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A next-generation probiotic: *Akkermansia muciniphila* ameliorates chronic stress–induced depressive-like behavior in mice by regulating gut microbiota and metabolites

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Abstract

Major depressive disorder (MDD) is a neurasthenic disease, which is the second-largest burden of disease globally. Increasing studies have revealed that depression is associated with abnormalities in gut microbiota and metabolites. Several species of bacteria have been classified as psychobiotics, which confer mental health benefits through interactions with commensal gut microbiota. Therefore, it is essential to identify new psychobiotics and elucidate their mechanisms in the treatment of depression. This study aims to evaluate the antidepressant effect of *Akkermansia muciniphila* (AKK) in a mouse model of depression induced by chronic restraint stress (CRS). C57BL/6 male mice were divided into three groups: mice subjected to CRS, mice not subjected to CRS, and mice treated with AKK for 3 weeks. Behavioral tests were performed, and hormone, neurotransmitter, and brain-derived neurotrophic factor (BDNF) levels were measured. Cecal microbiota was analyzed using 16S rRNA gene sequencing, and serum metabolites were detected using untargeted metabolomics. In addition, correlations between altered gut microbiota and metabolites with significant variations in serum associated with AKK ameliorating depression were analyzed using Pearson's correlation coefficient. The results revealed that AKK significantly ameliorated depressive-like behavior and restored abnormal variations in depression-related molecular (corticosterone, dopamine, and BDNF). Moreover, AKK altered chronic stress–induced gut microbial abnormalities. Untargeted metabolomics analysis revealed 23 potential biomarkers in serum that could be associated with the mechanisms underlying CRS-induced depression and the therapeutic effects of AKK. Pearson's correlation coefficient analysis revealed that AKK predominantly upregulated β-alanyl-3-methyl-L-histidine and edaravone to relieve depression. Furthermore, β-alanyl-3-methyl-L-histidine and edaravone exhibited the antidepressant phenotype in mice subjected to CRS. In conclusion, the study demonstrated that AKK ameliorates chronic stress–induced depressive symptoms in mice by regulating gut microbiota and metabolites.

Key points

- *AKK reduces depressive-like behaviors induced by chronic stress.*
- *AKK regulates the gut microbial structure and metabolomics of serum under the chronic stress.*
- *Antidepressant effect of AKK correlates with the increase of β-alanyl-3-methyl-l-histidine and edaravone.*

Keywords *Akkermansia* · Gut microbiota · Untargeted metabolomics · Stress · Depression

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Introduction

Major depressive disorder (MDD) is a neurasthenic disease that is characterized by depressed mood, diminished interests, impaired cognitive function, and vegetative symptoms, including disturbed sleep or decreased appetite (Otte et al. 2016). MDD severely influences personal work, learning, and social interaction, causing disability and suicide among patients. Being a clinically prevalent mental disorder, depression is

reported that 1 in 5 adults is affected by it throughout their lives, which is the second leading cause of disability in the USA (GBD 2016 Disease and Injury Incidence and Prevalence Collaborators 2017).

Based on the World Health Organization (WHO) report, approximately 350 million people suffered depression globally in 2015, and the prevalence rate was as high as 4.4% (Smith 2014). Additional research have predicted that depression will be the second-largest burden of disease globally by 2020 (Murray et al. 2013). Notably, the pathogenesis of depression has not been fully understood.

Over the last few decades, a few studies have implicated genetic factors, personality traits, and environmental factors in the pathogenesis of depression. Gut microbiota are considered a "second brain," which can regulate brain development and function (Franzosa et al. 2015). Moreover, gut microbiota and the central nervous system exchange information in two ways via nerve, endocrine, and immune pathways (Cryan and Dinan, 2012). Studies have demonstrated that gut microbes modulate the development of depression (Fung et al. 2017). While acknowledging a strong link between gut microbiota and depression, the precise microbial strain and mechanism of interaction between gut microbiota and metabolites remain obscure.

Akkermansia muciniphila, a gram-negative anaerobic bacteria, belonging to the phylum *Verrucomicrobia*, is a symbiotic bacterium widely distributed in the human intestinal mucus layer, accounting for 1–5% of human intestinal microorganisms (Derrien et al. 2008). The potential application of AKK as a next-generation probiotic drug has attracted the attention of researchers (Zhai et al. 2019; Hagi and Belzer 2021; Zhang et al. 2021). Several studies presently investigate the ability of AKK to ameliorate metabolic disorders (Depommier et al. 2019). Previous studies revealed that the abundance of AKK is inversely correlated with progeria, inflammatory bowel disease (IBD), colitis-associated colorectal cancer (CAC), and hypertension (Bárcena et al. 2019; Li et al. 2017; Png et al. 2010; Wang et al. 2020). In addition, AKK could exhibit a protective effect on neurological diseases such as epilepsy, amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and autism (Blacher et al. 2019; Li et al. 2019; Olson et al. 2018; Wang et al. 2011).

Recent studies reported that AKK is negatively correlated with depression (McGaughey et al. 2019). Nonetheless, the role of AKK in the treatment of depression and the underlying mechanism of anti-depression remain unclear. Therefore, this study aimed to investigate the effect of AKK on regulation of gut microbiota, metabolites, and the mechanism of reducing depression symptoms in mice.

Materials and methods

Animals

Male C57BL/6 mice (6–8 weeks) were purchased from the Comparative Medicine Centre of Yangzhou University (Approval ID: SCXK-(Su) 2011–0003). Animals were acclimatized for 7 days before experimental procedures began. The mice were maintained under standard laboratory conditions (temperature: 22 ± 2 °C; humidity: 50 ± 10%) for a 12-h light/12-h dark cycle and allowed to freely feed and drink. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Nanjing University of Chinese Medicine.

Treatment and chronic restraint stress

The mice were randomly divided into three groups: (1) control group ($n = 6$)—200 µl phosphate-buffered saline (PBS) was administered via gavage without application of chronic restraint stress (CRS) for 3 weeks; (2) CRS group ($n = 6$)—treated with 200 µl PBS via gavage and CRS applied for 3 weeks; (3) CRS + AKK group ($n = 6$)—200 µl (5×10^8 colony-forming unit (CFU) /mL) of AKK was administered via gavage and CRS applied for 3 weeks (Routy et al. 2018); (4) AKK group ($n = 6$)—200 µl (5×10^8 CFU/mL) of AKK was administered via gavage and without CRS applied for 3 weeks; (5) CRS + *Lactobacillus* L group ($n = 6$)—200 µl (5×10^8 CFU/mL) of *Lactobacillus* was administered via gavage and CRS applied for 3 weeks; (6) CRS + *Lactobacillus* H group ($n = 6$)—200 µl (5×10^9 CFU/mL) of *Lactobacillus* was administered via gavage and CRS applied for 3 weeks. The body weights and food intake levels were monitored every 3 days.

AKK (ATCC® BAA-835™) was cultured in brain–heart infusion liquid medium (supplemented with 0.1% mucin from porcine stomach) and anaerobically incubated at 37 °C. *Lactobacillus plantarum* (CICC® 23,133) was cultured in MRS (De Man, Rogosa and Sharpe) broth at 37 °C. Bacteria used for oral administration were prepared by suspending them in PBS. Suspensions of 10^9 CFU/mL were obtained using a spectrophotometer (NanoDrop 2000c, Thermo, Waltham, MA, USA) at an optical density of 600 nm.

CRS is a method of inducing depressive-like behavior in animals and it is widely used in depression-related

research because of its simplicity and superior reproducibility. CRS was conducted as previously described (Luo et al. 2014). An individual mouse was restrained in 50-ml conical centrifuge tube for 3 h daily. Small holes were made in the front part and the periphery of the centrifuge tubes to ensure that the mice could breathe normally while being immobilized. Details of the animal experimental procedures are presented in Fig. 1a.

Metabolite administration

To evaluate the effect of specific metabolites on behavioral phenotypes, β-alanyl-3-methyl-L-histidine was orally administered at a dose of 10 mg/mouse for 3 weeks (Kaneko et al. 2017), whereas edaravone was administered at a dose of 10 ml/kg per mouse for the last 7 days of restraint stress (Jangra et al. 2017).

Hepatic and renal function

Serum alanine aminotransferase (ALT); aspartate transaminase (AST); creatinine, urea, total cholesterol (TCHO); triglyceride; high-density lipoprotein cholesterol (HDL-C); and low-density lipoprotein cholesterol (LDL-C) were measured by automatic biochemical analyzer (Olympus AU640, Olympus Optical Co., Tokyo, Japan).

Behavioral tests

Open-field test

To examine the locomotor and exploratory activity, mice were placed in the center of an individual open-field arena (40 × 40 × 40 cm) with a video camera fixed at the top and allowed to freely explore the area for 5 min. The total distance traveled and time spent in the central zone was measured. Notably, the arena was thoroughly cleaned using 75% ethanol before each animal entered.

Tail suspension test

Mice were individually suspended 50 cm above the surface of a table with adhesive tape, which was placed 1 cm away from the tip of the tail for 6 min. A camera placed in front of the device was used to record the behavior of mice. Mice were considered immobile when they hung passively

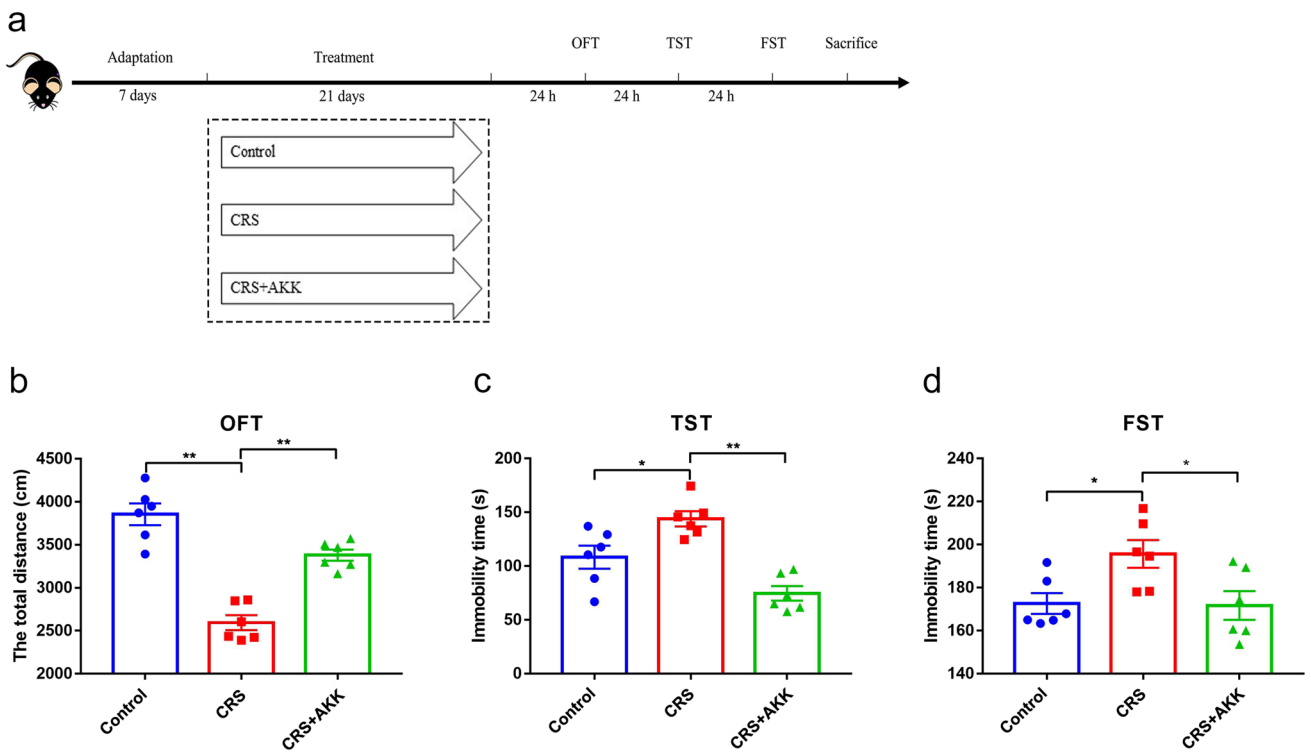

Fig. 1 AKK ameliorates CRS-induced depressive-like behavior in mice. **a** The timeline of the procedure. **b** Total distance covered by mice in open-field testing in 5 min. **c** Measurement of immobility time over the last 4 min during a 6-min testing time in tail suspension test. **d** Measurement of immobility time over the last 4 min during a 6-min testing time in forced swimming test. Data are presented as means ± SEM and $n = 6$/group; * and ** indicate $P < 0.05$ and $P < 0.01$

motionless. The software recorded the immobility time over the last 4 min of the 6-min testing period.

Forced swimming test

Mice were individually placed in clear Plexiglas cylinders (25 cm high; 10 cm in diameter) filled with water to a depth of 10 cm (23–25° C) for 6 min. A camera was mounted to record the immobility of mice. Immobility in this study was defined as mice floating passively in water without struggling. ANY-maze software (Stoelting, Wood Dale, IL, USA) was used to measure the immobility time over the last 4 min of the 6-min testing period.

Sample collection and preparation

Blood samples were drawn from eyeballs and serum was obtained via centrifugation at 3,000 rpm, 4 °C for 10 min after standing for 1 h. Subsequently, the serum was stored at − 80 °C until UHPLC-QE Orbitrap/MS analysis (1290, Agilent Technologies, Hilden, Germany). After the mice were euthanized, cecal samples were immediately collected in clean centrifuge tubes and placed in dry ice, and then stored at − 80 °C until microbiological analysis. Hippocampal tissues were weighed and subsequently frozen at − 80 °C for quantitative real-time PCR analysis (qRT-PCR).

Quantitative real-time PCR

Quantitative real-time PCR was performed to detect the RNA expression level of brain-derived neurotrophic factor (BDNF) in the hippocampus. Trizol extraction reagent (Invitrogen, Carlsbad, CA, USA) was used to extract hippocampal RNA. QRT-PCR was conducted on the 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Green Master Mix (Vazyme Biotech Co. Ltd., Nanjing, JS, China). Amplification reactions were run in the "no template control" mode, and all reactions were performed in triplicate, for each probe used. Cycle threshold (Ct) values were recorded and normalized to the *GAPDH* using the $2^{-\Delta\Delta Ct}$ method. All procedures were performed based on the manufacturer's instruction. The primers (Vazyme Biotech Co. Ltd., Nanjing, JS, China) used were as follows:

GAPDH forward: 5′-TCATACTTCGGTTGCATG AAGG-3′.

GAPDH reverse: 5′-ATGTCACGCACGATTTCC-3′.

BDNF forward: 5′-TCATACTTCGGTTGCATG AAGG-3′.

BDNF reverse: 5′-AGACCTCTCGAACCTGCCC-3′.

Determination of corticosterone, serotonin, and dopamine levels in serum

Corticosterone level was determined using commercial ELISA kits (Yi Fei Xue Biotechnology, Nanjing, JS, China) while serotonin and dopamine levels were determined using commercial ELISA kits (Jin Yibai Biological Technology Co. Ltd., Nanjing, JS, China) according to the manufacturer's instructions.

16S rRNA gene sequence analysis

Cecal and fecal samples were collected after the mice were euthanized for 16S rRNA gene sequence analysis. Microbial DNA was extracted using HiPure Soil DNA Kits (Magen, Guangzhou, GD, China) according to the manufacturer's protocols. The 16S rDNA targeting the V3-V4 region of the ribosomal RNA gene was amplified by PCR. Amplicons were excised from 2% agarose gels, purified using AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using ABI StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Purified amplicons were pooled in equimolar and paired-end sequenced (PE250) on an Illumina platform (Illumina, San Diego, CA, USA). Paired-end clean reads were merged as raw tags using fast length adjustment of short reads (FLSAH) with a minimum overlap of 10 bp and mismatch error rates of 2% (Magoč and Salzberg, 2011). The effective tags were clustered into operational taxonomic units (OTUs) with ≥ 97% similarity using the UPARSE pipeline (http://drive5.com/uparse/) (Edgar 2013). Comparisons of species between groups were performed using R software (v2.15.3, http://www.R-project.org). The analyses results of OTUs based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were inferred using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al. 2013). The Functional Annotation of Prokaryotic Taxa (FAPROTAX) database and associated software were used to generate the ecological function profiles of bacteria. The raw 16S rRNA sequence data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under accession number PRJNA707611.

Analysis of serum metabolomics using ultra high-performance liquid chromatography-Q exactive Orbitrap-mass spectrometry

Liquid chromatography-tandem mass spectrometry (LC–MS/MS) analyses were performed using a UHPLC system (1290, Agilent Technologies, Hilden, Germany) with a UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) coupled

to a Q Exactive benchtop Orbitrap mass spectrometer (Orbitrap MS, Thermo, Waltham, MA, USA).

The mobile phase A comprised of 0.1% formic acid in water for positive ionization mode, and 5 mmol/L ammonium acetate in water for negative ionization mode, and the mobile phase B comprised of acetonitrile. The elution gradient was set as follows: 0 min, 1% B; 1 min, 1% B; 8 min, 99% B; 10 min, 99% B; 10.1 min, 1% B; and 12 min, 1% B. The flow rate was set to 0.5 mL/min and the injection volume was 2 µL. The Q Exactive mass spectrometer was used because of its ability to acquire MS/MS spectra on an information-dependent acquisition (IDA) mode during the LC–MS/MS experiment. The data acquisition software (Xcalibur 4.0.27, Thermo, Waltham, MA, USA) continuously evaluated the full scan survey MS data in the IDA-based mode because it collects and triggers acquisition of MS/MS spectra depending on the preselected criteria. Electrospray ionization (ESI) source conditions were set as follows: sheath gas flow rate was 45 Arb, Aux gas flow rate was 15Arb, capillary temperature was 320 °C, full Ms resolution was 70,000, MS/MS resolution was 17,500, collision energy was 20/40/60 eV in normalized collisional energy model, spray voltage was 3.8 kV (positive mode) or − 3.1 kV (negative mode).

Full MS raw data files including retention time alignment, peak detection, and peak matching were converted to mzML format using ProteoWizard (https://proteowizard.sourceforge.io/), and processed using R package XCMS (v3.2, http://www.R-project.org). Afterwards, the data files were filtered based on the following criterion: sample numbers with metabolites that were less than 50% of all sample numbers in a group. Subsequently, normalization to an internal standard for each sample was conducted, and missing values were replaced by half of the minimum value observed in the dataset by default. The preprocessing results generated a data matrix comprising retention times (RTs), mass-to-charge ratio (m/z) values, and peak intensity.

Data analysis

Statistical analyses were performed using IBM SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 7 (GraphPad Software, Inc., La Jolla, CA, USA). All data were presented as mean ± standard error of the mean (SEM). Significant differences between the two groups were analyzed using Student's *t*-test, and multiple comparisons were analyzed using one-way ANOVA followed with Tukey's post hoc multiple comparisons test. Microbiota-related analyses were performed using Welch's *t*-test. The associations of serum metabolite intensities with genera levels were subsequently analyzed using Pearson's correlation coefficient. A P value of < 0.05 was considered statistically significant.

Data availability statement

The datasets generated during the current study are available from the corresponding author on reasonable request.

Results

Safety testing of AKK

We analyzed the serum levels of ALT, AST, creatinine, urea, TCHO, triglyceride, HDL-C, and LDL-C by animal biochemical analyzer. As shown in Supplemental Fig. S1a-h, compared to control group, single oral administration of AKK did not significantly affect the biochemical parameters of mice.

AKK ameliorates CRS-induced depressive-like behavior in mice

CRS increased the incidence of depressive-like behavior in mice. To investigate the ability of AKK in ameliorating depression in mice under stress conditions, AKK was orally administered to the mice for 3 weeks, then open-field test (OFT), forced swimming test (FST), and tail suspension test (TST) were performed. As shown in Fig. 1b–d, the total distance covered by the mice in the CRS group significantly reduced. However, mice treated with AKK exhibited a significant improvement in OFT. CRS results revealed that mice floated passively in water, in turn increasing immobility time of mice in FST. In contrast with the CRS group, AKK administration significantly decreased the immobility time of swimming. In TST, the immobility of the stress mice increased, while AKK significantly reduced immobility, indicating a higher activity rate than the CRS and normal groups. To verify the antidepressant effect of AKK on CRS mice again, we added two doses of *Lactobacillus* administration groups. As shown in Supplemental Fig. S2, the traveling distance in the CRS + AKK group, the CRS + *Lactobacillus* L group, and the CRS + *Lactobacillus* H group was significantly more than that in the CRS group ($P < 0.05$). In addition, administration of AKK or high dose of *Lactobacillus* significantly decreased the immobility times of mice in TST ($P < 0.01$, $P < 0.05$). The immobility times of swimming in the CRS + AKK group, CRS + *Lactobacillus* L group, and CRS + *Lactobacillus* H group were significantly decreased compared to those in the CRS group ($P < 0.01$, $P < 0.05$,

$P < 0.01$). Besides, AKK increased the body weight and food intake of CRS mice (Supplemental Fig. S3).

AKK regulates abnormal variations in hormone, neurotransmitter, and BDNF expression levels in CRS-induced mice

Hormone, neurotransmitters, and BDNF have been associated with the behavioral stress response. We analyzed the concentrations of hormone and neurotransmitters in the serum, as well as BDNF expression level in the hippocampus. As shown in Fig. 2a–d, CRS increased corticosterone concentration in serum, and decreased the concentration of dopamine and serotonin significantly. Moreover, BDNF expression at the mRNA level in the hippocampus of mice in the CRS group reduced significantly when compared to the control group ($P < 0.05$), whereas AKK inhibited CRS-induced increase in corticosterone concentration in serum, which in turn caused significant recovery in dopamine and BDNF levels when compared with the CRS group ($P < 0.01$). The results suggested that AKK could ameliorate depressive-like behavior in CRS-induced mice by regulating abnormal variations in the concentrations of hormone, neurotransmitters, and BDNF expression levels.

AKK regulates gut microbiota at the phylum and genus levels in CRS-induced mice by 16SrRNA gene sequencing

We evaluated the effects of AKK on the gut microbiota composition by sequencing bacterial 16S rRNA V3 + V4 region. A high-throughput pyrosequencing of the samples generated 1,149,582 raw tags, which were processed by QIIME (Caporaso et al. 2010). The tags were clustered into OTUs with ≥ 97% similarity using UPARSE pipeline (Kryukov et al. 2020). The analyses results revealed that the microbiota composition was altered significantly by CRS, and details are illustrated in Fig. 3 a and b. At the phylum level, AKK upregulated *Verrucomicrobia*, and downregulated *Epsilonbacteraeota*, *Patescibacteria*, *Chloroflexi*, and *Acidobacteria* when compared with the CRS group (Fig. 3c). At the genus level, AKK increased the relative abundance of *Akkermansia*, and decreased

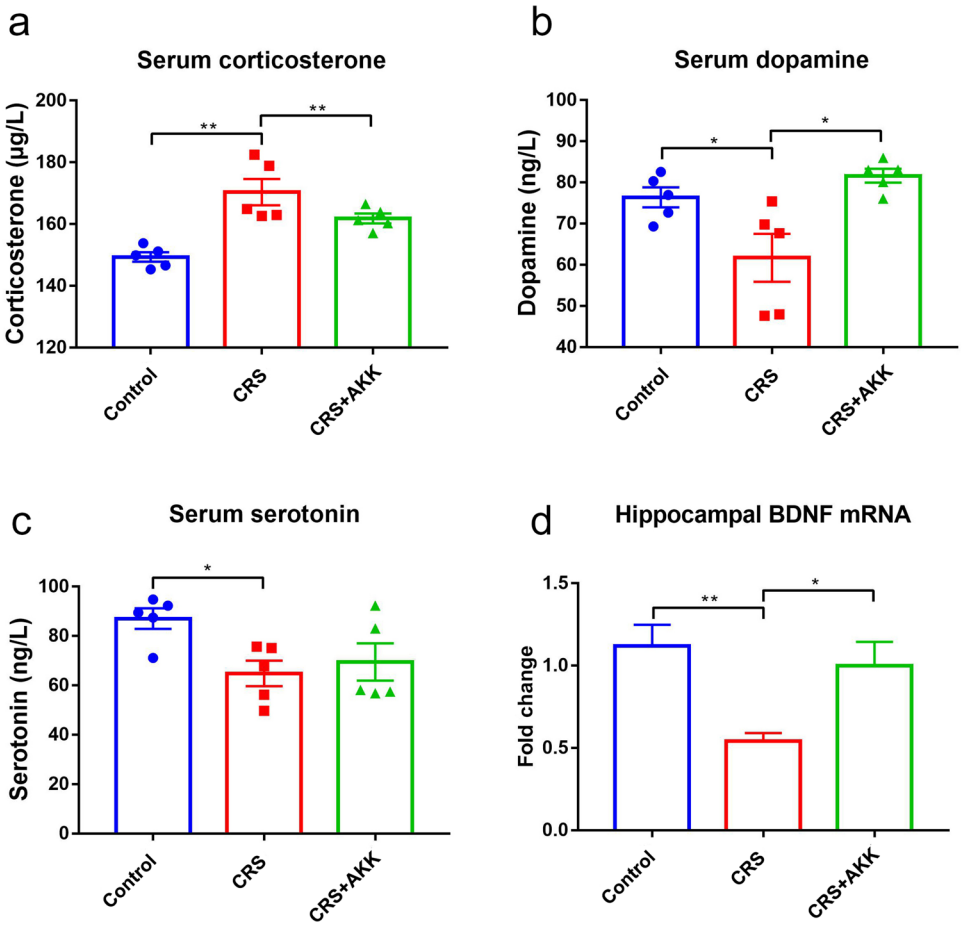

Fig. 2 AKK regulates abnormal variations in hormone, neurotransmitter, and BDNF levels in CRS-induced mice. **a** Corticosterone concentration in serum. **b** Dopamine concentration in serum. **c** Serotonin concentration in serum. **d** BDNF mRNA level in mice hippocampus. Data are presented as means ± SEM; * and ** indicate $P < 0.05$ and $P < 0.01$

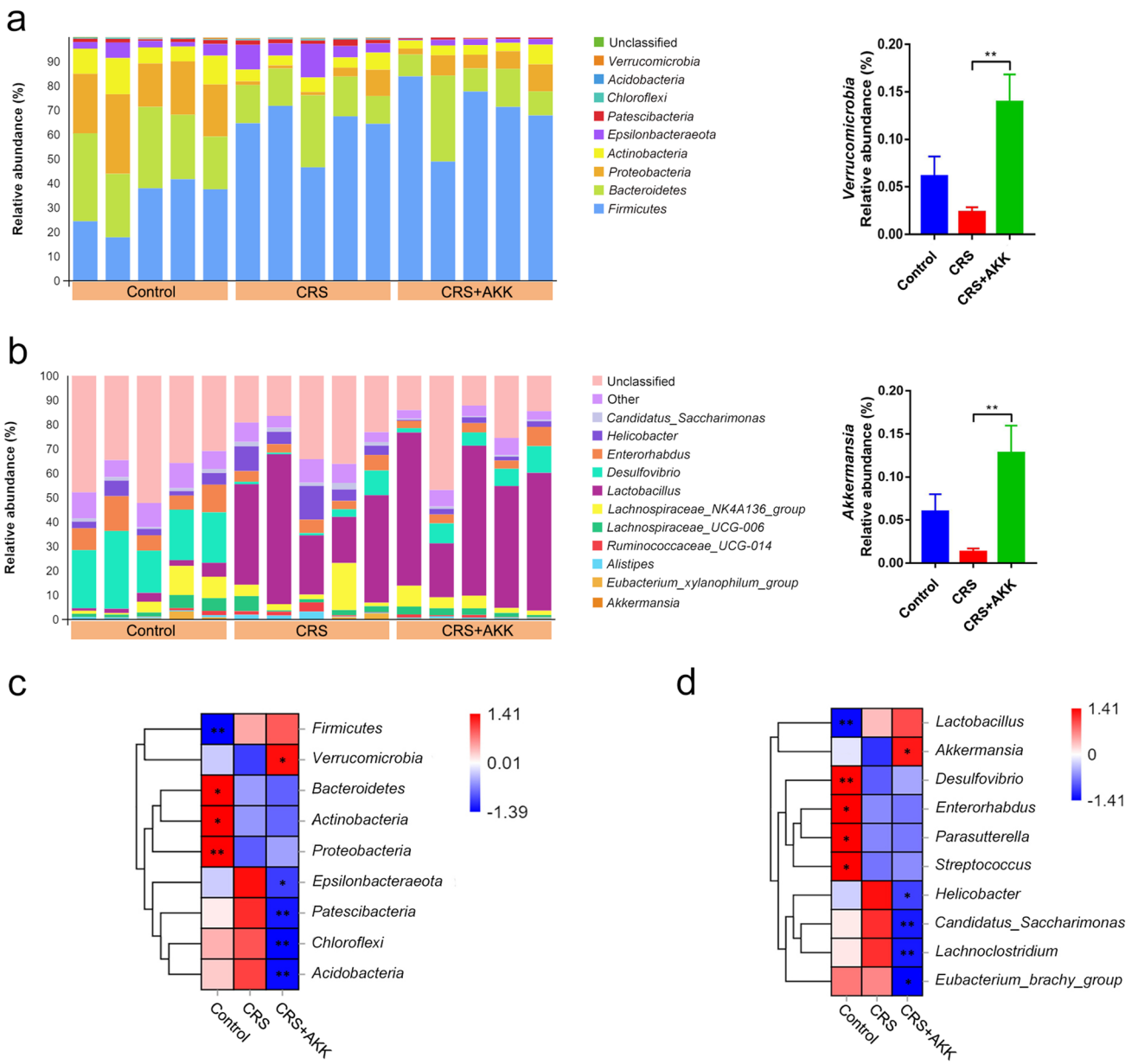

Fig. 3 AKK regulates gut microbiota in CRS-induced mice. **a**, **b** Relative abundance distribution of the most abundant microbial taxa at the phylum (**a**) and genus (**b**) levels. **c**, **d** The heat map representing the key taxa that were significantly altered in the control group vs the CRS group and the CRS group vs the CRS + AKK group at the phylum (**c**) and genus (**d**) levels through indicator species analysis. Values are presented as means ± SEM ($n = 5$). Differences were analyzed using *t*-test, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs the CRS group

the relative abundance of *Helicobacter*, *Candidatus_Saccharimonas*, *Eubacterium_brachy_group*, and *Lachnoclostridium* (Fig. 3d).

We examined the structure of gut microbiota using principal component analysis (PCA) and beta diversity using analysis of similarity (ANOSIM) (Lee et al. 2018; Ali et al. 2021). No significant difference was observed between the CRS group and the CRS + AKK group (Supplemental Fig. S4).

Predicted function of gut microbiota regulated by AKK

To evaluate variations in the functional capacities of intestinal bacterial community between the CRS and CRS + AKK groups, PICRUSt analysis was performed to determine KEGG pathways that are associated with intestinal microbiota. The KEGG pathway analyses revealed that pathways associated with neurodegenerative diseases were inhibited in the CRS + AKK group when compared to

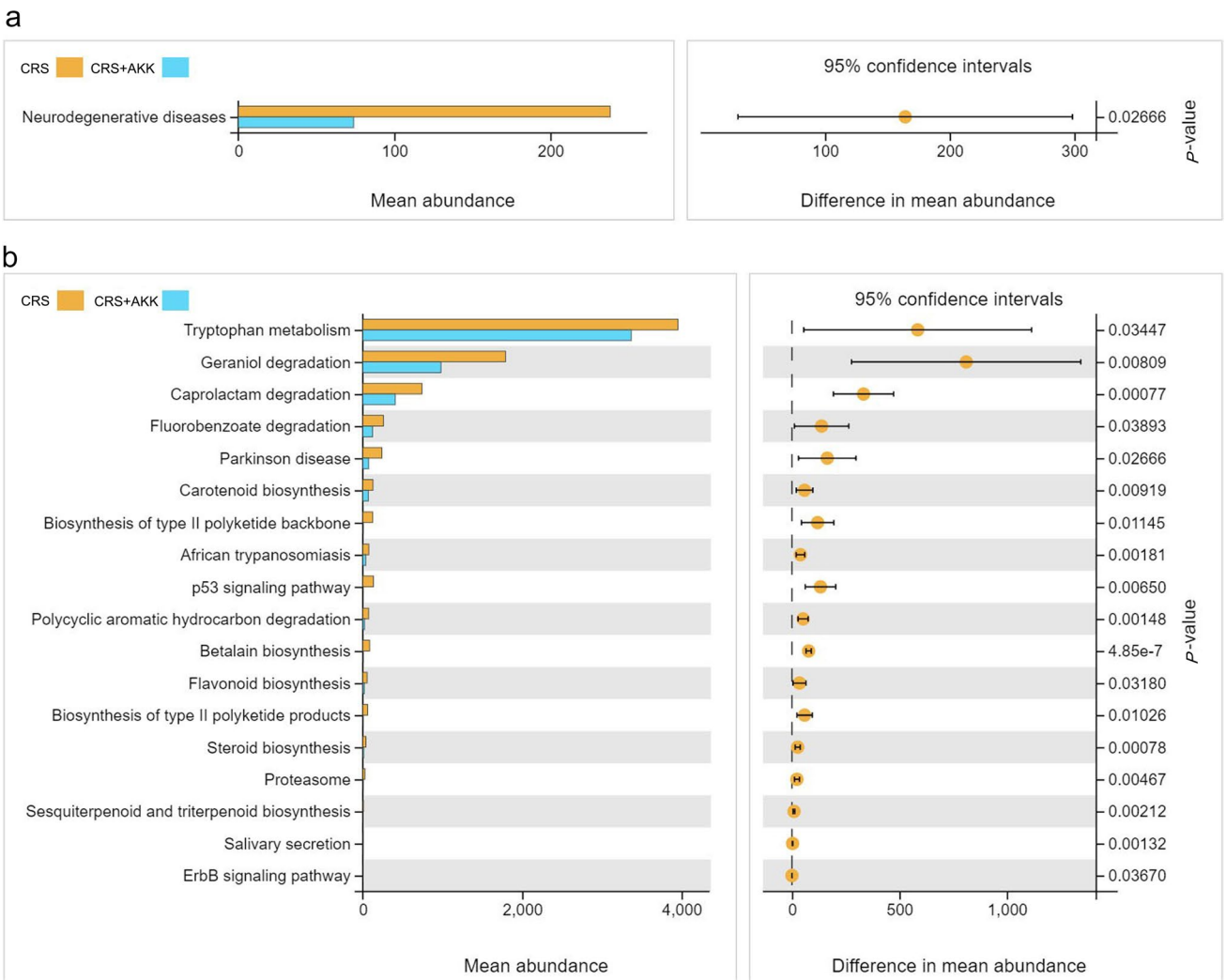

Fig. 4 Predicted metabolic functions of gut microbiota in the CRS and CRS + AKK groups. Second-level (**a**) and third-level (**b**) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways are shown in the extended error bar. *P*-values are shown on the right

the CRS group (Fig. 4a). The CRS + AKK group exhibited significantly lower relative abundances of genes involved in tryptophan metabolism, geraniol degradation, caprolactam degradation, fluorobenzoate degradation, and Parkinson's disease than in the CRS group, which was consistent with the KEGG pathway analysis results ($P < 0.05$) (Fig. 4b).

AKK regulates abnormal variations in serum metabolites in CRS-induced mice

UHPLC-QE Orbitrap/MS was used to analyze serum samples from the control, CRS, and CRS + AKK groups in the positive ion mode, which represented physiological status, pathological conditions, and intervening effects.

The metabolic profile of the CRS group was distinct from that of the control group (Fig. 5a). Furthermore, the CRS + AKK group differed from the CRS group, although it was closely similar to the control group. The results revealed that CRS induced considerable metabolic variations in the serum and that the variations could be modulated through administration of AKK.

Serum metabolites that were altered substantially were selected using *t*-test and variable importance in projection (VIP) values. We considered the variables that were far from the origin based on the *t*-test ($P < 0.05$) with a VIP ≥ 1 potential biomarkers associated with depression. A total of 95 variables, which contributed to the clustering of the *t*-test and VIP values based on the variations in serum metabolic profiles between the control and CRS groups, were treated as potential biomarkers.

After data processing using an in-house MS/MS database, 23 metabolites were identified (Fig. 5b). Based on the KEGG pathway enrichment analyses, differential

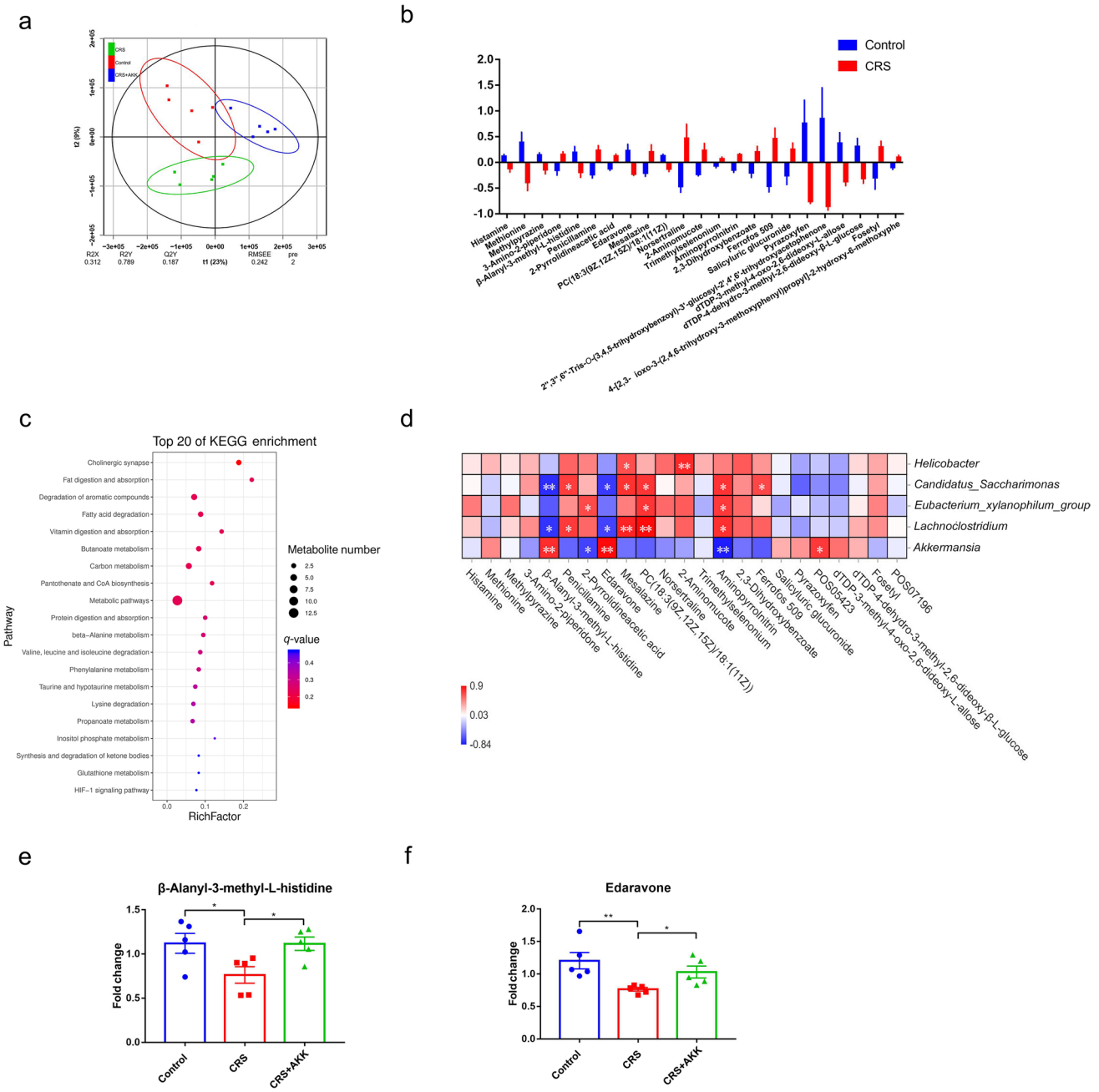

Fig. 5 AKK regulates abnormal variations in serum metabolites in CRS-induced mice. **a**Partial least squares-discriminant analysis score plot of serum samples collected from different groups in positive mode. **b** Significant variations in serum metabolites in the control and CRS groups are presented in the histogram. **c** Top 20 of the KEGG pathway enrichment analysis of differential metabolites between the CRS and CRS + AKK groups. **d** A correlation matrix heat map representing statistically significant correlation values between perturbed gut microbiota genera and altered serum metabolites in mice. Red squares represent significant positive correlations; green squares represent significant negative correlations. POS05423 and POS07196 represent 2″,3″,6″-Tris-*O*-(3,4,5-trihydroxybenzoyl)-3′-glucosyl-2′,4′,6′-trihydroxyacetophenone and {4-[2,3-dioxo-3-(2,4,6-trihydroxy-3-methoxyphenyl)propyl]-2-hydroxy-6-methoxyphenyl} oxidanesulfonic acid. **e** β-Alanyl-3-methyl-L-histidine expression in serum. **f** Edaravone expression in serum. * and ** indicate $P < 0.05$ and $P < 0.01$

metabolites between CRS and CRS + AKK groups were predominantly enriched in cholinergic synapse, fat digestion and absorption, degradation of aromatic compounds, fatty acid degradation, vitamin digestion and absorption, butanoate metabolism, carbon metabolism, pantothenate and CoA biosynthesis, metabolic pathways, and digestion and absorption (Fig. 5c). The analysis results revealed that AKK ameliorated depression by regulating the pathways.

A heat map of correlation was constructed to identify the potential link between altered gut microbiota and potential biomarkers in serum that are associated with depression ($P < 0.05$). The results revealed multiple significant associations between the perturbed gut microbiota and altered metabolites in mice with CRS-induced depression (Fig. 5d). The correlation analysis results revealed that AKK was positively correlated with 3 metabolites (β-alanyl-3-methyl-L-histidine, edaravone, and 2″,3″,6″-Tris-*O*-(3,4,5-trihydroxybenzoyl)-3′-glucosyl-2′,4′,6′-trihydroxyacetophenone), and negatively correlated with 2 metabolites (2-pyrrolidineacetic acid and aminopyrrolnitrin). β-Alanyl-3-methyl-L-histidine and edaravone were upregulated when compared with the CRS group after administration of AKK (Fig. 5e, f).

Administration of potential metabolites ameliorate CRS-induced depressive-like behavior, and hormone, neurotransmitter, and BDNF levels

To investigate the effect of specific metabolites regulated by AKK on depressive behavior phenotype, CRS-induced mice were treated with β-alanyl-3-methyl-L-histidine or edaravone. OFT results revealed that the total distance covered by mice treated with β-alanyl-3-methyl-L-histidine or edaravone significantly increased when compared with the CRS group (Fig. 6a). In addition, administration of β-alanyl-3-methyl-L-histidine or edaravone decreased the immobility time of mice in TST (Fig. 6b). However, both metabolites did not reduce the immobility time of mice in FST (Fig. 6c). We subsequently determined corticosterone, neurotransmitters, and BDNF levels, and the results revealed that β-alanyl-3-methyl-L-histidine and edaravone decreased corticosterone concentration significantly (Fig. 6d). Edaravone increased serotonin concentration considerably, whereas β-alanyl-3-methyl-L-histidine increased dopamine concentration (Fig. 6e, f). Both metabolites tended to restore BDNF expression level in the hippocampus, although the effect was not statistically significant (Fig. 6g).

Discussion

Depression is a psychiatric illness with elusive pathogenesis. The current clinical first-line antidepressants primarily include selective serotonin reuptake inhibitors (SSRI), and serotonin and norepinephrine reuptake inhibitors (SNRI), as well as norepinephrine and specific serotonin energy antidepressants (NaSSA). While alleviating the symptoms of depression, the antidepressants have side effects including nausea, vomiting, and sexual dysfunction (Rothmore 2020). Furthermore, a few antidepressants double the risk of suicidality and aggression in children and adolescents (Sharma et al. 2016). Thus, the development of safe and non-toxic alternatives to antidepressants is critical for the treatment of depression.

Probiotics are well-known for their regulatory activity on body weight, lipid metabolism, and immune response of the host. Nevertheless, increasing research on the gut-brain axis over the last few years has led to several studies on the effects of probiotics on mental state and cognitive functions. As a consequence, several animal experiments and clinical trials suggested that oral probiotics exhibited antidepressant effect to a certain extent (Pinto-Sanchez et al. 2017; Meyer and Vassar 2018; Liu et al. 2020; Tian et al. 2020).

AKK, a symbiotic bacterium of the mucus layer, is considered a potential probiotic. The probiotic effects of AKK, including metabolic modulation, immune regulation, and gut health protection, have been investigated extensively. Reports indicate that various neurological disorders including ALS, AD, and autism disrupt the abundance of AKK (Blacher et al. 2019; Li et al. 2019; Wang et al. 2011). Symptoms could be relieved by simply administering AD mice with AKK (Ou et al. 2020).

We demonstrated for the first time that orally administered AKK could ameliorate depressive-like behavior caused by exposure to chronic stress using a validated chronic restraint stress model of depression (Fig. 1).

AKK alleviates depression-like behaviors by affecting the levels of the monoamine neurotransmitter and BDNF

Several hypotheses have been posited in relation to the pathogenesis of depression; the monoamine neurotransmitter hypothesis has been supported by numerous studies (Haase and Brown 2015). Decreased levels of monoamine neurotransmitters in the central nervous system could cause depression (Castrén 2005). The classic monoamine neurotransmitters associated with the pathophysiology of depression include serotonin and dopamine (Hasler 2010). Our data corroborate with findings of previous studies, which demonstrated AKK increased serum concentrations of serotonin and dopamine in CRS-induced mice. Chronic stress could result in excessive activation of the HPA (hypothalamic–pituitary–adrenal) axis, causing an increase in circulating glucocorticoids, including increasing the corticosterone levels in rodents (Spencer and Deak 2017). We established that supplementing CRS-induced mice with AKK could ameliorate stress-induced serum corticosterone levels.

BDNF is a neurotrophin that modulates neuroplasticity in the brain, which regulates the pathogenesis of depression. It is widely distributed in the hippocampus and closely associated with the regeneration and repair of neurons. In addition, the BDNF activation pathway is associated with

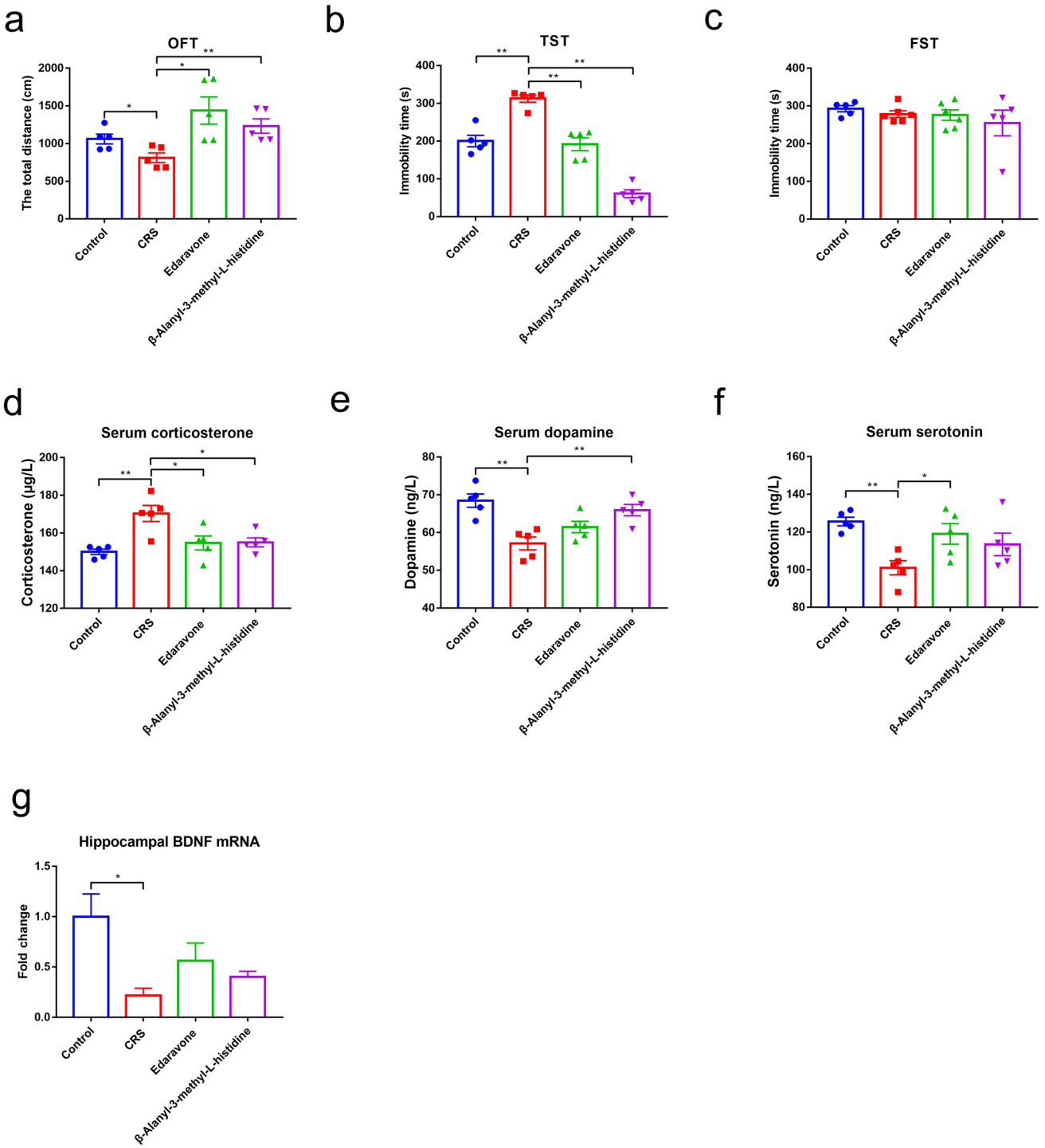

Fig. 6 Administration of potential metabolites ameliorate CRS-induced depressive-like behavior, and hormone, neurotransmitters, and BDNF levels. **a** Total distance covered by mice in open-field testing in 5 min. **b** Measurement of immobility time over the last 4 min during a 6-min testing time in tail suspension test. **c** Measurement of immobility time over the last 4 min during a 6-min testing time in forced swimming test. **d** Corticosterone concentration in serum. **e** Dopamine concentration in serum. **f** Serotonin concentration in serum. **g** BDNF mRNA level in mice hippocampus. Data are displayed as means ± SEM and $n = 5$/group; * and ** indicate $P < 0.05$ and $P < 0.01$

neurotrophic plasticity and synaptic plasticity in depression (Rakhit et al. 2005). mRNA levels of BDNF were decreased in the hippocampus of chronically stressed rats and depressed subjects (Bai et al. 2016; Duman and Monteggia, 2006). Generally, low BDNF protein and mRNA levels were observed in patients with MDD. Antidepressant treatment has been reported to increase the BDNF levels (Martinotti et al. 2016). CRS resulted in a decrease of BDNF levels in the hippocampus of mice, whereas the administration of AKK increased BDNF mRNA expression levels in the hippocampus (Fig. 2d), suggesting that AKK could enhance the synaptic signaling pathway and neuronal connections.

AKK improves depression by regulating the dysbiosis of the gut microbiota

Several strains have been demonstrated to possess antidepressant effects, which could be one of the mechanisms of modulating intestinal microecology (Moya-Pérez et al. 2017; Pinto-Sanchez et al. 2017; Tian et al. 2020). Therefore, we performed a comprehensive analysis of gut microbiota by sequencing the 16S rRNA gene. PCA and ANOSIM analysis results revealed that chronic stress exerted considerable effect on the overall gut microbiota composition. Nevertheless, AKK did not significantly influence variations in gut microbiota induced by chronic stress. AKK did not alter the stress-induced structure of gut microbiota, although prebiotics could decrease the diversity of gut microbiota due to selective proliferation of beneficial bacteria, and inhibit the growth of conditionally pathogenic bacteria (Slavin 2013). We further selected certain key taxa based on Welch's *t*-test results to analyze the effect of chronic stress on gut microbiota. Notably, not all the results were consistent with the findings of previous studies. For example, previous studies reported a decrease in the abundance of *Firmicutes*, whereas *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* increased considerably in patients with MDD (Jiang et al. 2015). In the present study, the tendency of the depressed mice was converse. The abundance of *Desulfovibrio* decreased in the Aβ-induced AD-like mice, although treatment with macromolecular yeast β-glucan could reverse the occurrence and confer benefit for the enhancement of Aβ-induced cognitive decline (Xu et al. 2020). The present study revealed that chronic stress could reduce the abundance of *Desulfovibrio*. However, the alteration of bacterial abundance was irreversible after administration of AKK. Our results revealed that AKK increased the abundance of *Verrucomicrobia* and *Akkermansia* substantially in CRS-induced mice, which could be attributed to the fact that AKK belongs to the phylum *Verrucomicrobia*, and genus *Akkermansia*.

Strikingly, the relative abundance of *Acidobacteria* "normalized" after administration of AKK. Notably, an increase in the abundance of *Acidobacteria* is rarely reported in patients with depression. However, a significant difference was observed in the results of our study. The observation could be a consequence of disease or temporary stress feedback. A previous study suggested that persistent pathogens such as *Helicobacter* could differentially influence mood disorders among women and men (Simanek et al. 2018). Here, the relative abundance of *Helicobacter* was decreased after AKK administration in CRS-induced mice.

We used PICRUSt to predict the functional capabilities of the microbial community. Differential gene expression and KEGG pathways between CRS and CRS + AKK groups were largely associated with neurodegenerative diseases, metabolism, and degradation (Fig. 4). Remarkably, pathways involved in neurodegenerative diseases, tryptophan metabolism, and geraniol degradation were downregulated considerably after AKK treatment. Tryptophan metabolism has been a therapeutic target in neurodegeneration (Platten et al. 2019). During infection, tryptophan metabolism is affected and gut microbiota activate indoleamine 2,3-dioxygenase (IDO), which degrades tryptophan via the kynurenine pathway and depleted tryptophan, thereby causing depression. After the production of kynurenine through tryptophan metabolism, quinolinic acid was produced under the mediation of enzymatic reaction, causing neurodegenerative changes. The maternal separation model of depression in rats fed with *Bifidobacterium infants* revealed reduced tryptophan metabolism, and the rats exhibited antidepressant ability (Desbonnet et al. 2008). Geraniol, with neuroprotection and anti-inflammation activities, has been demonstrated to exhibit antidepressant-like effect (Deng et al. 2015). Administration of AKK potentially reduced the degradation of geraniol to relieve depression.

AKK ameliorates depression by regulating metabolic disorders related to gut microbiota

Dysbiosis of the gut microbiota in mice with CRS was coupled with the alteration in serum metabolome. We used untargeted metabolomics to detect metabolites in the serum of mice to elucidate the underlying molecular mechanisms of depression in the CRS model by regulating gut microbiota. The results of metabolomics analyses revealed the various metabolic profiles of the control, CRS, and CRS + AKK groups, indicating that AKK could regulate the abnormal metabolic profiles of mice with CRS-induced gut microbiota dysbiosis. A total of 23 serum metabolites were identified as potential biomarkers implicated in gut microbiota dysbiosis between control and CRS groups based on the *t*-test and VIP values in the positive mode. Penicillamine is a thiol drug predominantly used in the treatment of Wilson's disease and rheumatoid arthritis, and adverse effects, which may include neuropathy, frequently occur during normal use of the drug (Pool et al. 1981). Mesalazine is a primary

treatment for IBD, and a multicenter trial revealed that it caused adverse effects of depression, although only in 5.4% of patients (Reinisch et al. 2010). β-Alanyl-3-methyl-L-histidine is also called anserine. Long-term consumption of fish stock reduces anxiety and modifies central amino acid levels including anserine in rats (Funatsu et al. 2015). A previous study revealed that anserine ameliorated neurovascular-unit dysfunction and spatial memory in an aged mouse model of Alzheimer's disease (Kaneko et al. 2017). A clinical research conducted in Japan investigated the association between serum concentrations of β-alanine, which is a metabolite of anserine, and the risk of dementia. The results of the study revealed that a high intake of anserine could be beneficial in the prevention of dementia (Hata et al. 2019). Edaravone, which exhibits a neuroprotective effect attributed to the potent-free radical scavenging activity, is largely used during the clinical acute phase of cerebral infarction adjuvant therapy (Lee and Xiang 2018). The present study has demonstrated that edaravone presents antidepressant-like activity. Studies using animal models have demonstrated that the antidepressant mechanism of edaravone is influenced by the expression of neurotransmitter turnover (Herbet et al. 2019), which could be coupled with inhibition of oxidonitrosative stress, neuroinflammation, and the endoplasmic reticulum stress cascade (Jangra et al. 2017). Furthermore, clinical trials have revealed that edaravone decreases depression severity in patients with symptomatic intracranial stenosis and has been associated with serum expression of sex hormones (Kong et al. 2020).

KEGG ontology enrichment analysis of the CRS and CRS + AKK groups (Fig. 5c) revealed that it was predominantly enriched in 9 pathways, which included cholinergic synapse, fat digestion and absorption, degradation of aromatic compounds, fatty acid degradation, vitamin digestion and absorption, butanoate metabolism, carbon metabolism, pantothenate, and CoA biosynthesis.

Accumulating evidence suggests that the cholinergic system plays a vital role in major depression and bipolar disorders. Specifically, previous studies established that scopolamine exerted rapid and sustained antidepressant effects on depressed humans (Dulawa and Janowsky, 2019). Furthermore, fluoxetine decreased cholinergic synaptic transmission and plasticity in established synapses to anti-depression (Getz et al. 2011). Fatty acid degradation is closely associated with depression; ω-3 polyunsaturated fatty acids (n-3 PUFAs) have been used as antidepressants in the treatment of MDD (Guu et al. 2019). Vitamins are essential nutrients; for instance, vitamin B or vitamin D deficiency triggers various disorders including depression symptoms (Milaneschi et al. 2014; Ghaleiha et al. 2016). Supplementing the diets of patients with MDD with vitamins confers beneficial effects on the symptoms (Sepehrmanesh et al. 2016). Pathway enrichment analysis results using non-targeted metabolomics to evaluate differential metabolites in a mouse model of depression induced by maternal separation (MS) demonstrated the significance of pantothenate and CoA biosynthesis. Therefore, the MS model increased susceptibility of rats to depression, which could regulate pantothenate and CoA biosynthesis (Cui et al. 2020). The pathway associated with pantothenate and CoA biosynthesis was enriched considerably in the KEGG pathways of MDD and bipolar depression (Ren et al. 2017). The results suggested that pantothenate and CoA biosynthesis could be key factors influencing depression. Based on the results of the metabolic pathway analyses, we hypothesized that the intervening effects of AKK against depression primarily occurred via regulation of the pathways.

Correlation analyses revealed that AKK exhibited a significant positive correlation with β-alanyl-3-methyl-L-histidine and edaravone, and administration of AKK restored the level of serum β-alanyl-3-methyl-L-histidine and edaravone in mice subjected to CRS (Fig. 5d–f). We therefore hypothesized that AKK ameliorates depression by increasing levels of β-alanyl-3-methyl-L-histidine and edaravone in serum. We conducted animal experiments to validate the antidepressant effects of β-alanyl-3-methyl-L-histidine and edaravone. The results revealed that β-alanyl-3-methyl-L-histidine and edaravone effectively alleviated CRS-induced depressive-like behavior (Fig. 6). In addition, administration of β-alanyl-3-methyl-L-histidine and edaravone increased dopamine, serotonin levels in serum, and BDNF expression in the hippocampus, particularly, significantly decreasing serum corticosterone levels in CRS-induced mice.

In conclusion, we demonstrated that AKK reduces CRS-induced depressive-like behavior in mice. The potential mechanisms involved influence hormone, neurotransmitter, and BDNF levels, as well as resulting in modifications in gut microbiota and serum metabolism. The findings of the present study could provide potential interventions of coping with stress-induced depression disorder with regard to gut microbiome and metabolomics. Besides, we provided a novel psychobiotic as a therapeutic strategy for depression. Nonetheless, further studies should be conducted to elucidate the association between depression and administration of AKK.

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Author contribution Y. D. designed the study, performed the experiments, analyzed the data, and wrote the manuscript. F. B., T. C., G. P. S., Z. Y. F., Z. L. D., and R. W. helped with performed experiments and analyzed data. Y. G. C. and X. M. Y. helped with design and review of the manuscript. S. M. Z., Q. W., and J. Y. Z contributed analytical tools. All authors read and approved the manuscript.

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Declarations

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare no competing interests.

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