (OTU) clustering and taxonomic analysis were performed using Genomics workbench v.22.0 (CLC Bio, Denmark). The sequences were trimmed, merged, and clustered into OTUs at 97% sequence similarity based on the SILVA v.32 database using CLC

Microbial Genomics Module. Alpha diversity metrics were calculated using the *phyloseq* package in R software based on rarefied OTU counts. The beta diversity index was defined as the difference between the total number of species in the 2 groups and the number of species common to both groups. The exploratory principal coordinate analysis of beta (between-sample) diversity was performed based on the Bray-Curtis measure of dissimilarity. For the hierarchical cluster analysis, Bray-Curtis metrics and complete linkage clustering were implemented. LEfSe analysis was performed to detect bacterial taxa with significantly different abundance between the control and sleep deprivation groups; significance was indicated if the linear discriminant analysis value was >2.0 with $p < 0.05$.

Statistical analysis

Values are presented as mean ± standard error of the mean. Student's *t* tests were performed for comparisons between the control and sleep deprivation groups by using GraphPad Prism 8.0.1 software. Heat maps were plotted via R version 4.0.3 (R Foundation for Statistical Computing), which showed the Spearman's rank correlation coefficient between abundance of bacterial taxa and gene /protein levels. Differences were considered significant at $p<0.05$.

Ethical Approval

The Taipei Medical University Institutional Animal Care and Use Committee (IACUC/IACUP) approved all animal procedures (approval no. LAC-2020-0048). All procedures were conducted in accordance with the Taiwan code of practice for the care and use of animals for scientific purposes.

RESULTS

CSD significantly causes attenuated growth status and delayed onset of puberty in juvenile rats

Body weight and pubertal timing in rats were monitored during experiment. The results revealed that CSD for 4 weeks resulted in a significant decrease in body weight (Figure 1A superior and inferior section) and a delay in pubertal timing in female and male rats (Figure 1B superior and inferior section). Biochemical characteristics and organs/tissues weight analysis revealed that CSD causes significantly attenuated blood levels of total protein and albumin in female and male rats, but significantly increased levels of triglyceride and AST in male rats (Table S1); in addition, CSD significantly attenuated weights of muscle in female rats and significantly attenuated brain, muscle epididymal white adipose tissue, liver, kidney, seminal vesicle, and epididymis in male rats (Table S2). Taken together, CSD for 4 weeks significantly attenuates growth status and delayed onset of puberty in female and male rats.

CSD increases antioxidant enzyme activities in reproductive organs in female and male rats

To determine the effects of CSD on antioxidant responses, we investigated the antioxidant enzyme activities in the colon and reproductive organs. In colon organ, we observed that the antioxidant enzymes as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities were not significantly changed between CF and SDF rats (Figure 2A, left panel); however, antioxidant enzymes (CAT, SOD, and GPx) in SDM rats was significantly higher than those of CM rats (Figure 2A, right panel). In reproductive organs, we observed that both CAT and SOD activities in SDF rats were significantly higher those of CF rats (Figure 2B, left panel); in addition, CAT, SOD and GPx activities in CM rats were significantly higher than those of SDM rats (Figure 2B, right panel). Taken together, CSD causes an increase in antioxidant enzymes activities in reproductive organs of both sexes; however, antioxidant enzyme activities in colon organs have only increases in male rats.

CSD causes inflammation in the colon, reproductive organs and circulatory system in female and male rats

Next, we investigated whether CSD causes an inflammatory response in female and male mice's colon, reproductive organs, and circulatory systems. Therefore, we determined protein levels of lipopolysaccharide binding protein (LBP), IL-1β, IL-6, and TNF-α in the circulatory system and mRNA levels of those in the colon and reproductive organs. The results revealed that the protein levels of LPS, IL1-β, IL-6, and TNF-α in the plasma of SDF rats were significantly higher than those of CF rats (Figure 3 A, superior section). In contrast, the protein levels of IL-6 in SDM rats' plasma were significantly higher than that of CM rats (Figure 3A, inferior section). In the colon, mRNA levels of *TNF-α* in SDF rats were significantly higher than those of CF rats; mRNA levels of *IL-1β* in SDM rats were significantly higher than those of CM rats (Figure 3B). In reproductive organs, mRNA levels of *IL-1β* in SDF rats were significantly higher than those of CF rats; mRNA levels of *TNF-α* in SDM rats were significantly higher than those of CM rats (Figure 3C). Overall, CSD causes inflammation in the colon, reproductive organs and circulatory system, especially in female rats.

CSD alters gut microbiome composition

Next, we investigated the association between CSD and the composition of the gut microbiome. First, α-diversity (including shannon and simpson index) analysis indicated the sleep deprivation groups was significantly lower than that of control groups in both sexes (Figure S1). Second, β diversity analysis indicated the distinct clustering the microbiome compositions between the control and the sleep-deprived groups, and the result revealed the significant difference between CF and SDF groups, as well as CM and SDM groups (Figure 4A). Furthermore, the relations between specific bacterial taxa and sleep deprivation in both sexes were determined by

using LEfSe analysis. The predominant bacteria at the genus level were Muribaculaceae, Prevotellaceae UCG-001, and Ruminococcaceae UCG-005 in the SDF group, and Prevotellaceae NK3B31, Ruminococcaceae UCG-010, Eubacterium coprostanoligenes, and Shuttleworthia in the SDM group (Figure 4B).

Correlation among abundant genera, pubertal timing, antioxidant enzyme activity, and inflammatory cytokines

Abundant genera were involved in pubertal timing in female and male rats, and the results revealed that g_Ruminococcaceae_UCG-005 was positively correlated with vaginal opening day in SDF group, whereas g_Roseburia was negatively correlated with vaginal opening day in CF group (Table S3). In addition, g_Prevotellaceae_NK3B31_group was positively correlated with preputial separation day in SDM group, whereas g_Lachnospiraceae_A2, g_Ruminiclostridium_9, g_Clostridium_sensu_stricto_1 and g_Clostridiales_vadinBB60_group_Uncultured were negatively correlated with preputial separation day in CM group (TABLE S4).

The heat maps were shown the correlations between abundant genera and antioxidant enzyme activity (Figure 5A and B) as well as the correlations between abundant genera and inflammation (Figure 5C and 5D). The results revealed the abundant genera in the SDF and SDM groups were positively correlated with antioxidant enzyme activity and inflammation; in contrast, the abundant genera in the CF and CM groups had were negatively correlated with antioxidant enzyme activity and inflammation.

DISCUSSION

The present study's findings suggest that CSD causes delayed puberty onset and attenuated body weight in juvenile rats. Moreover, we observed inflammation and gut microbial taxonomies alterations in the colon and subsequently affected reproductive organs. To repair damage caused

by CSD, future interventional or mechanistic studies should focus on treating oxidative stress and gut dysbiosis.

Studies have shown that sleep-deprived rats have lower body weight than those of control rats (Everson & Szabo 2011; Koban et al. 2008; Lai et al. 2022), but our study indicated likewise. Nonetheless, in clinical studies, sleep deprivation was found to be associated with weight gain (Schmid et al. 2008; Taheri et al. 2004).

Body weight may be positively correlated with the onset of puberty as being malnourished is related to a delay in the onset of puberty in children (Parent et al. 2003). Other clinical studies have shown that a higher body mass index is associated with an earlier onset of puberty (Deng et al. 2018; Liu et al. 2021; Seo et al. 2020).

In this study, we showed that sleep deprivation increases the levels of free radicals, which induce antioxidant responses. Increased antioxidant enzyme activity, marked by CAT, SOD, and GPx, was observed in the colon and testes of the rats in the SDM group. Increased antioxidant enzyme activity, marked by CAT and SOD, was observed in the ovaries of the rats in the SDF group. Findings on levels of oxidative stress due to sleep deprivation have differed between studies. Lungato et al. observed increased levels of SOD in splenocytes as a result of sleep deprivation (Lungato et al. 2013). Nonetheless, Gao et al. reported significantly lower levels of antioxidant enzyme activity in sleep-deprived rats (Gao et al. 2019). Lower levels of antioxidant enzyme activity in the organs occur due to uncompensated oxidative stress – when enhanced free radicals eventually damage the antioxidant enzymes, resulting in decreased antioxidant responses, as shown in studies (Lungato et al. 2013; Villafuerte et al. 2015); therefore, these results suggested that our study on sleep deprivation caused moderate levels of toxic reactants, which led to an increase in antioxidant enzyme activities.

In addition, we observed elevated circulating LBP and proinflammatory cytokines (including IL-1β, IL-6, and TNF-α) levels (Figure 4A), LPS, a major component of the gram-negative bacteria outer membrane, is known as endotoxin, which causes endotoxemia when it released into the bloodstream (Gnauck et al. 2016; Meng et al. 2021). It binds to LBP, which eventually results in the production of cytokines and other proinflammatory mediators (Guha & Mackman 2001; Meng et al. 2021); therefore, LBP may regulate IL-1β, IL-6 and TNF-α. In addition, the mRNA levels of *TNF-α* and *IL-1B* significantly increase in the colon of the SDF and SDM groups, respectively (Figure 4B); the mRNA levels of *IL1-B* and *TNF-α* also significantly increase in the reproductive organs of the SDF and SDM groups, respectively (Figure 4C). Previous studies have shown that sleep deprivation causes inflammation (Lai et al. 2022; Mullington et al. 2010), these results are consistent with ours. Some ex vivo studies also revealed that production of TNF-α, IL-1β and IL-6 induced by LPS increases during sleep deprivation (Garbarino et al. 2021). A previous study showed that inflammation in the hypothalamus upregulates the *IL-1B* gene expression in the hypothalamus, reduces *GnRH* mRNA levels, and as a consequence, reduces LH release (Haziak et al. 2018); this result supports our findings on how inflammation in sleep-deprived rats is associated with a delay in the onset of puberty, especially in female rats.

After the rats had undergone 4 weeks of sleep deprivation, the richness and diversity of their gut microbiomes were significantly decreased regardless of sex.

Previous literature has demonstrated that CSD influences gut microbiome composition in both human and animal studies. One human study revealed that sleep disturbance for two days causes significant increase in Firmicutes-Bacterioides ratio (positive correlation with obesity), higher abundances of the families Coriobacteriaceae and Erysipelotrichaceae, and lower abundance of Tenericutes in young individuals (Benedict et al. 2016); in addition, another animal study indicated

sleep disruption for four weeks causes an increase of food intake, visceral white adipose tissue and systemic inflammation via changes in gut microbiomes (characterized by the preferential growth Lachnospiraceae and Ruminococcaceae and a decrease of Lactobacillaceae families)(Poroyko et al. 2016). Therefore, CSD causes white adipose tissue or systemic inflammation via gut microbiome alterations; these results are consistent with ours.

Previous studies have shown that f_Muribaculaceae attenuates obesity and is related to body weight loss (Hou et al. 2020; Lagkouvardos et al. 2019). At the genus level, we observed an increased abundance of Prevotellaceae UCG-001, which is positively correlated with the AMPK (AMP-activated protein kinase) activation signaling pathway (Song et al. 2019), and Ruminoccocaceae UCG-005, which has been reported to alleviate obesity (Zhang et al. 2019; Zhao et al. 2017). Thus, the increased abundance of these bacterial taxa may explain reduced body weight in the SDF group. Additionally, a higher abundance of g_Shuttleworthia in the SDM group was found to be correlated with inflammation (Du et al. 2022; Li et al. 2022). As a result, our findings regarding the abundance of bacterial taxa, increased levels of antioxidant enzyme activity, and proinflammatory markers after 4 weeks of sleep deprivation are consistent with those of other studies.

A limitation of our study is that we did not obtain data on hormone, kisspeptin, or GnRH levels at the onset of puberty. Unlike researchers in previous studies, we did not use electroencephalography (EEG) to measure sleep deprivation (Huber et al. 2000; Mohammed et al. 2011); however, results from our study and results from other studies appear to be consistent and independent of the use of EEG (Barf et al. 2012; Koban et al. 2008; Mohammed et al. 2011). Ours is the first study to investigate the association between CSD and the onset of puberty in juvenile rats.

In summary, we demonstrated that CSD increases antioxidant enzyme activity and inflammation as well as alternation of gut microbiome plays an important role in antioxidant enzyme activity and inflammation. Future studies are suggested to use prebiotics/postbiotic intervention to change the gut microbiome or treat with some compounds to reduce proinflammatory cytokines to reverse sleep deprivation-related alterations in the onset of puberty.

CONCLUSIONS

CSD causes delayed onset of puberty, as well as an increase in the levels of proinflammatory cytokines in the colon, reproductive organs, and circulatory system in both sexes; in addition, CSD also causes altered gut microbiome in both sexes. Therefore, CSD causes delayed onset of puberty in juvenile rats through an inflammatory response and alternation of gut microbiome.

AUTHOR CONTRIBUTIONS

Study conceptualization, S-Y H., Y-C C.; Animal and Cellular experiment, S.P.G., S-L.L., G-A.L.,T-H.T.; data collection, S.P.G., T-H.T., data analyses and interpretation, S.P.G., N.N.N., C-Y.L., C-T S., S-Y H., Y-C C.; drafting the manuscript, S.P.G., J-W H., Y-C C.; All authors have read and approved the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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DATA AVAILABILITY

The data underlying the current study are available from the corresponding author upon reasonable request.

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Figure legends

Figure 1. Sleep deprivation decreases body weight and delays the onset of puberty in both sexes. Sleep deprivation-treated groups consistently have lower body weight compared to control groups. Sleep deprivation delays vaginal opening and preputial separation in female and male rats, respectively. Dots represent group means ± SEM (n = 6 rats/group). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Figure 2. Sleep deprivation increases levels of free radical which induces elevated antioxidant enzyme activities.

(A) The comparison of antioxidant enzyme activities in colon of rats in the control and sleep deprivation groups is shown, (B) The comparison of antioxidant enzyme activities in reproductive organs (female: ovary, male: testis) of rats in the control and sleep deprivation groups is shown. Data are presented in as mean ± SEM (n = 6 rats/group). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Figure 3. Sleep deprivation causes an increase in LBP and proinflammatory cytokines levels in circulation, colon and reproductive organs in female and male rats. (A) Comparison of protein levels of LBP, IL-1β, IL-6 and TNF-α in the circulatory system between control and sleep deprived groups in female (superior section) and male (inferior section) rats. (B) Comparison of mRNA levels of *IL-1β*, *IL-6* and *TNF-α* in colon between control and sleep deprived groups in female (left section) and male (right section). (C) Comparison of mRNA levels of *TNF-α*, *IL-1β* and *IL-6* in

499 reproductive organs between control and sleep deprived groups in female (left section) and male
500 (right section) rats. Data are presented as mean ± SEM (n=6 in each group), *p<0.05; **p<0.01;
501 ***p<0.001.
502
503 **Figure 4.** Sleep deprivation alters gut microbiota composition in the sleep deprivation-treated rat
504 groups.
505 (A) The comparison of β-diversity patterns of rats in the control and sleep deprivation groups is
506 shown. (B) The comparison of abundant bacterial taxa of rats in the control and sleep deprivation
507 groups is shown. Different colors of linear discriminant analysis effect size (LEfSe) indicate the
508 group in which clade was most abundant (n = 6 rats/group). Significant bacterial genera were
509 determined by Kruskal-Wallis test (p < 0.05) with LDA score greater than 2.
510

Figure 5. Abundant bacterial taxa in sleep deprivation rats were associated with elevated antioxidant enzyme activities and proinflammatory mRNA levels.

(A-B) Heat map depicting associations between abundant bacterial taxa and antioxidant enzyme activities of rats in the control and sleep deprivation groups is shown. (C-D) Heat map depicting associations between abundant bacterial taxa and proinflammatory mRNA expression levels of rats in the control and sleep deprivation groups is shown. *p* value is determined with spearman's correlation; $^{*}p<0.05$

Figure 1

Sleep deprivation decreases body weight and delays the onset of puberty in both sexes

Sleep deprivation-treated groups consistently have lower body weight compared to control groups. Sleep deprivation delays vaginal opening and preputial separation in female and male rats, respectively. Dots represent group means ± SEM (n = 6 rats/group). *p<0.05; **p<0.01; ***p<0.001.

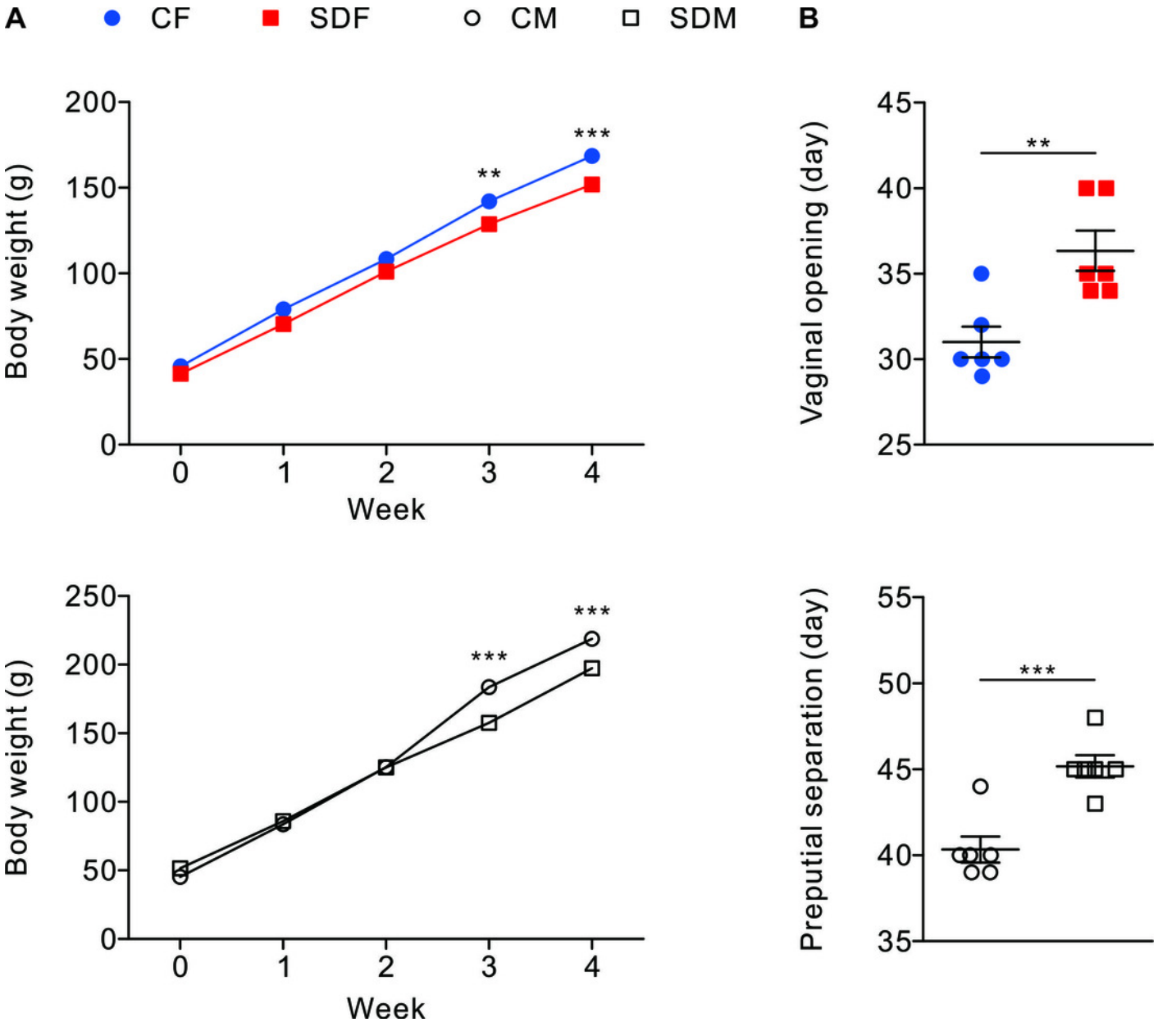


Figure 2

Sleep deprivation increases levels of free radical which induces elevated antioxidant enzyme activities.

(A) The comparison of antioxidant enzyme activities in colon of rats in the control and sleep deprivation groups is shown, (B) The comparison of antioxidant enzyme activities in reproductive organs (female: ovary, male: testis) of rats in the control and sleep deprivation groups is shown. Data are presented in as mean ± SEM (n = 6 rats/group). *p<0.05; **p<0.01; ***p<0.001.

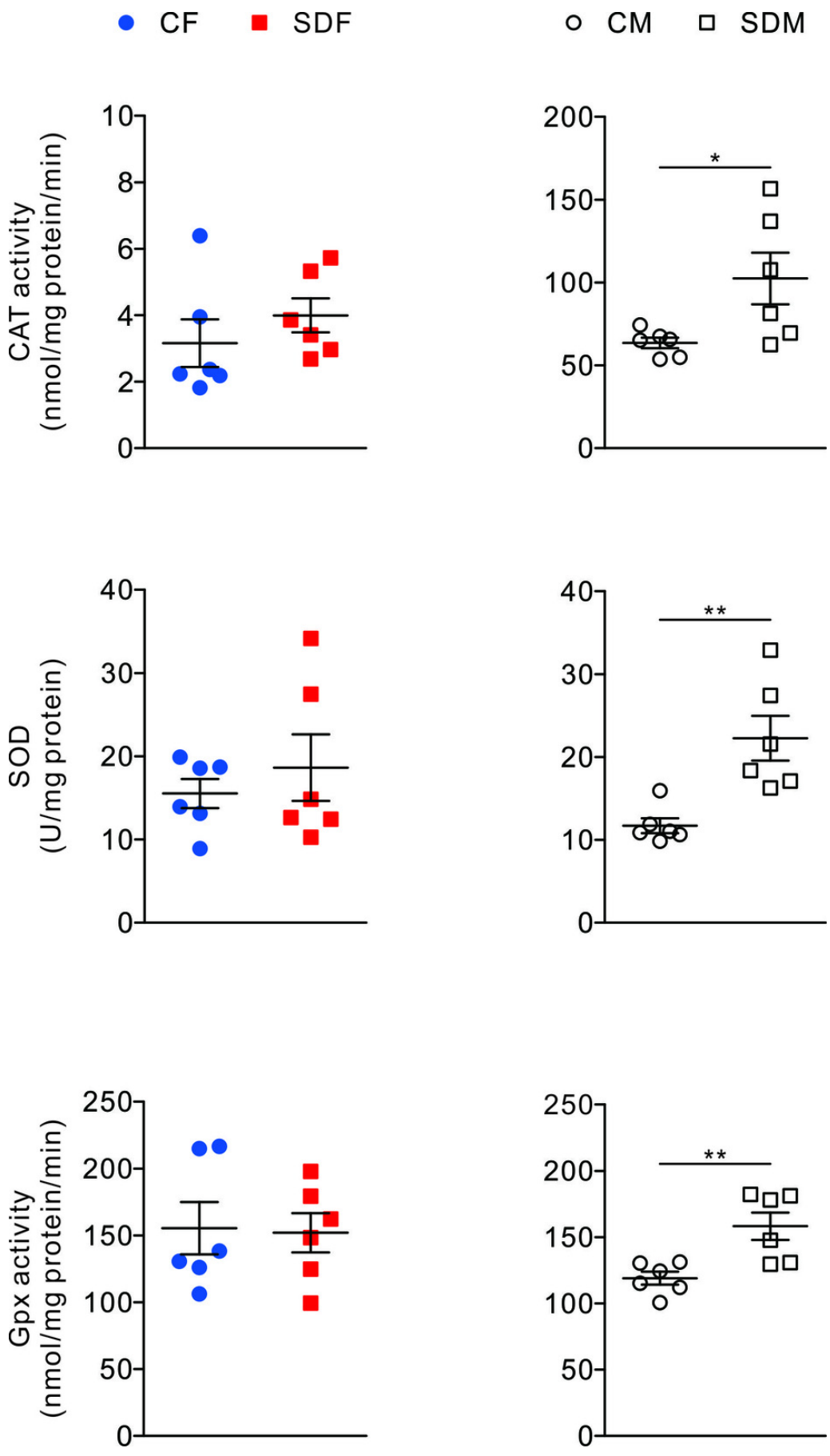

CF
SDF
CM
SDM
CAT activity
(nmol/mg protein/min)
SOD
(U/mg protein)
Gpx activity
(nmol/mg protein/min)
*
**
**

Figure 3

Sleep deprivation increases levels of free radical which induces elevated antioxidant enzyme activities

(A) The comparison of antioxidant enzyme activities in colon of rats in the control and sleep deprivation groups is shown, (B) The comparison of antioxidant enzyme activities in reproductive organs (female: ovary, male: testis) of rats in the control and sleep deprivation groups is shown. Data are presented in as mean ± SEM (n = 6 rats/group). *p<0.05; **p<0.01; ***p<0.001.

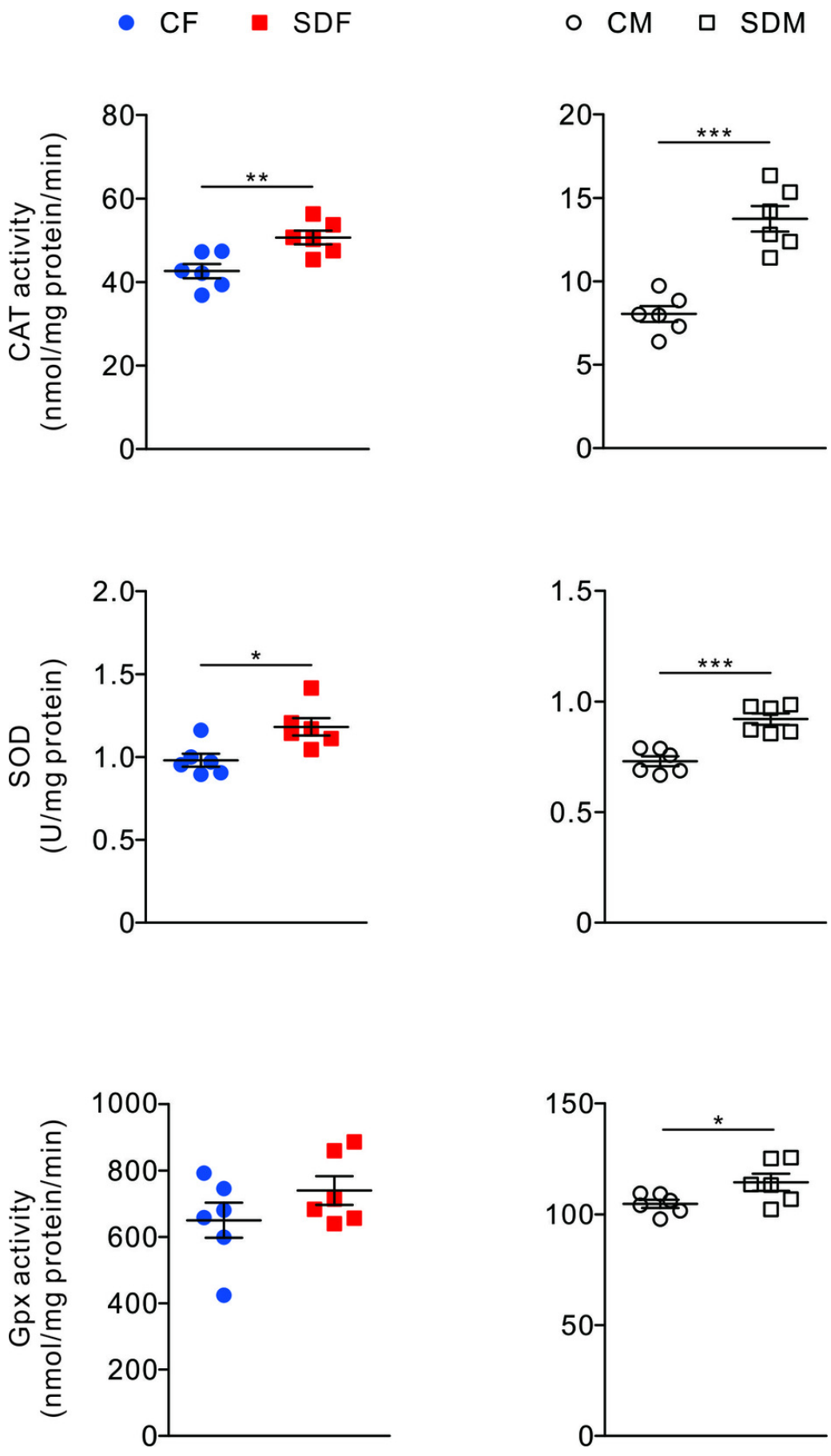
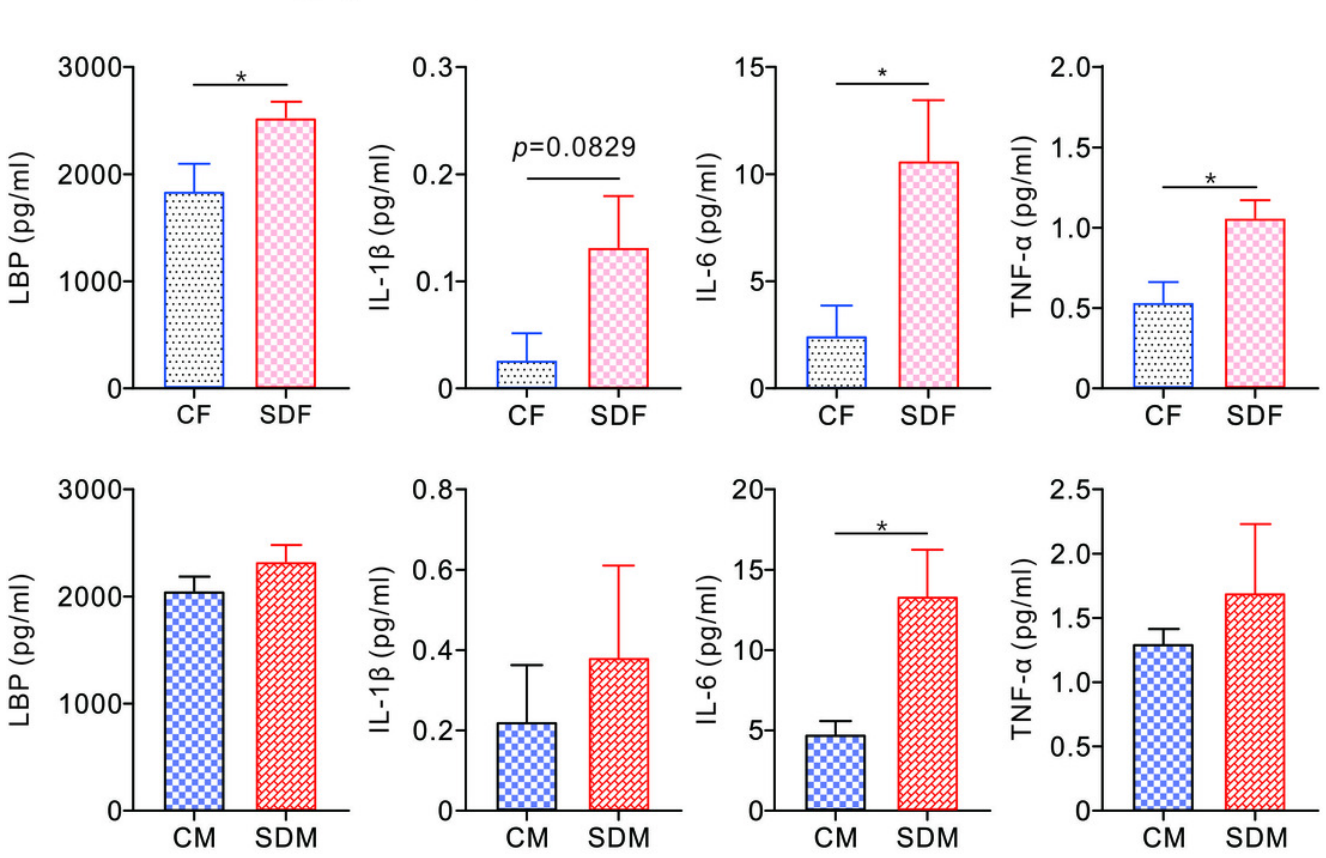
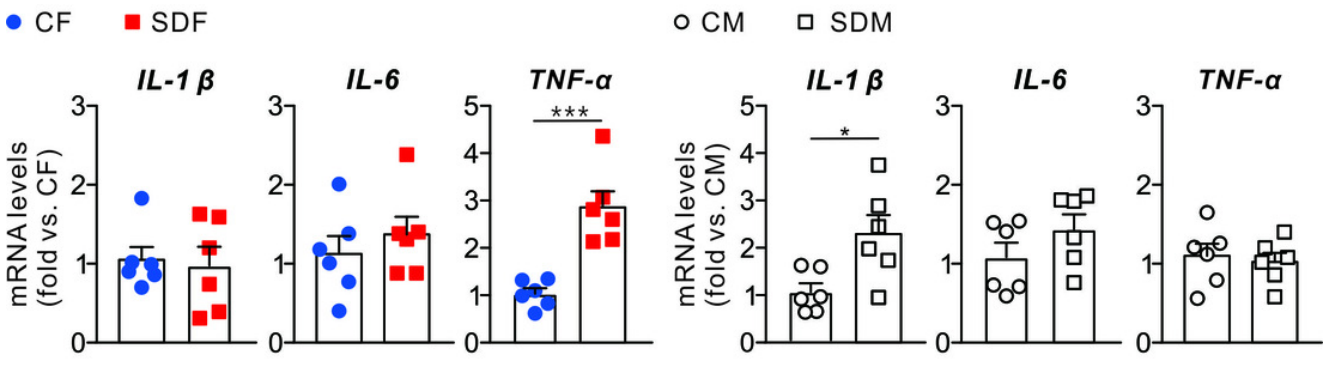

CF
SDF
CM
SDM
CAT activity
(nmol/mg protein/min)
80
60
40
20
0
**
20
15
10
5
0
***
SOD
(U/mg protein)
2.0
1.5
1.0
0.5
0
*
1.5
1.0
0.5
0
***
Gpx activity
(nmol/mg protein/min)
1000
800
600
400
200
0
150
100
50
0
*

Figure 4

Sleep deprivation causes an increase in LBP and proinflammatory cytokines levels in circulation, colon and reproductive organs in female and male rats

(A) Comparison of protein levels of LBP, IL-1β, IL-6 and TNF-α in the circulatory system between control and sleep deprived groups in female (superior section) and male (inferior section) rats. (B) Comparison of mRNA levels of *IL-1β*, *IL-6* and *TNF-α* in colon between control and sleep deprived groups in female (left section) and male (right section). (C) Comparison of mRNA levels of *TNF-α*, *IL-1β* and *IL-6* in reproductive organs between control and sleep deprived groups in female (left section) and male (right section) rats. Data are presented as mean ± SEM (n=6 in each group), *$p<0.05$; **$p<0.01$; ***$p<0.001$.


A Proinflammatory cytokines
LBP (pg/ml)
IL-1β (pg/ml)
IL-6 (pg/ml)
TNF-α (pg/ml)
p=0.0829
CF
SDF
CM
SDM


B Proinflammatory cytokine genes expression in colon
CF
SDF
CM
SDM
IL-1 β
IL-6
TNF-α
mRNA levels (fold vs. CF)
mRNA levels (fold vs. CM)

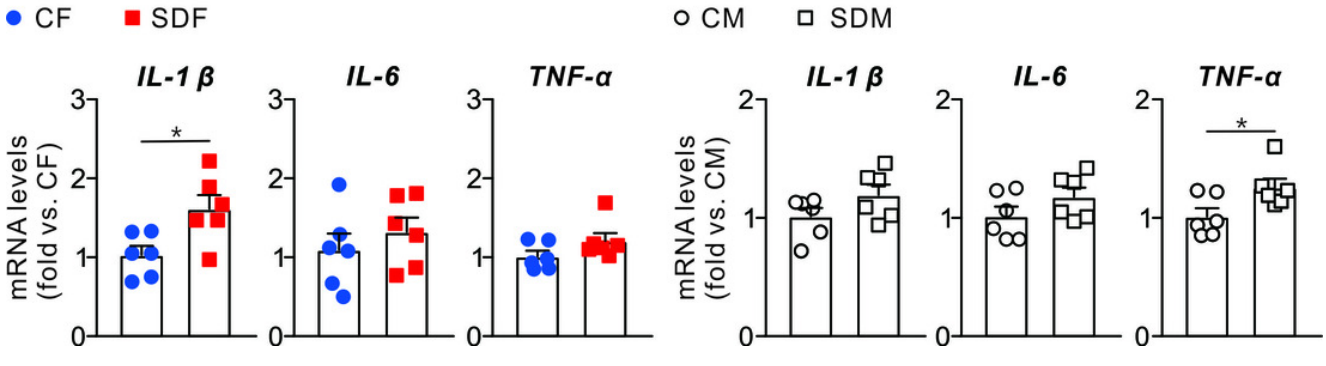

C Proinflammatory cytokine genes expression in reproductive organs
CF
SDF
CM
SDM
IL-1 β
IL-6
TNF-α
mRNA levels (fold vs. CF)
mRNA levels (fold vs. CM)

Figure 5

Sleep deprivation alters gut microbiota composition in the sleep deprivation-treated rat groups

(A) The comparison of β-diversity patterns of rats in the control and sleep deprivation groups is shown. (B) The comparison of abundant bacterial taxa of rats in the control and sleep deprivation groups is shown. Different colors of linear discriminant analysis effect size (LEfSe) indicate the group in which clade was most abundant (n = 6 rats/group). Significant bacterial genera were determined by Kruskal-Wallis test ($p < 0.05$) with LDA score greater than 2.

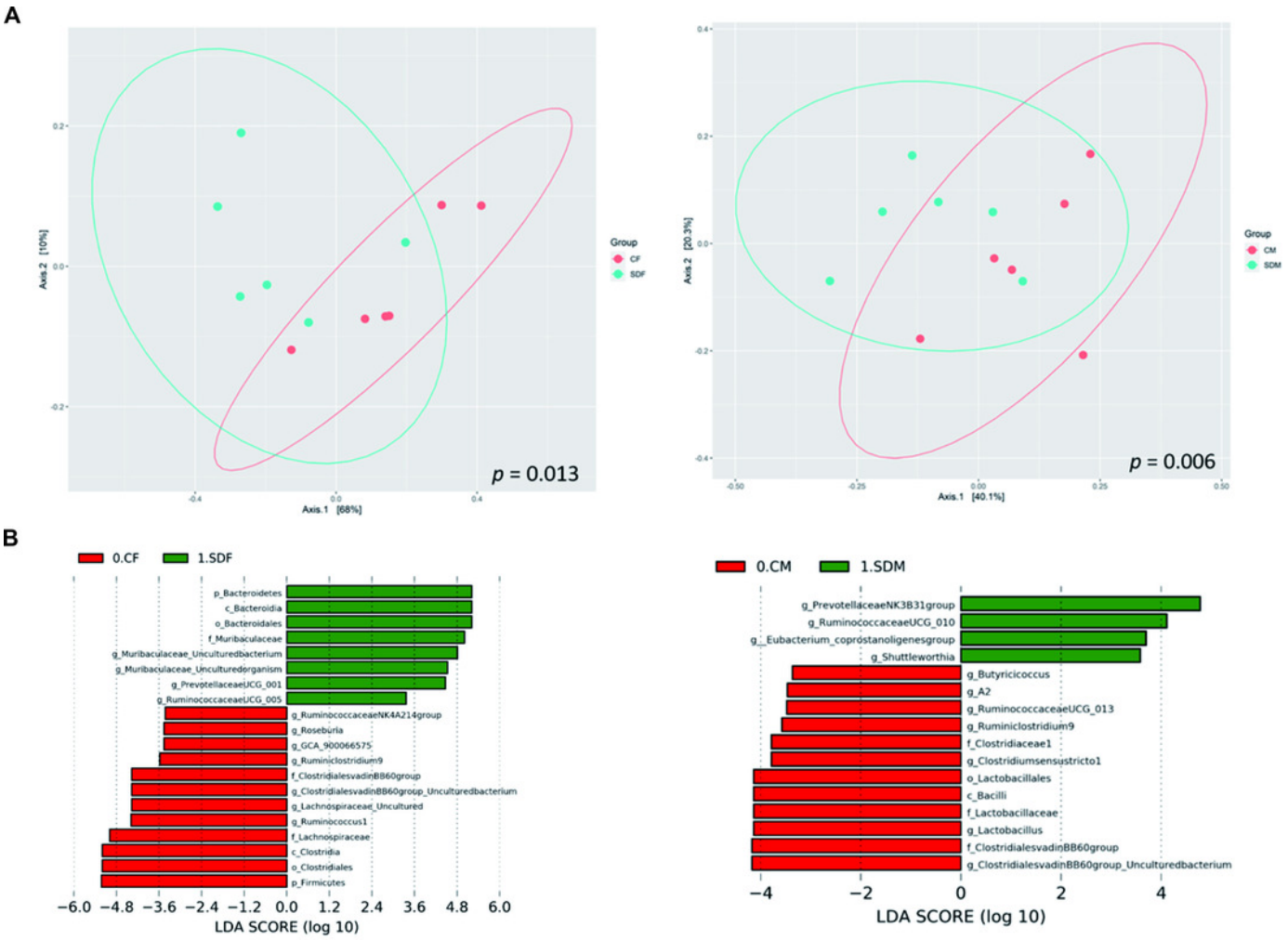


Figure 6

Abundant bacterial taxa in sleep deprivation rats were associated with elevated antioxidant enzyme activities and proinflammatory mRNA levels

(A-B) Heat map depicting associations between abundant bacterial taxa and antioxidant enzyme activities of rats in the control and sleep deprivation groups is shown. (C-D) Heat map depicting associations between abundant bacterial taxa and proinflammatory mRNA expression levels of rats in the control and sleep deprivation groups is shown. *p* value is determined with spearman's correlation; $*p<0.05$

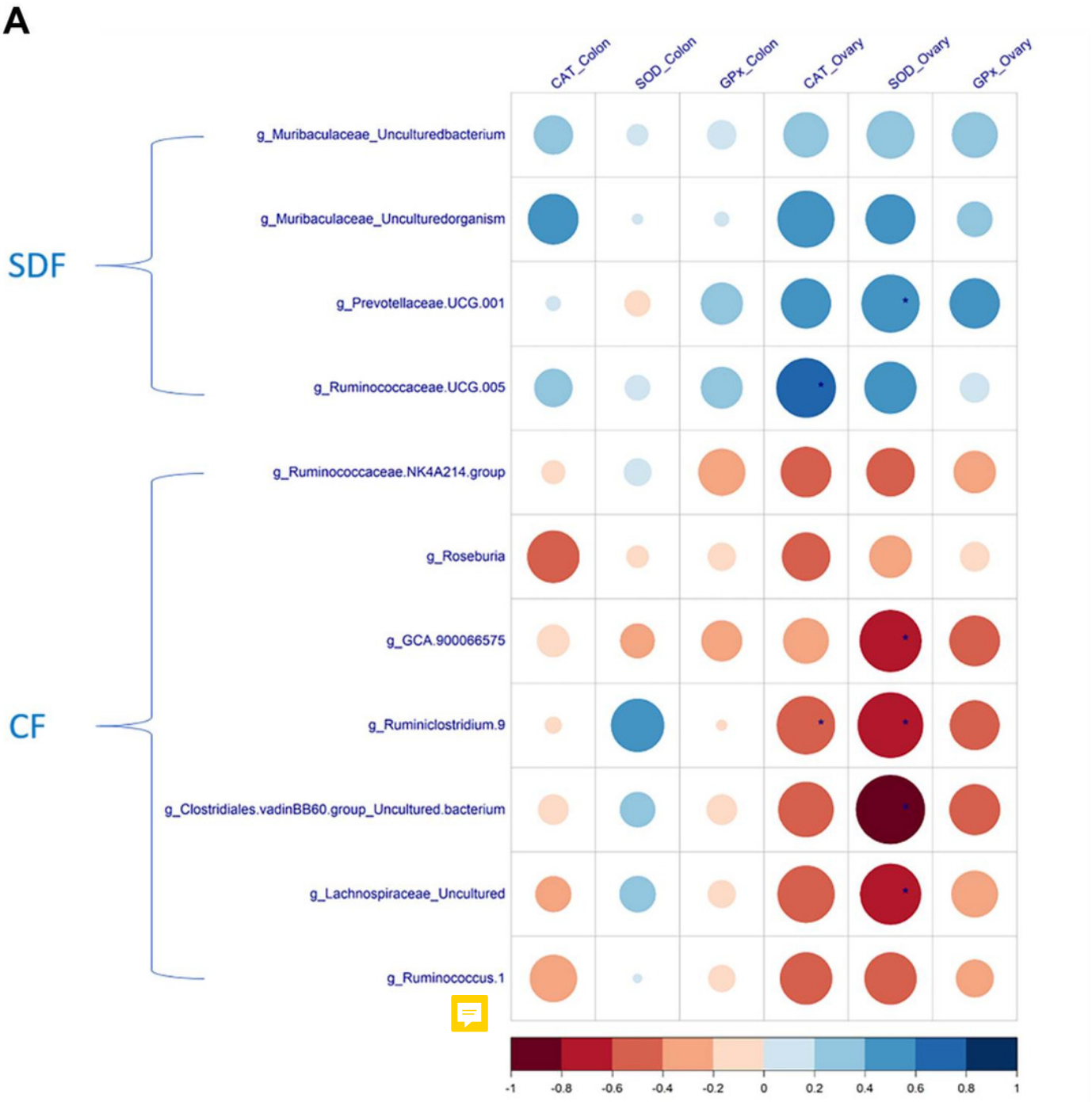

A
CAT_Colon
SOD_Colon
GPx_Colon
CAT_Ovary
SOD_Ovary
GPx_Ovary
SDF
CF
g_Muribaculaceae_Unculturedbacterium
g_Muribaculaceae_Unculturedorganism
g_Prevotellaceae.UCG.001
g_Ruminococcaceae.UCG.005
g_Ruminococcaceae.NK4A214.group
g_Roseburia
g_GCA.900066575
g_Ruminiclostridium.9
g_Clostridiales.vadinBB60.group_Uncultured.bacterium
g_Lachnospiraceae_Uncultured
g_Ruminococcus.1
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1

Figure 7

Abundant bacterial taxa in sleep deprivation rats were associated with elevated antioxidant enzyme activities and proinflammatory mRNA levels

(A-B) Heat map depicting associations between abundant bacterial taxa and antioxidant enzyme activities of rats in the control and sleep deprivation groups is shown. (C-D) Heat map depicting associations between abundant bacterial taxa and proinflammatory mRNA expression levels of rats in the control and sleep deprivation groups is shown. *p* value is determined with spearman's correlation; *$p<0.05$

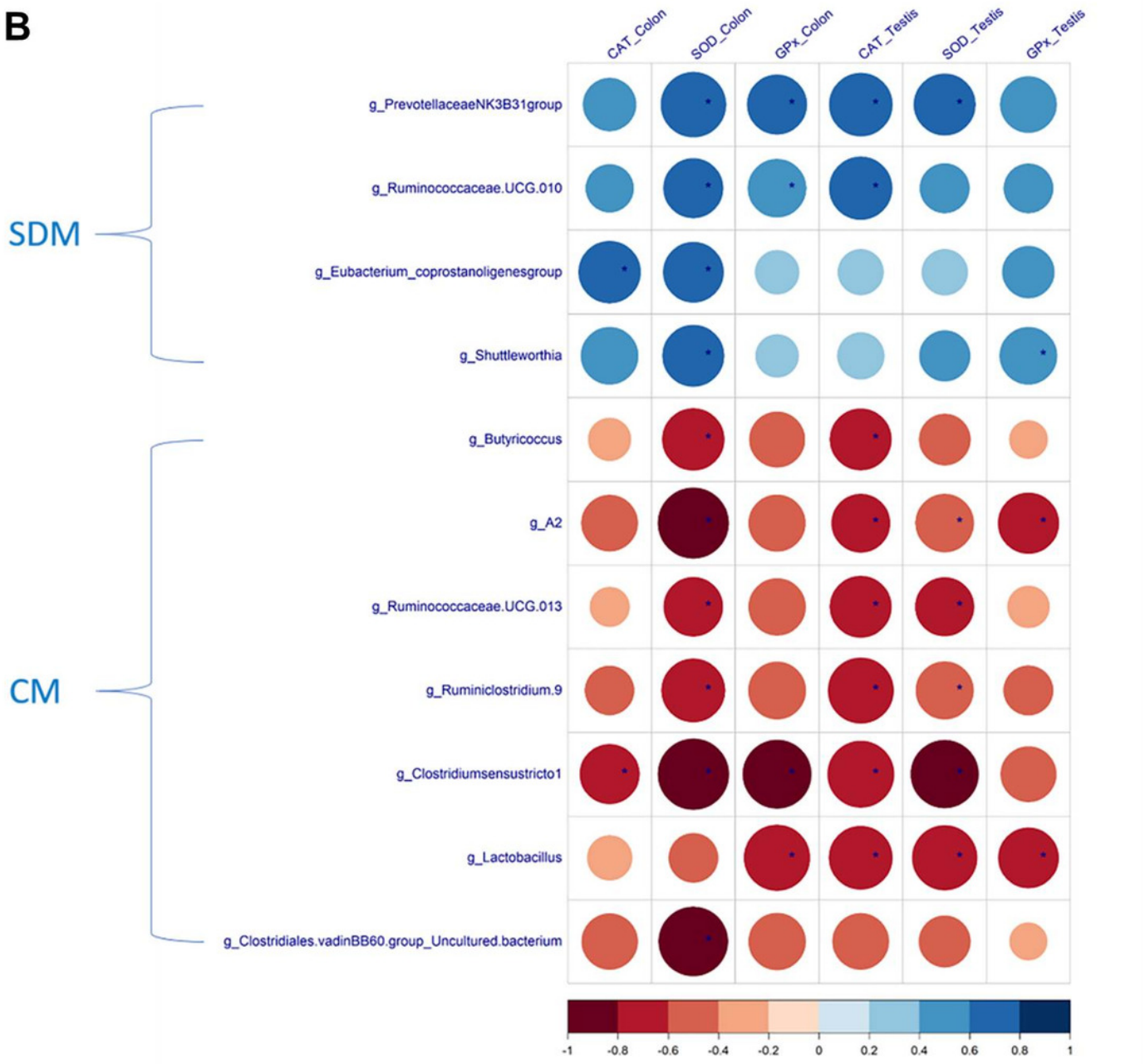

B
CAT_Colon
SOD_Colon
GPx_Colon
CAT_Testis
SOD_Testis
GPx_Testis
SDM
CM
g_PrevotellaceaeNK3B31group
g_Ruminococcaceae.UCG.010
g_Eubacterium_coprostanoligenesgroup
g_Shuttleworthia
g_Butyricoccus
g_A2
g_Ruminococcaceae.UCG.013
g_Ruminiclostridium.9
g_Clostridiumsensustricto1
g_Lactobacillus
g_Clostridiales.vadinBB60.group_Uncultured.bacterium
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1

Figure 8

Abundant bacterial taxa in sleep deprivation rats were associated with elevated antioxidant enzyme activities and proinflammatory mRNA levels

(A-B) Heat map depicting associations between abundant bacterial taxa and antioxidant enzyme activities of rats in the control and sleep deprivation groups is shown. (C-D) Heat map depicting associations between abundant bacterial taxa and proinflammatory mRNA expression levels of rats in the control and sleep deprivation groups is shown. *p* value is determined with spearman's correlation; *$p<0.05$

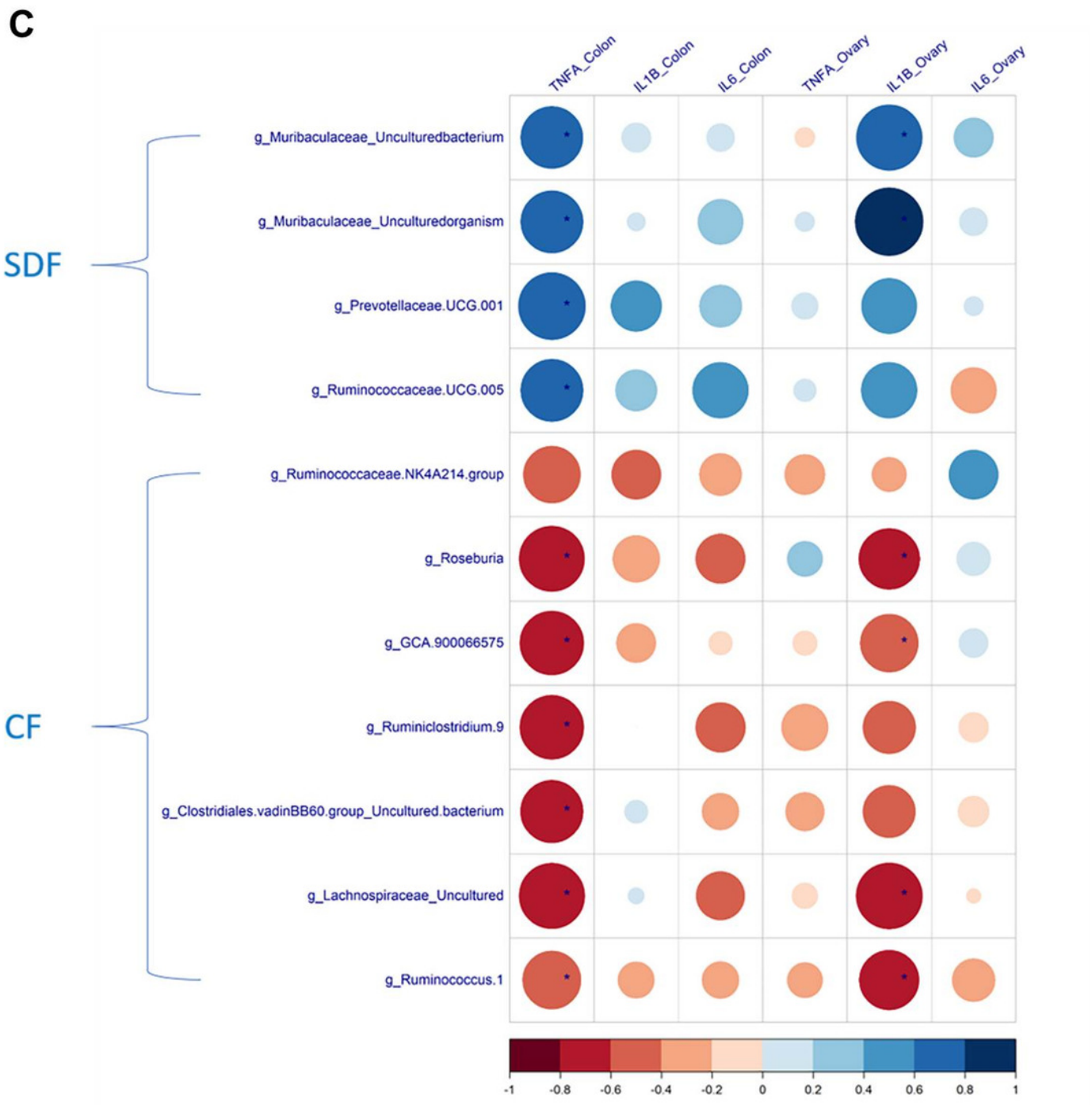
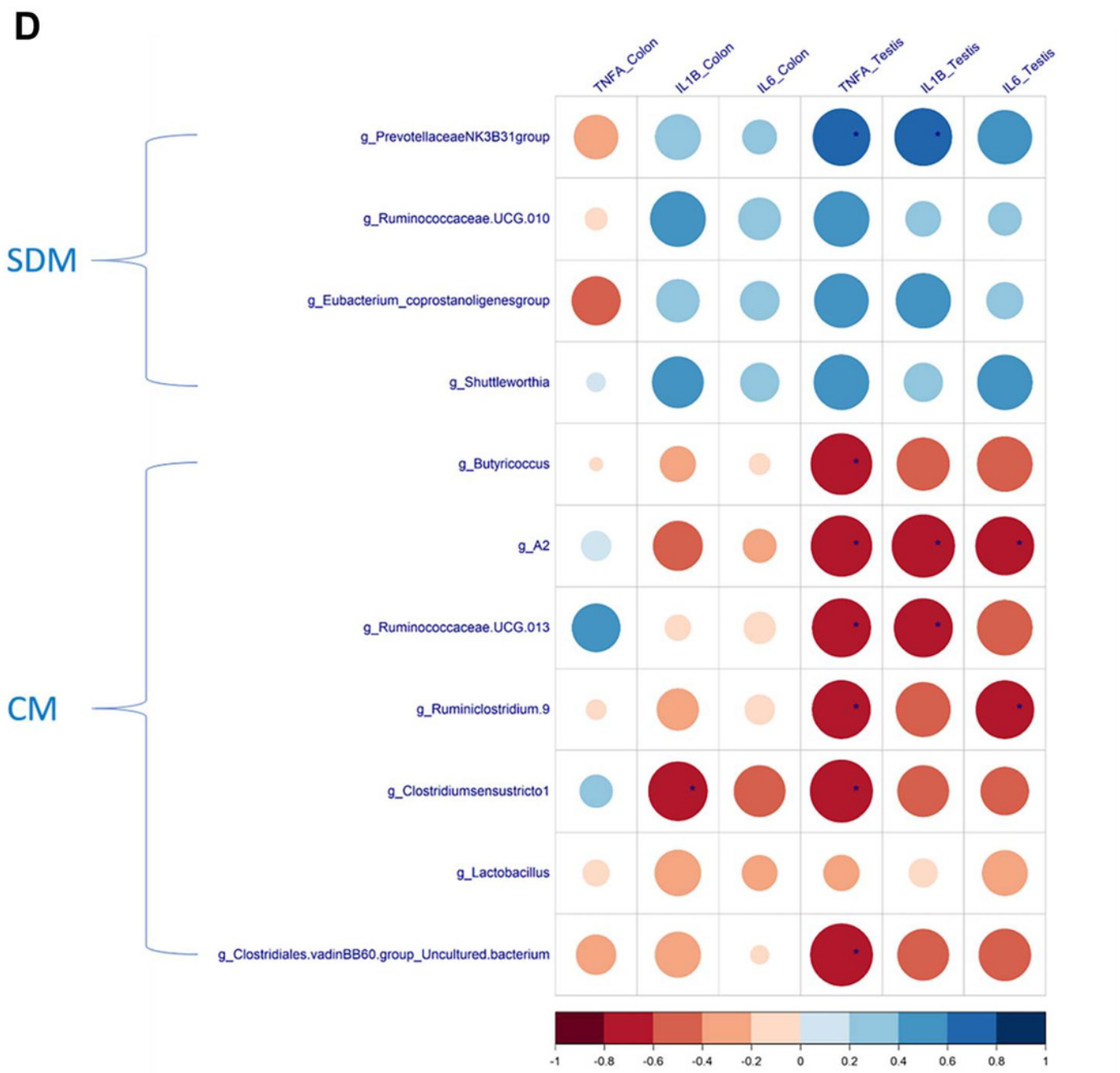

c
TNFA_Colon
IL1B_Colon
IL6_Colon
TNFA_Ovary
IL1B_Ovary
IL6_Ovary
SDF
CF
g_Muribaculaceae_Unculturedbacterium
g_Muribaculaceae_Unculturedorganism
g_Prevotellaceae.UCG.001
g_Ruminococcaceae.UCG.005
g_Ruminococcaceae.NK4A214.group
g_Roseburia
g_GCA.900066575
g_Ruminiclostridium.9
g_Clostridiales.vadinBB60.group_Uncultured.bacterium
g_Lachnospiraceae_Uncultured
g_Ruminococcus.1
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1

Figure 9

Abundant bacterial taxa in sleep deprivation rats were associated with elevated antioxidant enzyme activities and proinflammatory mRNA levels

(A-B) Heat map depicting associations between abundant bacterial taxa and antioxidant enzyme activities of rats in the control and sleep deprivation groups is shown. (C-D) Heat map depicting associations between abundant bacterial taxa and proinflammatory mRNA expression levels of rats in the control and sleep deprivation groups is shown. *p* value is determined with spearman's correlation; $*p<0.05$


D
TNFA_Colon
IL1B_Colon
IL6_Colon
TNFA_Testis
IL1B_Testis
IL6_Testis
SDM
CM
g_PrevotellaceaeNK3B31group
g_Ruminococcaceae.UCG.010
g_Eubacterium_coprostanoligenesgroup
g_Shuttleworthia
g_Butyricoccus
g_A2
g_Ruminococcaceae.UCG.013
g_Ruminiclostridium.9
g_Clostridiumsensustricto1
g_Lactobacillus
g_Clostridiales.vadinBB60.group_Uncultured.bacterium
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1