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Full Paper

Effects of subchronic and mild social defeat stress on the intestinal microbiota and fecal bile acid composition in mice

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The gut microbiota plays a crucial role in both the pathogenesis and alleviation of host depression by modulating the brain-gut axis. We have developed a murine model of human depression called the subchronic and mild social defeat stress (sCSDS) model, which impacts not only behavior but also the host gut microbiota and gut metabolites, including bile acids. In this study, we utilized liquid chromatography/mass spectrometry (LC/MS) to explore the effects of sCSDS on the mouse fecal bile acid profile. sCSDS mice exhibited significantly elevated levels of deoxycholic acid (DCA) and lithocholic acid (LCA) in fecal extracts, leading to a notable increase in total bile acids and 7α-dehydroxylated secondary bile acids. Consequently, a noteworthy negative correlation was identified between the abundances of DCA and LCA and the social interaction score, an indicator of susceptibility in stressed mice. Furthermore, analysis of the colonic microbiome unveiled a negative correlation between the abundance of CDCA and *Turicibacter*. Additionally, DCA and LCA exhibited positive correlations with *Oscillospiraceae* and *Lachnospiraceae* but negative correlations with the *Eubacterium coprostanoligenes* group. These findings suggest that sCSDS impacts the bidirectional interaction between the gut microbiota and bile acids and is associated with reduced social interaction, a behavioral indicator of susceptibility in stressed mice.

Key words: psychological stress, bile acids, depression model, feces, deoxycholic acid, lithocholic acid

INTRODUCTION

Depression is a major mood disorder that affects the health of people throughout their lifetimes, is a leading cause of disability around the world, and contributes greatly to the global burden of disease (WHO, 2021: https://www.who.int/news-room/fact-sheets/detail/depression). The gut–microbiota–brain axis is a complex network involving multiple biological systems, including the nervous, endocrine, and immune systems. This network enables bidirectional communication between gut bacteria and the brain and plays a crucial role in the overall health of the host, including mental state, and has been associated with mental and neurodegenerative disorders [1]. Stress, depression, and anxiety are highly co-morbid conditions and have overlapping biological mechanisms and manifestations [2]. The process of responding to psychological stress and restoring homeostasis involves dynamic regulation of the body's stress response systems [3]. Analysis of a mouse stress model revealed that the gut microbiota is involved in these stress response systems [4, 5].

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Chronic social defeat stress (CSDS) is widely used to develop murine models of human depression. It is based on the resident intruder paradigm between an elder resident aggressor male ICR mouse and a younger intruder C57BL/6 J mouse. The standard CSDS protocol consisted of 5 to 10 min of physical attack per day and subsequent sensory contact between the resident and intruder for 10 days [6]. It is known that C57BL/6 J mice display less aversive behavior in response to the aggressor compared with BALB/c mice. Our research group found that BALB/c mice exhibited higher susceptibility to CSDS due to the downregulation of claudin-1, a tight junction protein that regulates the permeability and barrier function of intestinal epithelial cells [7]. Another murine stress model was developed using subchronic and mild social defeat stress (sCSDS), in which the attack time was reduced by 30 sec each day, starting from 5 min [8]. Mice exposed to CSDS (CSDS mice) exhibited social deficit-associated behaviors and body weight loss, while those exposed to sCSDS (sCSDS mice) showed increased water intake and higher body water contents [8]. An elevated level of taurocyamine, an inhibitor of taurine transporters, was observed in the liver in sCSDS mice, and this may result in the dysfunction of taurine-induced osmoregulation following an increase in body water composition [9, 10]. Using omics approaches, we have demonstrated that sCSDS significantly reduces the expression of genes responsible for the immune response in the ileum [11]. Additionally, we have found that a lectin microarray analysis showed decreased reactivity of the distal intestinal mucosa to fucose-specific lectins and that the expression levels of *Fut1* and *Fut2* genes are downregulated in the distal ileum [12]. Among the metabolites analysed by capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS), the most abundant metabolite in the cecum of sCSDS mice was cholic acid (CA), which was significantly accumulated compared with the levels in the cecum of control mice [11]. Due to the sensitivity limitations of CE-TOFMS, only CA was detected among bile acids in the metabolome analysis.

Bile acids are originally identified as micelle-forming surfactants that promote solubilization and absorption of lipids. The primary bile acids, including CA, are synthesized from cholesterol in the liver and are conjugated with either glycine or taurine to increase their solubility before being stored in the gallbladder. Bile acids secreted into the intestine are transformed by the gut microbiota into unconjugated bile acids, which are further converted into secondary bile acids. The primary bile acids, CA and CDCA, are converted by the gut microbiota into the secondary bile acids DCA and LCA, respectively, via 7α-dehydroxylation [13]. Another process that occurs in the gut microbiota is the conversion of the hydroxy group in the β-configuration at the 6-position to the α-configuration, which converts β-muricholic acid (MCA) to ω-MCA in mice. It is suggested that different microbiota are involved in the 7α-dehydroxylation and conversion from β-MCA to ω-MCA [14]. While 95% of the intestinal bile acids are reabsorbed in the enterohepatic circulation, the remaining 5% are excreted in feces [15].

Besides participating in the digestion and absorption of fat, secondary bile acids modified by the gut microbiota have also been identified as pleiotropic signaling molecules that regulate various physiological and pathological processes [13, 16, 17]. The interaction between bile acids and the gut microbiota changes the bile acid composition and modulates signaling via the nuclear bile acid receptor farnesoid X receptor (FXR) and the plasma membrane receptor G protein-coupled bile acid receptor 1 (GPBAR1/ TGR5) [17]. FXR is primarily activated mainly by the primary bile acids, chenodeoxycholic acid (CDCA), and to a lesser extent by the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (LCA) [18, 19], while the most potent ligands for GPBAR1 are LCA and DCA [17]. Activated FXR induces the expression of *Fgf15* (murine *FGF19*), which in turn triggers a signaling cascade that inhibits the expression of *Cyp7a1*, encoding a rate-limiting enzyme for bile acid biosynthesis from cholesterol [20]. In fact, our previous study revealed a significant increase in gene expression of *Fgf15* in the distal ileum of sCSDS mice on day 13 after an sCSDS period (days 1–10) [11]. Feedback inhibition of *Cyp7a1* transcription by FXR is one of the most important mechanisms in bile acid homeostasis [21]. Furthermore, FXR negatively regulates Slc10a2/ASBT, the main bile acid transport system in ileal enterocytes [22]. Thus, bile acids are important components that regulate the gut microbiota population through antimicrobial activity and control of gene expression [23, 24] and through systemic functions by their metabolites.

Recent studies have shown that bile acids play a significant role in connecting the gut microbiome and the brain [25, 26]. We hypothesized that fecal profiles of individual bile acids could be utilized as biomarkers to assess the psychological stress-induced behavioral impairments and subsequent changes of gut microbiota in an animal model. This study aimed to investigate the effect of sCSDS on fecal bile acids and the gut microbiota and to explore their relationships.

MATERIALS AND METHODS

Materials

Bile acid standards, 5β-cholanic acid-3α, 7α, 12α-triol (CA), 5β- cholanic acid-3α, 12α-diol (DCA), 5β-cholanic acid-3α-ol (LCA), 5β-cholanic acid-3α, 7α-diol (CDCA), 5β-cholanic acid-3α, 6α-diol (hyodeoxycholic acid; HDCA), 5β-cholanic acid-3α, 7β-diol (ursodeoxycholic acid; UDCA), 5β-cholanic acid-3α, 12α-diol N-(carboxymethyl)-amide (glycodeoxycholic acid; GDCA), 5β-cholanic acid-3α, 7β-diol N-(carboxymethyl)-amide (glycoursodeoxycholic acid; GUDCA), 5β-cholanic acid-3α-ol N-(carboxymethyl)-amide (glycolithocholic acid; GLCA), 5β-cholanic acid-3α, 7α, 12α-triol N-(2-sulphoethyl)-amide (taurocholic acid; TCA), 5β-cholanic acid-3α-ol N-(2-sulphoethyl)-amide (taurolithocholic acid; TLCA), 5β-cholanic acid-3α, 12α-diol N-(2-sulphoethyl)-amide (taurodeoxycholic acid; TDCA), and an internal standard, 23-nor-5β-cholanic acid-3α, 12α-diol (nordeoxycholic acid; NDCA) were purchased from Steraloids (Newport, RI, USA). Standards of MCAs were not used in this study.

Animals

All animal procedures were conducted in accordance with the animal experimentation guidelines of the National Agriculture and Food Research Organization (Ibaraki, Japan). The protocol was approved by the Animal Care Committee, National Institute of Livestock and Grassland Science (NILGS, Ibaraki, Japan; permit number: 14113017). Male C57BL/6JJmsSlc (C57BL/6) mice (7 weeks of age) and male Slc:ICR (ICR) mice (older than 5 months of age) were purchased from SLC Japan (Shizuoka, Japan) and

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reared in the animal facility of the NILGS. C57BL/6 mice had received *ad libitum* reverse osmosis-purified drinking water and a semi-purified diet (AIN-93G, Oriental Yeast, Tokyo, Japan). Food and water were weighed every day to monitor daily consumption of food and water in the C57BL/6 mice. Body weight was also weighed every day to calculate body weight gain (BWG).

Experimental design of sCSDS

The introduction of a C57BL/6 mouse into the cage of an ICR mouse resulted in the resident attacking the intruder. The duration of physical contact was limited to 5 min after the first attack bites on day 1, after which the duration was reduced stepwise by 0.5 min per day (sCSDS mice, n=7) [8, 9, 27]. After the physical contact, the ICR mice and intruder C57BL/6 mice were kept in the same cages, separated by a perforated acrylic plate, which enabled sensory contact while preventing physical contact for 24 hr. Control C57BL/6 mice were housed in cages in the same manner, that is, on either side of the divider, for 10 days without any physical contact (control mice, n=6). To evaluate the social behaviors of the C57BL/6 mice after exposure to sCSDS for 10 days (days 1–10) and the control mice, a social interaction (SI) test was performed on day 10, as described previously [8, 9]. SI scores (% of target absent) were estimated as 100 × (duration of time spent in interaction zone in the presence of the ICR mouse / duration of time spent in interaction zone in the absence of the ICR mouse), as described by Krishnan *et al.* [28]. On day 11, the mice were euthanized under anesthesia, and spleens and thymuses were removed and weighed. Colon contents were collected for metagenome analysis and kept at −80°C until use.

Bile acid analysis

Analysis of bile acids in feces was performed according to Hagio *et al.* [29]. Briefly, fresh feces were collected on day 7 during bedding replacement to minimize the impact on the inherent effects of sCSDS and were stored at −80°C until use. The feces were lyophilized in an evaporator, and 100 mg aliquots were ground in a mortar, to which 1 mL of ethanol and 50 µL of 500 µM NDCA (25 nmol) in methanol as an internal standard were added. The ethanol extract of the freeze-dried feces was evaporated and dissolved in methanol. Bile acids in the solution were purified by solid extraction with an HLB cartridge (Waters, Milford, MA, USA) based on the manufacturer's instructions. Each sample was analyzed using an ACQUITY UPLC system connected to an Xevo TQD by a ZSpray ion source (Waters) to identify the following bile acids: CA, DCA, LCA, CDCA, HDCA, UDCA, GDCA, GUDCA, GLCA, TCA, TLCA, and TDCA. The individual bile acid concentrations were calculated as normalized values by using NDCA as the internal standard.

DNA isolation and 16S rRNA metagenomic analysis

DNA from colon contents was prepared from precipitates using a QIAamp DNA Stool Mini Kit (QIAGEN). Pyrosequencing of 16S ribosomal RNA (rRNA) genes was conducted using the GS-FLX 454 platform with Titanium chemistry at Macrogen Inc. (Seoul, South Korea), as described previously [11]. Nucleotide sequence data reported are available in the DDBJ Sequenced Read Archive (DRA) under the BioProject PRJDB15491 (BioSamples SAMD00595560−SAMD00595570). In addition, previous nucleotide sequence data of fecal samples [11] were obtained from the DDBJ Sequenced Read Archive under the BioProject DRA004574 (BioSamples SAMD00035497−SAMD00035521) and analyzed with the present data.

The sequencing data were processed with the QIIME 2 pipeline using the SILVA 128 SSU database [30–32]. First, the read sequences were clustered into amplicon sequence variants (ASVs) by DADA2 [33] and identified taxonomically. Samples were then classified into three clusters by principal coordinate analysis (PCoA) and beta-diversity analysis.

Statistical analysis

Food intake (FI), water intake (WI), spleen weight, and thymus weight were analyzed by unpaired Student's t-test using the JMP Pro version 16 statistical software (SAS Institute Inc., Cary, NC, USA). Body weight gain data were analyzed by Welch's t-test because the variances between groups were not equal. Data are reported as means ± SE. Values with a p-value less than 0.05 were considered statistically significant.

The representative ASVs, accounting for more than 0.5% of the total reads and detected in 3 or more mice, were selected. Spearman's rank correlation coefficient (r_s) was calculated between each representative ASV and each of the five types of bile acids as well as between each representative ASV and the SI score, and these correlations were used to generate a heat map using R. Spearman's rank correlation coefficient was also calculated between each of the five types of bile acids and the SI score.

RESULTS

Effects of sCSDS on social behaviors

Throughout sCSDS, the direct attack time of the ICR mice was consistently reduced by 30 sec each day, starting from 5 min. Two animals in the sCSDS group with SI scores above 100 were considered resilient mice. Therefore, these two animals were excluded from the subsequent analysis. Further comparisons were made between the control group and the sCSDS-susceptible group. The results of the SI test showed that mice in the sCSDS group showed significantly lower SI scores compared with the mice in the control group (Fig. 1). Comparisons of SI scores, physical and physiological changes, and bile acid contents in the feces of all mice, including the resilient mice, are presented in Supplementary Fig. 1, Supplementary Table 1, and Supplementary Table 2, respectively.

Body weight gain, food intake, and water intake

Daily BWG, FI, and WI were monitored to assess the impact of sCSDS on overall body condition. On day 11 after the sCSDS period, the weights of the spleen and thymus were also measured (Table 1). The mice in the sCSDS group exhibited a tendency ($p<0.1$) for higher BWG, average WI, and a lighter thymus weight compared with the mice in the control group, as observed in a previous study [9].

Effects of sCSDS on bile acid contents

Fresh feces were collected from the C57BL/6 mice at day 7 and analyzed for various bile acid levels. Thirteen bile acid standards, including NDCA added as an internal standard, were used to analyze the samples, and seven bile acids were detected and quantified (Table 2). Among these bile acids, the fecal content of DCA and LCA, along with the 7α-dehydroxylated secondary

bile acids, in the sCSDS group was significantly higher than that in the control group. The total bile acid content per 100 mg of dried feces was 53.2 nmol in the control group and 120 nmol in the sCSDS group, reflecting a significantly higher overall bile acid content in the sCSDS group (Table 2).

The primary bile acids CA and CDCA exhibited substantial variability and did not show significant differences in the sCSDS group. Moreover, the Spearman's rank correlation coefficient between SI and DCA showed negative correlation (r_s=−0.68, p<0.05). In the cases of other bile acids, CA, CDCA, HDCA, and LCA, the calculated absolute values of r_s were less than the critical value ($|r_s|$<0.618) from Spearman's rho table. This suggests activated conversion from primary to 7α-dehydroxylated secondary bile acids within the sCSDS group.

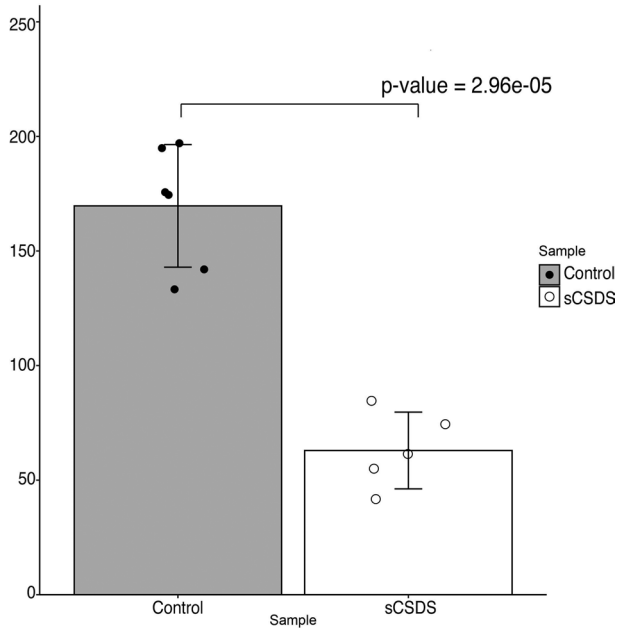

Fig. 1. Effects of subchronic and mild social defeat stress (sCSDS) on SI scores. SI scores (%) were estimated as described in the Materials and Methods. Two mice considered resilient mice (SI scores >100) in the sCSDS group were excluded from this figure. The sCSDS mice (n=5) showed significantly lower values than the control mice (n=6). **p=2.96e-05 vs. control. Data are expressed as the mean ± standard error of the mean (SEM).

Effects of sCSDS on the colon microbiota

The effect of 10 days of exposure to sCSDS on microbiota in colon contents was explored using 16S rRNA gene sequencing, and 254 ASVs were identified. We calculated Spearman's rank correlation coefficients between the abundances of 27 representative ASVs and bile acids, as well asbetween the abundances of the 27 representative ASVs and the SI score. These 27 ASVs, each accounting for more than 0.5% of the total reads and detected in 3 or more mice, were selected for the analysis. The results are presented as a heatmap in Fig. 2.

Among the primary bile acids, CDCA exhibited a negative correlation with ASV19_*Turicibacter* (r_s=−0.75, p<0.05), while CA did not show any significant correlation with the 27 ASVs. Conversely, the secondary bile acid DCA demonstrated positive correlations with ASV6_*Lachnospiraceae* (r_s=0.71, p<0.05) and ASV7_*Oscillospiraceae* (r_s=0.72, p<0.05) and a negative correlation with ASV11_*Eubacterium coprostanoligenes* group (r_s=−0.72, p<0.05). LCA exhibited positive correlations with ASV7 (r_s=0.72, p<0.05) and ASV22_*Roseburia* (r_s=0.69, p<0.05) and a negative correlation with ASV11 (r_s=0.88, p<0.01). SI showed strong positive correlations with ASV11 (r_s=0.88, p<0.01) and ASV16_*Akkermansia* (r_s=0.71, p<0.05) and negative correlations with ASV6 (r_s=−0.78, p<0.01) and ASV7 (r_s=−0.70, p<0.05). ASVs that exhibited positive correlations with DCA were inversely correlated with the SI score, while ASV16 displayed a notably high correlation solely with the SI score. These findings suggest that altered gut microbiota induced by sCSDS may influence the conversion to 7α-dehydroxylated secondary bile acids.

To investigate the impact of sCSDS on the colonic microbiota, PCoA based on weighted and unweighted UniFrac distances was performed. The control mice and sCSDS mice were divided into different clusters by PCoA of the β-diversity of the colonic microbiota (Fig. 3). PCoA based on the weighted UniFrac distance revealed that the ASV belonging to *Bacteroides* contributed most positively to the PC2 distribution, while the ASVs belonging to *Lachnospiraceae*_NK4A136_group and *Faecalibaculum* contributed most negatively to the PC2 distribution. PCoA based on the unweighted UniFrac distance revealed that the ASVs belonging to *Oscillospiraceae* contributed most positively to the PC1 distribution, while the ASVs belonging to *Lachnospiraceae* negatively contributed to the PC1 distribution.

Table 1. Physical and physiological changes in mice induced by subchronic and mild social defeat stress (sCSDS)

Measured items	Group		t-test
	Control ± SE	sCSDS ± SE	Group
Average body weight gain (BWG) (g/day)[a]	0.142 ± 0.037	0.281 ± 0.060	p=0.0508
Average food intake (FI) (g)[a]	2.68 ± 0.048	2.75 ± 0.047	p=0.150
Average water intake (WI) (g)[a]	3.39 ± 0.151	3.74 ± 0.123	p=0.08
Spleen (mg)[b]	69.9 ± 6.17	72.4 ± 6.71	p=0.786
Thymus (mg)[b]	31.3 ± 1.56	26.4 ± 1.99	p=0.0808

[a]average of sCSDS period
[b]weights were measured on day 11 after the sCSDS period.
FI, WI, and the weights of the spleen and thymus were analyzed using an unpaired Student's t-test. BWG was analyzed using Welch's t-test.
SE: standard error.

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DISCUSSION

We previously identified CA as the predominant cecal metabolite of sCSDS mice compared with control mice [11], and this prompted the present study to obtain detailed profiles of fecal bile acids other than CA using LC/MS. Unexpectedly, there was no significant difference in fecal CA contents between the control and sCSDS groups. There were clear differences in the stress duration and organs examined between the previous and present study. Cecum contents were analyzed on day 10 in the previous study, whereas feces were analyzed on day 7 in the present study. It is possible that the conversion of primary bile

Table 2. Bile acid contents in feces of mice that suffered 7 days of stress

	Control mice nmol/100 mg feces ± SE	sCSDS mice nmol/100 mg feces ± SE	t-test group
CA	1.30 ± 0.473	1.55 ± 0.463	p=0.72
CDCA	7.50 ± 2.00	13.1 ± 2.21	p=0.093
DCA	32.9 ± 2.63	83.2 ± 17.5	p=0.012
LCA	10.72 ± 1.59	20.2 ± 2.54	p=0.0095
HDCA	1.17 ± 0.31	2.91 ± 0.78	p=0.052
UDCA	n.d.	n.d.	n.t.
GDCA	n.d.	n.d.	n.t.
GUDCA	n.d.	n.d.	n.t.
GLCA	n.d.	n.d.	n.t.
TCA	n.d.	n.d.	n.t.
TLCA	0.164 ± 0.04	0.0985 ± 0.017	p=0.19
TDCA	0.0592 ± 0.0092	0.0444 ± 0.0048	p=0.21
NDCA[a]	25.0	25.0	
Total	53.8 ± 4.86	121 ± 22.1	p=0.0098
Primary bile acids	8.80 ± 2.27	14.6 ± 2.31	p=0.11
7a-dehydroxylated secondary bile acids	45.0 ± 3.51	106 ± 20.3	p=0.0095

[a]internal control.
sCSDS: subchronic and mild social defeat stress; SE: standard error; n.d.: not detected; n.t.: not tested.

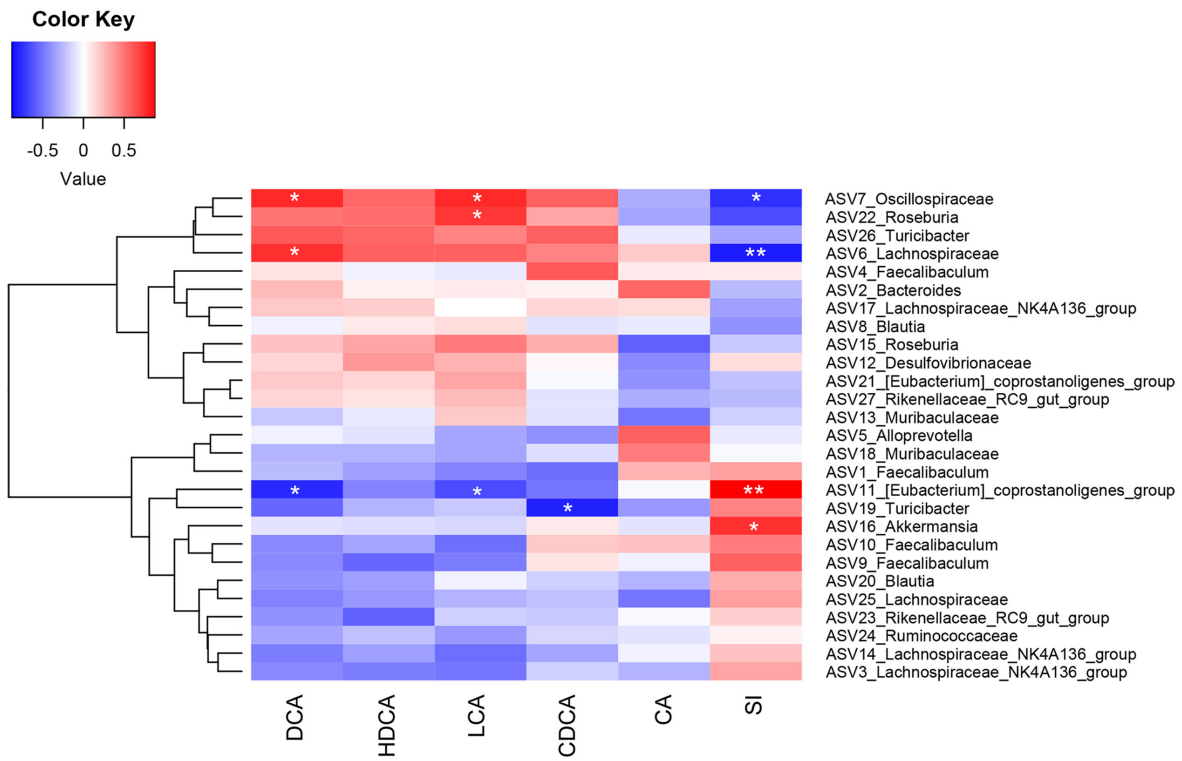

Fig. 2. Heat map of correlation analysis between bile acids/SI and amplicon sequence variants (ASVs) in colon contents. Among the ASVs accounting for more than 0.5% of the total reads, those detected in three or more mice were selected; the Spearman's rank correlation coefficient (r_s) between the ratio of ASVs present and each bile acid or SI score was calculated. *Significant correlation at p<0.05 ($|r_s|$ >0.618), based on Spearman's rho table. **Significant correlation at p<0.01 ($|r_s|$ >0.755), based on Spearman's rho table.

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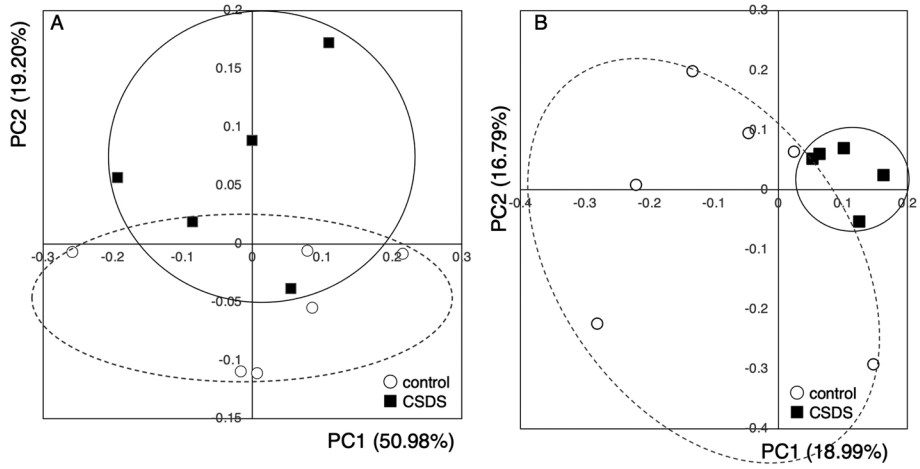

Fig. 3. Principal coordinate analysis (PCoA) plots. A: weighted UniFrac distances; B: unweighted UniFrac distances.

acids to secondary bile acids occurred during the transition from the cecum to the colon due to the distinct microbiota compositions in these regions. This study found significantly higher levels of 7α-dehydroxylated secondary bile acids, DCA, LCA, and total bile acids in the fecal contents of sCSDS mice compared with control mice (Table 2).

We found that sCSDS significantly affected the levels of bile acid, as well as the microbiota and metabolites present in the cecum contents and feces [11]. To assess the consistency of our findings across different experiments, we compared the microbiota of the control and sCSDS mice from both our current study and the previous study [11], which was conducted using the same sCSDS protocol. Despite being performed at different facilities, both experiments on sCSDS revealed similar changes in the fecal and colonic microbiota. We merged nucleotide sequence data from fecal samples of control and sCSDS mice collected on day 0 (before sCSDS) and day 11 (after sCSDS) from the previous study with the current data collected on day 11. When we analyzed the data, we discovered that the fecal microbiota structure clustered into three groups: a day 0 group, control group, and sCSDS group, as shown in Supplementary Fig. 2.

The loading factors of ASVs contributing positively to PC1, PC2, and PC3 are presented in Supplementary Table 3, while those contributing negatively to PC1, PC2, and PC3 are listed in Supplementary Table 4. We found that PC1 was responsible for the distribution between the period before sCSDS (day 0) and after sCSDS (day 11). The ASVs corresponding to *Alloprevotella* (ASV31/OTU2), *Muribaculaceae* (ASV32/OTU3), and *Phocaeicola sartorii* (ASV33/OTU43) showed a positive involvement in the distribution, while those corresponding to *Faecalibaculum rodentium* (ASV1/OTU1), *Phocaeicola vulgatus* (ASV2/OTU13), and *Clostridia*_UCG-014 (ASV33/OTU4) were negatively involved. On the other hand, PC3 was found to contribute to the distribution between the control group and the sCSDS group. The top three ASVs that positively contributed were *F. rodentium* (ASV35/OTU1), *Bacteroides* (ASV34/OTU13), and *Clostridia*_UCG-014 (ASV30/OTU4). On the other hand, ASVs corresponding to *P. vulgatus* (ASV2/OTU13), *F. rodentium* (ASV1/OTU1), and *Lachnospiraceae* (ASV6/OTU7) showed negative involvement in the distribution.

In this study, we revealed that the amounts of DCA and LCA in the feces of sCSDS mice were higher than those of control mice and positively correlated with the abundances of colonic microbiota, positively correlated with the ASVs of *Roseburia* (ASV22), *Oscillospiraceae* (ASV7), and *Lachnospiraceae* (ASV6; Fig. 2), and negatively correlated with SI. Consistent with our results, recent studies identified that some of the bacteria involved in 7α-dehydroxylation were affiliated with the families *Oscillospiraceae* [34] and *Lachnospiraceae* [35].

Elevated levels of intestinal secondary bile acids were also observed in chronic unpredictable mild stress (CUMS) progression, another animal depression model. CUMS also significantly increased the secondary bile acid levels in the feces, serum, and liver in ICR mice [36]. CUMS-induced alteration of the bile acid profile was positively correlated with *Ruminococcaceae*_UCG-010, *Ruminococcus*, and *Clostridia*_UCG-014, which may be involved in the conversion of DCA in the intestine. Even though susceptibility to psychological stress varies by mouse strain background [7] and diet composition [25], psychological stress generally alters the gut microbiota and increases the levels of secondary bile acids. Using omics approaches, we found that social defeat stress causes a decrease in immune response-related genes in the ileum. This alteration in gene expression would lead to a shift in the microbiota balance in the gut [11]. Our study also discovered that the gene expression of the fucosyltransferases *Fut1* and *Fut2* decrease only in the distal ileum, leading to a reduction in fucosylated glycoproteins in the distal intestinal mucosa. Fucosylated glycoproteins serve as energy/carbon sources, adhesion sites, or regulators of gene expression for intestinal bacteria. Fut2-deficient mice are characterized by significantly reduced α1,2-fucosylation in the intestinal mucosa and disruption of the gut microbiota, termed dysbiosis [37]. Therefore, the stress-induced reduction of fucosylated glycans in the intestinal epithelium may cause changes in the intestinal microbiota [12].

This study investigated the increase in 7α-dehydroxylated secondary bile acids in feces on day 7, which was in the middle of sCSDS loading. On the other hand, the gene expression of *Fgf15* was analyzed using the ileum, which was collected on day 13 after the end of the sCSDS period. Thus, it is thought that the

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increase in *Fgf15* expression was likely an attempt to suppress the synthesis of bile acids that increased with sCSDS loading. To clarify the effect of sCSDS on bile acid regulation, it would be necessary to have data for throughout the time course of the fecal and liver bile acid analysis as well as for liver and small intestinal gene expression.

In conclusion, sCSDS loading has a significant impact on gene expression, the gut microbiota, and metabolites in the intestine, leading to changes in bile acid metabolism. sCSDS mice showed an increase in fecal 7α-dehydroxylated secondary bile acids, predominantly DCA and LCA, with a concomitant increase in total bile acid content. Changes in the gut microbiota induced by sCSDS, particularly the increase in the abundances of *Oscillospiraceae* and *Lachnospiraceae* and the decrease in the *E. coprostanoligenes* group, were found to be linked with increased fecal excretion of DCA and LCA and a decrease in social interaction. Further insight could be gained by analyzing fecal, blood, and liver bile acids along with the gut microbiota during sCSDS loading.

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