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Integrated multi-omics profiling highlights the diet-gut-brain axis in low-calorie diets promoted novelty-seeking behavior

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ABSTRACT

The foods that we eat are closely linked to the development and function of neurophysiology, affecting mood, cognition, and mental health. Yet, it is not known whether and how dietary patterns affect brain function and mood. Here, we explored the impact of various diets on the behavior of mice. Low-calorie (LC) diet-fed mice exhibited increased novel exploratory behaviors, including novelty to new foods, objects, and environments. The host transcriptome sequencing showed an increase of *Areg* in the cerebral cortex of mice fed with LC, and IMPC showed that *Areg* knock-out mice exhibited significantly decreased exploration of novel environments. According to the metagenomic sequencing results, a significant increase in the levels of s_Schaedlerella and s_1XD8-76 was observed after LC feeding. Integrated analysis of microbiota metabolites and host transcriptomics suggested that 68 differential metabolites in LC-fed mice were associated with upregulation of *Areg* expression. This study demonstrates the powerful impact of LC feeding on the restoration of gut microbiota and the improvement of novelty-seeking behavior. In addition, this study supports the idea that microbiota-associated metabolites can modulate host gene transcription, which provides a link between dietary patterns and their impact on the emotional and cognitive centers of the brain.

1. Introduction

The food we consume, once digestive enzymes have broken down the food, shapes how we feel, how we think, how we respond to stress, how we remember things, how we age, and so on (Bremner et al., 2020; Firth et al., 2020; Melzer et al., 2021). Diet is becoming an essential tool in mental health and psychiatric care (Vauzour et al., 2017; Jennings et al., 2020; Lachance and Ramsey, 2015). Over the last few decades, researchers have discovered that the brain and diet are interconnected in a much broader way than previously thought, influencing everything from hunger levels to mood to the energy required for cognitive processes (Köster and Mojet, 2015; Collins and Stafford, 2015).

Dietary patterns are the sum of food types and quantities (proportions) and are key to good nutrition and health. People's diets are largely determined by geography, economics, culture, and personal preferences. For example, the Western diet is high in meat, while the East Asian diet is high in grain. Two of the most popular healthy diets are the ketogenic diet (KD) and the Mediterranean diet (Mt). The Mt consisting of daily consumption of fruits and vegetables, whole grains, pulses, nuts, fish, white meat, and olive oil, has attracted considerable attention for possible effects on mental health, in particular a reduced risk of depression (Ventriglio et al., 2020; Yin et al., 2021). The KD is a diet high in fat, adequate protein, and very low in carbohydrates (Zhu et al., 2022), the role of the diet in brain therapy for mental disorders has also been highlighted (Sethi and Ford, 2022; Nagpal et al., 2019). A low-calorie eating plan is a structured diet that limits daily calorie intake, usually for weight loss. Now, as a potential adjunctive intervention to prevent and/or treat cancer, calorie restriction is gaining attention (Vidoni et al., 2021). However, the effects of low-calorie diets on mood and behavior have not yet been reported.

The exploration of the physical and social environment and the search for novelty are essential for the discovery of new food resources, for the assessment of possible dangers and a better understanding of the world around us (Ahmadlou et al., 2021). However, the effect of dietary patterns on novelty-seeking behavior has not been reported to date. Here, we examined the effects of different dietary patterns on mouse

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behavior. In addition to the two dietary patterns, Mt and KD, the effects of dietary patterns such as calorie restriction and high proportions of the three main nutrients - sugar, protein, and fat - on novelty exploration behavior were also investigated. The results showed that a low-calorie (LC) diet was effective in promoting novelty-seeking behavior in mice. An LC diet limits the daily calories consumed, which is metabolically beneficial. Further data showed an increase of AREG in the cerebral cortex of mice fed with LC, and *Areg* knock-out mice exhibited significantly decreased exploration of novel environments in the SHIRPA and dysmorphology test. To further uncover the potential regulatory pathways of LC feeding in mice, a series of analyses were performed, including microbiota metagenome sequencing and non-target metabolic assessments of the gut, host transcriptomic analysis, and integrative analysis. An understanding of the relationship between diet and behavior could have important implications for developing dietary strategies to help the brain develop and function healthily.

2. Experimental methods and reagents

2.1. Isolation and culture of primary cortex neurons

As in our earlier work, the P0 mouse cortex was isolated (Xing et al., 2021). In brief, 6 mouse E15 embryos were used to isolate primary cortical neurons. Cortices were dissected in ice-cold HBSS (Gibco) and immersed in 0.125% trypsin-EDTA (Invitrogen) at 37 °C for 15 min. Viable cells were grown on glass bottom dishes that had been coated with poly-D-lysine (Sigma) and were cultured at 37 °C in 5% CO_2 in the Neurobasal medium (Invitrogen). The medium was supplemented with 2% B27.

2.2. Immunostaining

Cultured neurons from the cortex were grown on glass-bottom dishes (NEST). Neuronal cells were fixed in 4% paraformaldehyde after treatment with 100 ng/mL AREGs for 24 h, followed by permeabilization with 0.5% saponin for 10 min. After thorough washing in PBS, cells were incubated with antibody against MAP2 (Merck Millipore, AB5622) in blocking buffer (5% BSA, 0.05% saponin in PBS) at 4 °C overnight. The cells were then incubated with Alexa Fluor 488-labeled secondary antibody (Invitrogen, A-11008) in a blocking buffer for 1 h at room temperature. The cells were subsequently washed and analyzed by confocal microscopy.

2.3. Microscopy

A confocal microscopy (LSM880; Zeiss) with a camera with a 60 × oil objective was utilized to capture fluorescence images. A 405 nm and a 488 nm laser were used for excitation. Images were processed using the Zen Blue 2 software (Zeiss).

2.4. Western blotting

Cortical lysis was performed in cold RIPA buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1 mM PMSF). The buffer contained a complete protease-inhibiting cocktail (Roche). Cortex was homogenized, sonicated, and centrifuged at 12,000 rpm for 10 min at 4 °C. SDS-PAGE was used to separate 20 μg of total proteins, followed by blotting and probing with AREG (Proteintech, 16036-1-AP) and Tubulin antibodies (Proteintech, 11224-1-AP). Image J software (National Institutes of Health) was used for quantification of Western blots.

2.5. Mouse strains and behavioral assays

All animal experiments, the care of animals and protocol were approved by the ethics committee of Yunnan Agricultural University. All mice were obtained from Vital River Laboratory Animal Technology Co., China. C57BL/6 mice, aged 5–6 weeks, were randomized into different experimental groups and given the following diets for a period of 5 weeks: control diet (Ctr), low-calorie diet (LC) (Lien et al., 2021), ketogenic diet (KD) (Lien et al., 2021), Mediterranean diet (Mt) (Park et al., 2024), high-fat and sugar diet (HFHS) (Vellers et al., 2017; Fritz et al., 2018), and high protein diet (HP) (Zhong et al., 2022). After initiation of the experimental diets, control mice received the average daily chow provided, while calorie-restricted mice received 60% of the control daily chow (Lien et al., 2021).

2.5.1. Novelty suppression feeding

Following a previous study, the NSF was performed (Siopi et al., 2023). Briefly, 24 h before the behavioral test, the mice were placed on food restriction. In an arena (40 × 40 cm), a single food pellet was placed in the center. Pellet-eating latency was recorded for each mouse (5 min) and food intake was measured.

2.5.2. Novel object recognition test (NOR)

Slightly modified protocols published in the literature (Zhang et al., 2023) were used in our study. The behavioral observation apparatus (VanBi-OFM, Shanghai VanBi Technology Co. Ltd., China) was used for testing in the behavioral procedure room. The observation box was 40 × 40 cm. Cylinders A and B (red, diameter 3 cm, height 6 cm) and cylinder C (yellow, diameter 5 cm, height 8 cm) were used as recognition objects. Each object had sufficient weight for stability. Both probe and test trials were used in the NOR procedure. First, the mice were familiarized with the empty box for 10 min. Subsequently, the sample trial consisted of object A and object B presented opposite to each other and separated by 14 cm. The duration of the exploration period for each mouse was 10 min. During testing, the mouse was positioned between two objects. During the test trial, object B is replaced by object C, a new object for them. At the end of each trial, the objects and the box were cleaned with 75% ethanol to avoid olfactory cues. Videotapes were used to record the behavior of the mice.

2.5.3. Open field test

Mice performed the open field test after 1 h of habituation to the behavioral testing room (Ji et al., 2023). Each mouse had 10 min of free movement in the box, beginning at the center of the box. After 10 min, mouse was removed and returned to home. A 75% ethanol solution was then used to clean the arena. Movement distances and the time spent in each zone were recorded. Mice were screened to validate novel ingestion and other behavioral experiments based on the Open Field test.

2.6. Multi-omics profiling

2.6.1. Metagenomic sequencing

The DNeasy PowerSoil tool kit (QIAGEN, Valencia, CA, USA) was used to extract microbial genomic DNA from mouse feces. A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) and electrophoresis sugar gel were used to measure the quantity and quality of extracted DNA. A metagenomic shotgun library was constructed using the Illumina TruSeq Nano DNA LT Library Preparation Kit from the DNA samples used for quality testing. PE 150 strategies of each library were sequencing on the Illumina HiSeq X-10 platform (Illumina, San Diego, CA, USA) by Personal Biotechnology Co., Ltd. (Shanghai, China). Fast QC, an original analysis read, was used for quality control. Cutadapt (V1.2.1) (NBIS, Uppsala, Sweden) was used to remove the sorting adapter from the sorting reads. A sliding window algorithm for ambiguous base removal removed low quality reads. The sequencing reads were mapped to the host genomic reads and the host genomic reads were deleted. A macro-genome was then constructed for each sample by recombining the quality-filtered reads.

2.6.2. Untargeted metabolomics analysis

Mouse fecal samples were subjected to high-resolution non-targeted metabolomic analysis. Ultra-high-performance chromatography-tandem time-of-flight mass spectrometry (UHPLC-TOF-MS) was used for metabolite detection in samples by Personal Biotechnology Co. Dwell Time, Molar Mass (Molar Mass Deviation <10 ppm), Secondary Fragmentation Spectrum, and Impact Energy were used to identify metabolic products in biological samples, and evaluation results were rigorously reviewed and manually confirmed.

2.6.3. RNAseq analysis

Total RNA was extracted from the mouse cortex using Trizol reagent (Invitrogen/Life Technologies). Concentration, quality, and identity were determined using the NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The mRNA was purified from total RNA utilizing poly-t oligosaccharide-linked magnetic beads. Random oligonucleotides and superscript II were used for the synthesis of first-strand cDNA. DNA polymerase I and RNase H are used to synthesize the second strand cDNA. The remaining overhanging ends are converted to blunt ends using exonuclease/polymerase activity. The enzyme is then removed. Library segments were purified to select cDNA fragments with a preferred length of 400–500 bp using the Ampure XP system (Beckman Kurt, USA). The Illumina PCR primer mix was used to selectively enrich DNA fragments with binding molecules at both ends in 15 cycles of PCR. An Agilent 2100 Biological Analysis System (Agilent) was used for purification and high-sensitivity DNA analysis (Agilent). Corrections for multiple testing were made using the Benjamini and Hochberg method, and p-values of less than 0.05 were regarded as significant.

2.7. Statistical analysis

Data are presented as mean ± standard error (SEM). For statistical comparison of multiple groups of samples, one-way ANOVA followed by Dunnett's post hoc test was used. The nonparametric test was used to determine statistically significant differences between the two groups. Prism (GraphPad) statistical analysis software was used for all statistical analyses.

3. Results

3.1. LC-fed mice promote food novelty-seeking exploration

Here, we investigated the effects of different dietary patterns on mouse novelty-seeking behavior. C57BL/6J mice were exposed to control (Ctr), low-calorie (LC), ketogenic diet (KD), Mediterranean (Mt), high-fat and high-sugar (HFHS), and high-protein (HP) diets (Table 1). In the novelty suppression feeding test, the evaluation of novelty feeding behavior was determined by the latency to the first bite and the amount of food consumed (Fig. 1A). The KD-fed mice showed an increased latency and the HFHS-fed mice showed a decreased food intake, while the LC-fed mice exhibited both a significantly shorter latency and increased food intake, compared to the control animals (Fig. 1B–C). The results suggest that LC feeding promotes novelty feeding behavior.

To confirm our findings further, we repeated the experiment with mice that had been pre-screened using the open-field test (Fig. 2A). Consistent with the previous results, LC-fed promoted novelty feeding behavior, as evidenced by a significantly reduced feeding latency and increased food intake (Fig. 2B–C). Hunger, like curiosity, can be an intrinsic motivational drive for novel food exploratory behavior, while

Table 1
Nutrient composition of different dietary patterns.

Diet	Control		ketogenic diet		Low-calorie diet		high-fat & high-sugar		Mediterranean diet		high-protein diet	
	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%
Protein	18.13%	18.00%	14.53%	8.70%	31.38%	31.00%	19.55%	18.00%	5.28%	40.00%	73.55%	73.00%
Carbohydrate	65.45%	65.00%	1.67%	1.00%	41.09%	40.60%	56.47%	52.00%	44.84%	45.00%	10.08%	10.00%
Fat	7.61%	17.00%	67.03%	90.30%	12.78%	28.40%	14.48%	30.00%	6.64%	15.00%	7.61%	17.00%
Total		100%		100%		100%		100%		100%		100%
kcal/gm	4.03		6.68		4.05		4.34		3.99		4.03	
Ingredient	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%
Casein, 80 mesh	177.34	709.36	87.09	348.34	305.42	1221.67	177.34	709.36	50.00	200.00	719.21	2876.85
Soybean isolate protein									347.00	1388.00		
L-Cystine	2.66	10.64	1.31	5.23	4.58	18.33	2.66	10.64	3.00	12.00	10.79	43.15
Corn Starch	408	1632.00		0.00	229.3	917.20		0.00	208	832.00		0.00
Maltodextrin	132.00	528.00	9.16	36.64	70	280.00	132.00	528.00	132.00	528.00	0.00	0.00
Sucrose	100	400.00		0.00	90	360.00	378	1512.00	100	400.00	90	360.00
Cellulose	50	0.00	50	0.00	83.3	0.00	50	0.00	50	0.00	50	0.00
Soybean Oil	75.56	680.00	25	225.00	126.22	1136.00	133.33	1200.00	66.67	600.00	75.56	680.00
Cocoa Butter			382.75	3444.79								
Mineral Mix S10022G	35	0.00			58.3	0.00	35	0.00	35	0.00	35	0.00
Mineral Mix S10026B			50	0.00		0.00						
Vitamin Mix V10037	10	40.00			16.7	66.80	10	40.00	10	40.00	10	40.00
Vitamin mix,V10001C			1	4.00		0.00						
Choline Bitartrate	2.50	0.00	2.00	0.00	4.20	0.00	2.50	0.00	2.00	0.00	2.00	0.00
FD&C Red Dye #40	0	0.00	0.05	0.00	0	0.00	0	0.00	0.025	0.00	0	0.00
FD&C Yellow Dye #5	0	0.00	0	0.00	0.05	0.00	0	0.00	0.025	0.00	0.025	0.00
FD&C Blue Dye #1	0	0.00	0	0.00	0	0.00	0.05	0.00	0	0.00	0.025	0.00
Total	993.06	4000.00	608.31	4064.00	988.02	4000.00	920.83	4000.00	1003.67	4000.00	992.56	4000.00

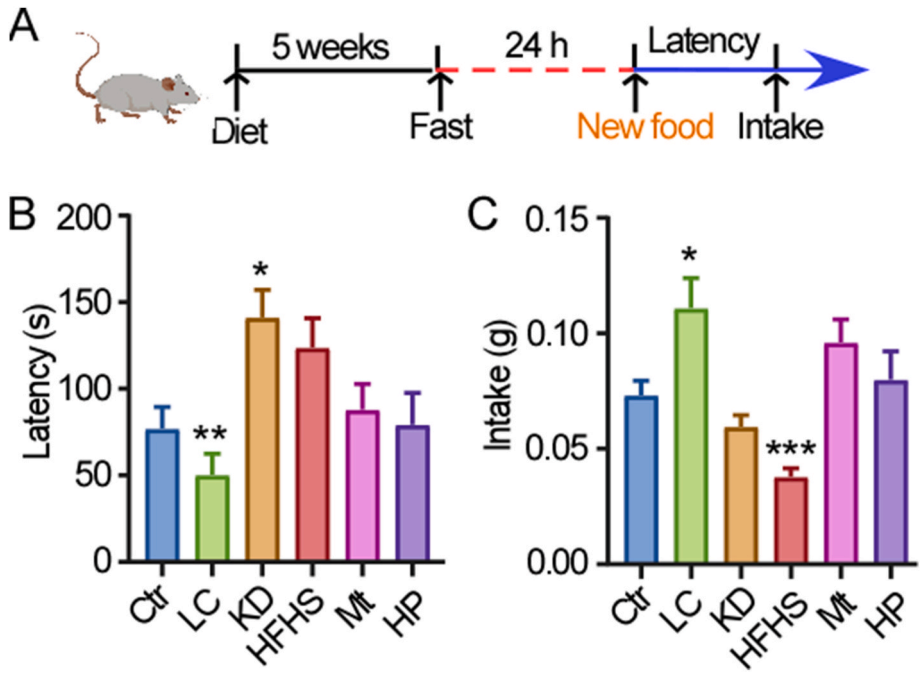

Fig. 1. LC-fed mice promote food novelty-seeking exploration. (A) Diagram of the flow chart of animal treatments. (B–C) Quantification of the first bite latency (B) and food consumption (C) in mice fed with control (Ctr), low-calorie (LC), ketogenic diet (KD), Mediterranean (Mt), high-fat and high-sugar (HFHS), and high-protein (HP) diets (n = 10–12). Data are expressed as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

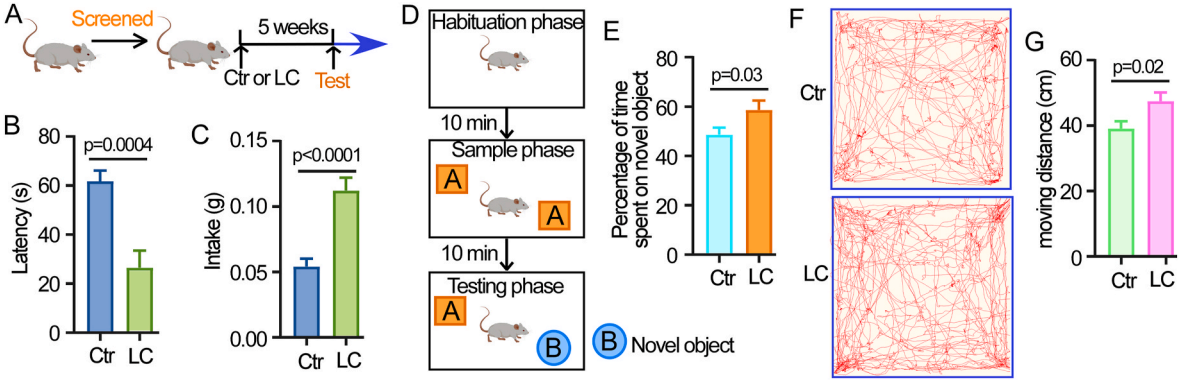

Fig. 2. LC-fed mice promote novelty-seeking exploration. (A) Diagram illustrating the treatment of the mice used in Fig. 2. The mice were pre-screened using an open-field test. (B–C) Quantifying latency to the first bite (B) and the amount of food consumed (C) in selected mice fed with a control or LC diet (n = 12–15). (D) Schematic of the novel object recognition test. (E) Quantification of the percentage of time spent on novel objects by selected mice fed with a control or LC diet (n = 12–15). (F) Representative locomotor trajectory and (G) distance traveled quantification of selected control and LC-fed mice (n = 12–15). Data are expressed as mean ± SEM. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

only curiosity is the motivational drive to explore and investigate new objects and new environments. To distinguish motivational drive from hunger or curiosity, we further tested the effect of LC-fed on exploratory behavior with novel objects and novel environments. The novel object recognition test was used to measure novel object-seeking behavior (Fig. 2D). Data showed that the percentage of time spent on the novel object increased in the LC-supplemented groups compared with the control group (Fig. 2E), indicating that LC feeding enhanced novelty object-seeking. The open-field test is a common measure of exploratory behavior in mice. Compared to the control group, LC-fed also showed a significant increase in open-field locomotor parameters (Fig. 2F–G). Taken together, these data suggest that the LC diet was effective in promoting novelty-seeking behavior in mice.

3.2. *AREG is involved in the cortex of LC-fed mice*

We further determined the neurological molecular mechanism by which the LC diet promotes novelty-seeking. The cortex was reported to regulate thoughts, emotions, and behaviors (Friedman and Robbins, 2022). Therefore, we performed RNA sequencing (RNA-seq) to analyze the cortex of control and LC-fed mice to study transcriptional regulation. At the transcriptional level, the second most upregulated gene is amphiregulin (*Areg*) (Fig. 3A–B). Differential gene analysis of the KEGG signaling pathway showed that the signaling pathway regulated by Top1 in the cortex is the neuroactive ligand-receptor interaction (Fig. 3C), and AREG is a neuroactive ligand. Therefore, we further validated the protein level of AREG in the cerebral cortex of mice fed with LC using Western blot analysis (Fig. 3D–E). Together, these data suggest that the LC diet feeding upregulated the level of cortical AREG in mice.

AREGs are secreted as membrane-bound precursors which, following

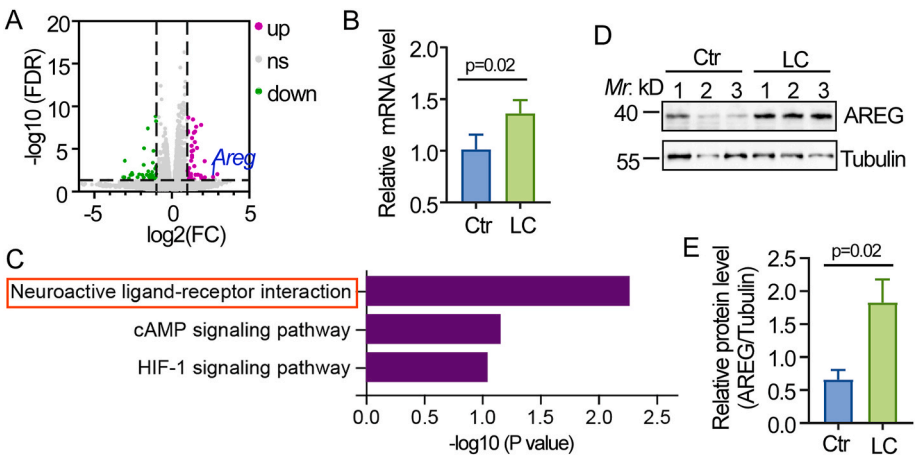

Fig. 3. AREG is upregulated in the cortex of LC-fed mice. (A) Gene transcriptional changes in the cortex of control or LC-fed mouse by RNAseq. (B) In vivo quantitative determination of mRNA level of Areg. (C) The KEGG pathway enrichment analysis was performed on the genes with the most significant changes. The names of the KEGG pathways are shown on the left and the corresponding P values are shown on the right. (D–E) Protein expression (D) and quantification (E) of control or LC-fed mouse cortex (n = 3). Data are expressed as mean ± SEM. *, $p < 0.05$.

proteolysis by cell membrane proteolytic enzymes, act as autocrine or paracrine factors (Singh et al., 2022). Knockout of *Areg* in mice showed significantly reduced exploration of novel environments in the combined SHIRPA and dysmorphology test (Fig. 4A), according to data downloaded from the International Mouse Phenotyping Consortium (IMPC). AREG is an epidermal growth factor receptor (EGFR) ligand (Singh et al., 2016), which is involved in neural progenitor cell differentiation, neuronal growth and function (Romano and Bucci, 2020). Neuron is the material basis of novel exploratory activity, and the complexity of neuron growth is an important indicator of neuronal function. We have therefore investigated the effect of AREG on the growth of neurites and the complexity of neurites. The results showed that cortical neurons treated with AREG showed longer neuronal dendrites as well as more branches (Fig. 4B–D), indicating that AREG promotes the complexity of the neurons. Taken together, AREG may mediate the increased novelty-seeking behavior by promoting neuronal growth and function.

3.3. Altered gut microbiota in LC-fed mice revealed by microbial diversity analysis

Over the past few years, the human gut microbiome has emerged as a potential target for modulating health and disease (de Vos et al., 2022; Fan and Pedersen, 2021). The gut microbiota could be shaped by a variety of factors, with diet being one of the most important influences (Zmora et al., 2019; Rinninella et al., 2019). To investigate how LC feeding increases novelty-seeking behavior by modulating the community structures of the microbiome, we performed metagenome sequencing samples from mice feces in the control and LC treatment groups. The Circos plot shows changes in the composition of the gut flora in control and LC-fed mice (Fig. 5A). Furthermore, LC feeding increased alpha diversity with a significant increase in Chao, Goods coverage, and Simpson ND Pielou_e index (Fig. 5B). The beta diversity of the gut microbiota was also assessed by Principal Coordinate Analysis (PCoA) (Fig. 5C), which showed the distinct clusters between the control and LC-fed groups. The distinct top 10 microbes between the control and LC-fed groups were shown in (Fig. 5D), with an upregulation of s_Schaedlerella and s_1XD8-76 and a decrease in s_Faecalibaculum and s_Filiplasma by LC. We performed functional clustering of the strains by PCoA, and the gut microbes of mice in the control and LC-fed groups showed different clustering (Fig. 5E), suggesting altered gut microbial function. We also used Linear discriminant analysis effect size (LEfSe) analysis to search for different functions between the two groups, and the evolutionary branching exhibits the difference of metabolic pathways differences between the groups (Fig. 5F).

3.4. Gut microbiota Modulates Areg upregulation

To investigate the regulation of gene expression in the mouse cortex by gut microbes, Pearson's correlation analysis was performed between the 46 up-regulated genes of the mouse cortex as determined by RNA-Seq and the top 50 microbes from the metagenomics. The significance

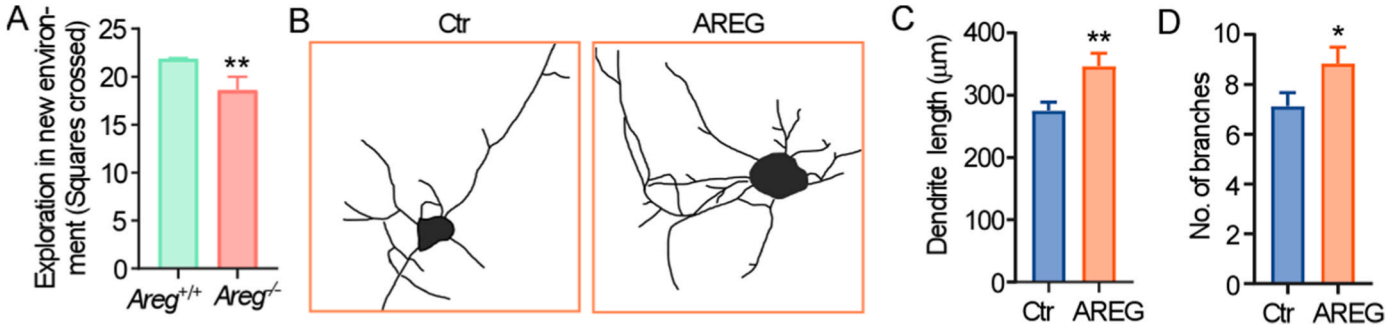

Fig. 4. Deficiency of *Areg* impaired novelty-seeking exploration. (A) The quantification of the points of the control mice (n = 5563) and the *Areg* knockout mice (n = 16) in the combined SHIRPA and dysmorphology test. (B) The representative morphology of primary neurons was examined after treatment with or without 100 ng/mL of AREG for 24 h, followed by labeling with the Map2 antibody. The quantification of the length of dendrites (C) and the number of branches (D) of (B). 25 neurons were analyzed in each group. Data are expressed as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$.

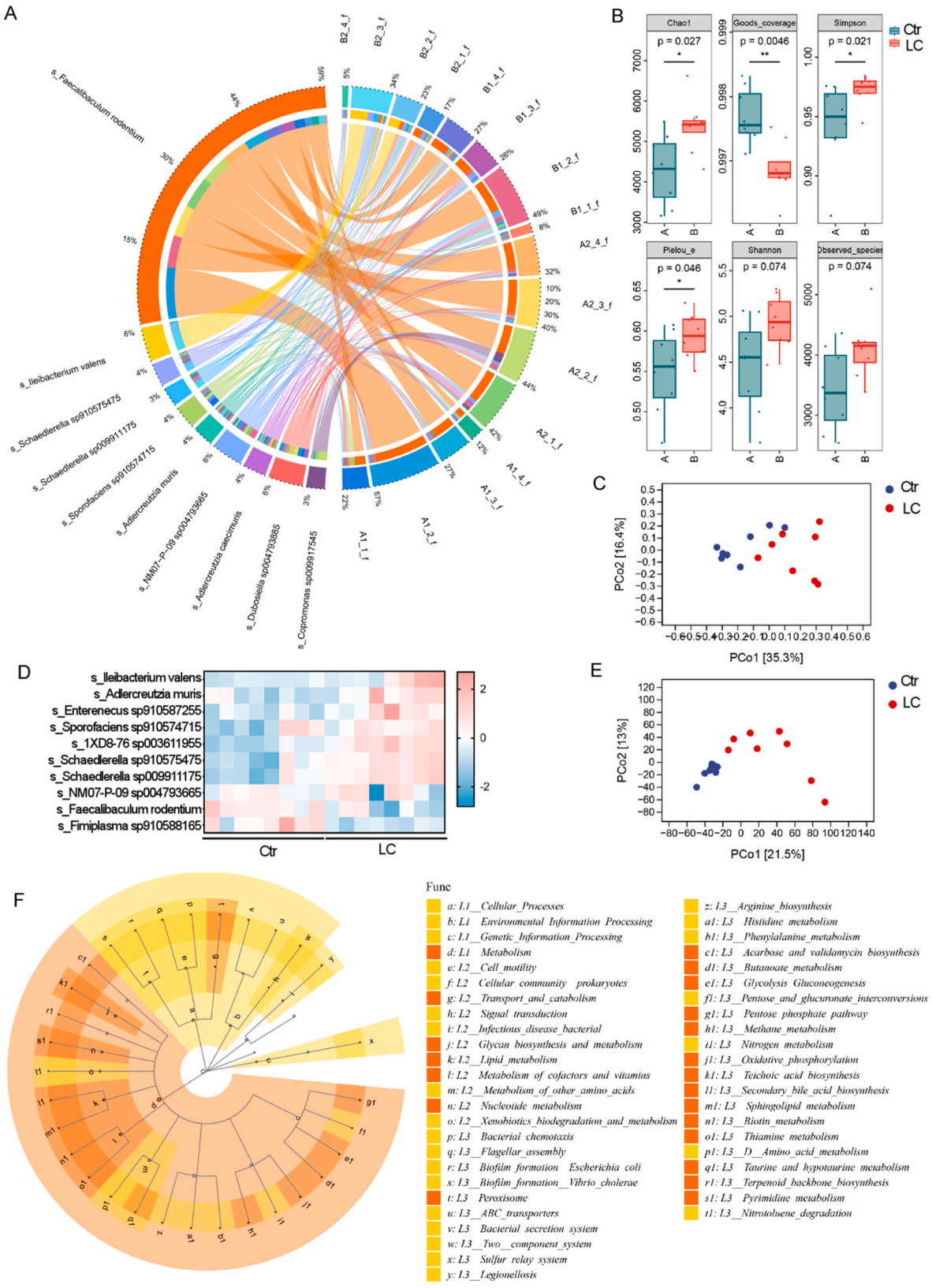

Fig. 5. Microbial diversity analysis reveals changes in gut microbiota in LC-fed mice. (A) Analysis of species abundance of intestinal flora from control or LC-fed mice. (B) The alpha diversity index of microbes from fecal samples was obtained from control or LC-fed mice. (C) The CoA plot shows the microbes differences between samples from the control group (blue) and the LC-fed mouse group (red). (D) Heat map analysis of the microbiota in fecal samples from control or LC-fed mice. (E) The CoA plot shows the differences in microbes' function between control or LC-fed mice. (F) LEfSe analysis is used to search for the differences in microbial functions. n = 8 biologically independent animals per group.

of each bacterium correlation to the upregulation of Areg has been marked in the graphs. The results showed that the upregulation of Areg by LC feeding was significantly positively associated with 9 strains and negatively associated with 3 strains (Fig. 6A). We also analyzed the abundance of the 12 microbes in the control and LC-fed groups. The results showed that there were 4 strains (g_Merdisoma, g_Schaedlerella, g_1XD8-76, and g_UBA3282) positively associated with Areg upregulation with a significant increase in abundance and 1 strain, g_CAG-485, negatively associated with *Areg* upregulation with a significant decrease in abundance (Fig. 6B). We hypothesized that these strains may

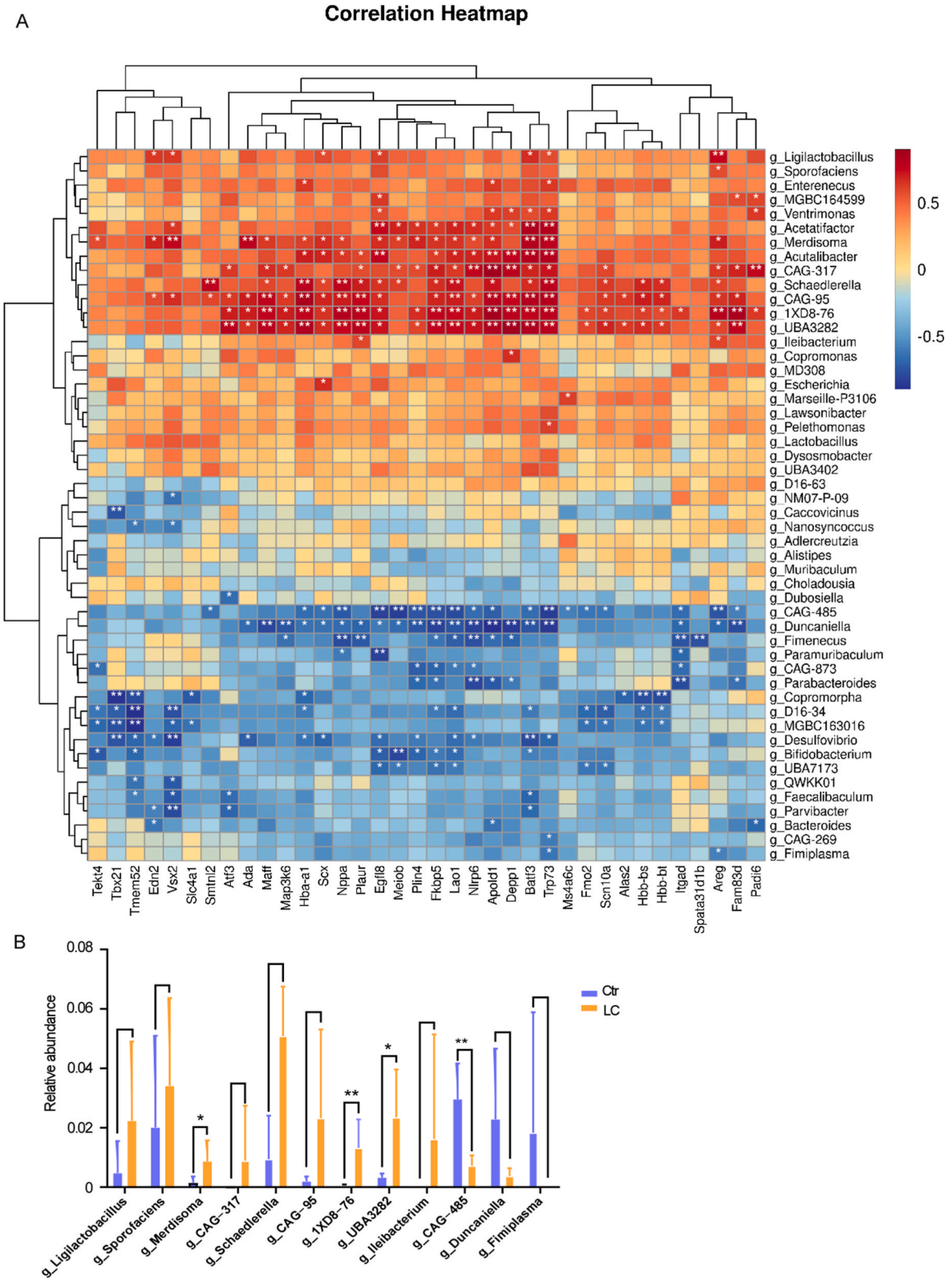

Fig. 6. Gut microbiota modulates Areg upregulation. (A) Heat map of correlation analysis between Areg upregulation by LC feeding and top 50 gut microbiota. (B) The abundance of microbiota which were associated with *Areg* regulation. Data are expressed as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

be responsible for the regulation of AREG gene expression and we would like to further explore which metabolites these strains regulate this process through.

3.5. Changes in gut metabolites in LC-fed mice revealed by metabolomics analysis

For this purpose, we performed untargeted metabolomics analyses of fecal samples from control and LC-fed mice after 35 d of feeding. Classification of gut metabolites revealed that 21.5% of the differential compounds were carboxylic acids and derivatives, 15.4% were fatty acids and 6.6% were organooxygen compounds (Fig. 7A). PCoA analysis showed a significant difference in gut metabolite composition between control and LC-fed mice (Fig. 7B). The differential metabolites were listed and clustered in (Fig. 7C), including 74 down-regulated and 37 up-regulated. KEGG pathway analysis revealed enrichment in amino acid metabolism, lipo-metabolism, and ABC transport. (Fig. 7D).

3.6. Gut metabolites modulate Areg upregulation

To explore the regulation of *Areg* by gut metabolites, Pearson's correlation analysis was used to perform the correlation analysis between the differential metabolites and Areg. The results showed that *Areg* upregulation by LC feeding was positively associated with 20 up-regulated metabolites and negatively associated with 48 down-regulated metabolites, respectively. We further analyzed the correlation of these 68 differential metabolites with the 5 differential microbes upregulated by *Areg* and found that the expression of 62 of these metabolites correlated to varying degrees with these five strains (Fig. 8). The microbiota, g_1XD8-76 and g_UBA3282, were significantly associated with almost all metabolite alterations associated with *Areg* upregulation (Fig. 9) and may play a more dominant role in mediating metabolite alterations.

4. Discussion

Over the past few decades, researchers have discovered that the brain and diet are much more interconnected than previously thought,

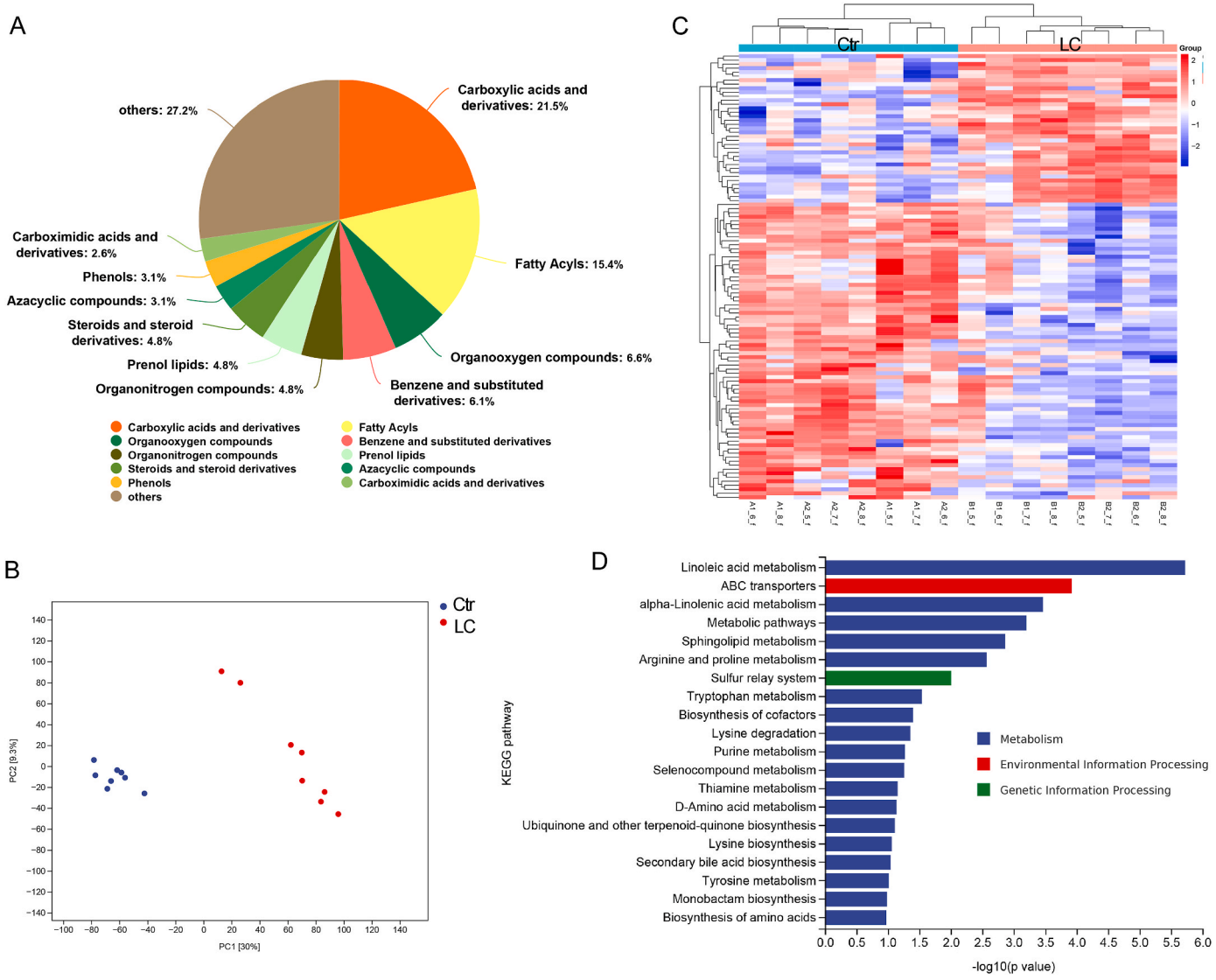

Fig. 7. Metabolomics analysis reveals changes in gut metabolites in LC fed-mice. (A) Classification of compounds with different gut metabolites in the metabolome database. (B) PCoA plot showing gut metabolomic differences between control or LC-fed mice. (C) Fecal metabolite heat map analysis from control or LC-fed mice. (D) Significantly altered gut metabolites were used for KEGG pathway enrichment analysis. The KEGG pathway names are shown on the left and the p-values on the right. n = 8 biologically independent animals per group.

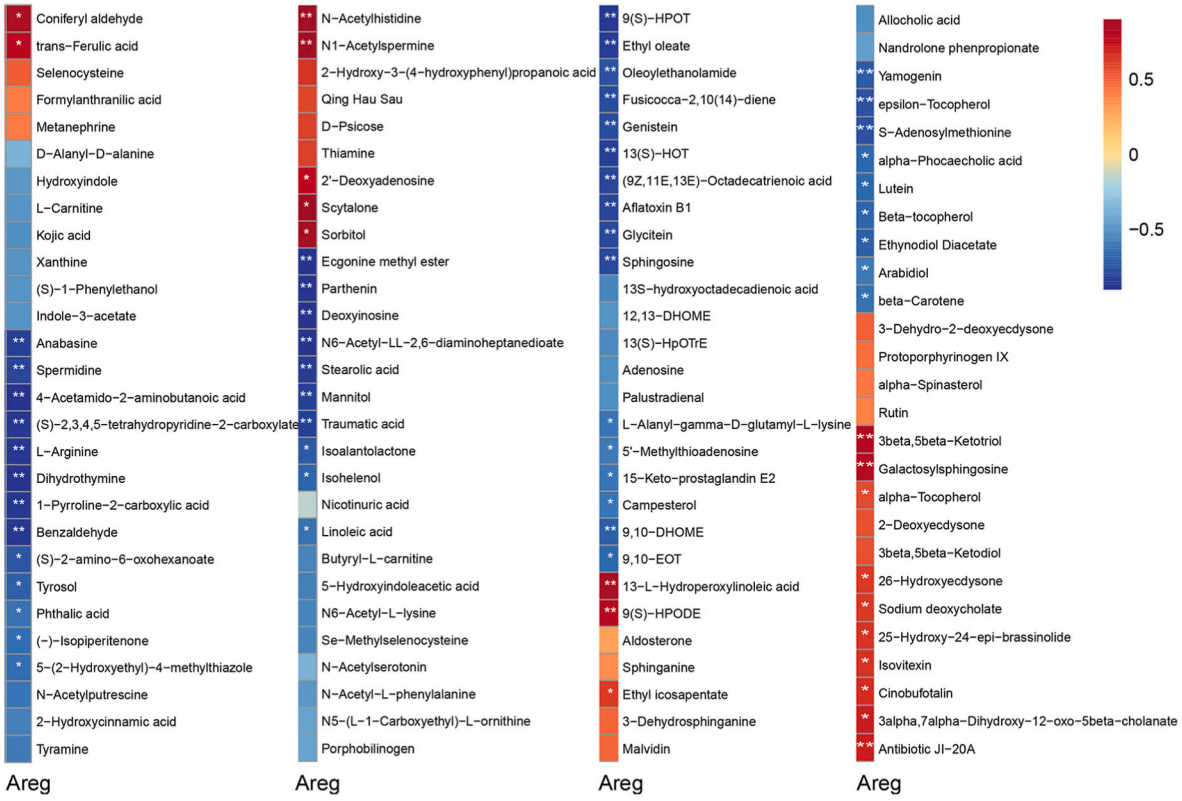

Fig. 8. Gut metabolites Modulates Areg upregulation. Heat map of correlation analysis between *Areg* upregulation by LC feeding and differential metabolites.

affecting everything from hunger levels and mood to energy requirements for cognitive processes (Gómez-Pinilla, 2008; Grosso, 2021). There have been reports on the effects of calorie restriction on weight loss and cancer resistance (Wang et al., 2023; Xiao et al., 2024), but the effects of calorie restriction on mood changes and behavior have not yet been reported. The study tested the effects of the Mt and KD diets and the effects of caloric restriction and the three main nutritional components, sugars, proteins, and fats, upon novelty exploration. Mice fed KD had an increased latency, HFHS had reduced feeding and LC had both significantly shorter latency and increased feeding. Further data showed an increase in AREG in the cerebral cortex of the LC-fed mice, and *Areg* knock-out mice have shown a marked reduction in the exploration of novel environments in the combined SHIRPA and dysmorphology test. In conclusion, we propose that AREG is upregulated in the cerebral cortex of LC-fed mice and drives the exploration of novelty that links dietary patterns to their effects on the emotional and cognitive centers of the brain.

Considering that the gut microbiota is one of the major modalities through which diet has important and diverse effects on host physiology, we aimed to characterize the gut microbiota and metabolic profile and to investigate the underlying mechanism and correlation between gut metabolites and transcriptional regulation in LC-fed mice through multi-omics analyses to understand the mechanisms of LC diet in the upregulation of novelty-seeking behavior. Metagenomic and metabolic profiles revealed that LC feeding caused significant changes in bacterial structure, function, and metabolism. Furthermore, we used integrative microbiota/metabolite analyses with host cortical tissue transcriptome sequencing to explore the possible pathway of neuronal action. Transcriptome analysis revealed that LC feeding interacts with neuroactive ligands and receptors. The most prominent KEGG pathway in the cortex of LC-fed mice is neuroactive ligand-receptor interaction, and the gene that up-regulates TOP2, *Areg*, is a neuroactive regulatory ligand gene. Therefore, to further analyze the correlation between differential strains and differential metabolites associated with *Areg* up-regulation, we analyzed the strains and differential metabolites of Top50 associated with *Areg* up-regulation. The results indicated that five differential strains, particularly g_1XD8-76 and g_UBA3282, may ultimately influence *Areg* upregulation in the cerebral cortex by regulating multiple metabolites.

In conclusion, our data suggest a diet-gut microbiota-brain axis mediating increased novelty-seeking behavior in LC-fed mice. Our study provides a link between the patterns of diet and the effects they have on the emotional and cognitive centers of the brain induced by gut microbiota. Diet and brain function have been the subject of much research over the past decade, with theories suggesting that diet affects brain function and possibly mood and behavior, mental health, and illness (Bremner et al., 2020). The structure and function of gut microbes and metabolites, areas of the brain, and neuropeptides that have been implicated in both mood and appetite are likely to play a role in mediating this relationship. Our ability to develop dietary strategies to promote healthy brain development and function will be enhanced by a better understanding of how diet regulates the emotional and cognitive centers of the brain.

CRediT authorship contribution statement

Shuangping Wang: Data curation, performed the research, All authors analyzed and interpreted the data. **Ling-Yan Su:** Data curation, designed the research, performed the research, All authors analyzed and interpreted the data. **Junquan Chen:** Data curation, performed the research, All authors analyzed and interpreted the data. **Yang Tian:** designed the research. **Hejiang Zhou:** Writing – original draft, designed the research, wrote the paper.

Ethical Statement

All animal experiments, the care of animals and protocol were approved by the ethics committee of Yunnan Agricultural University.

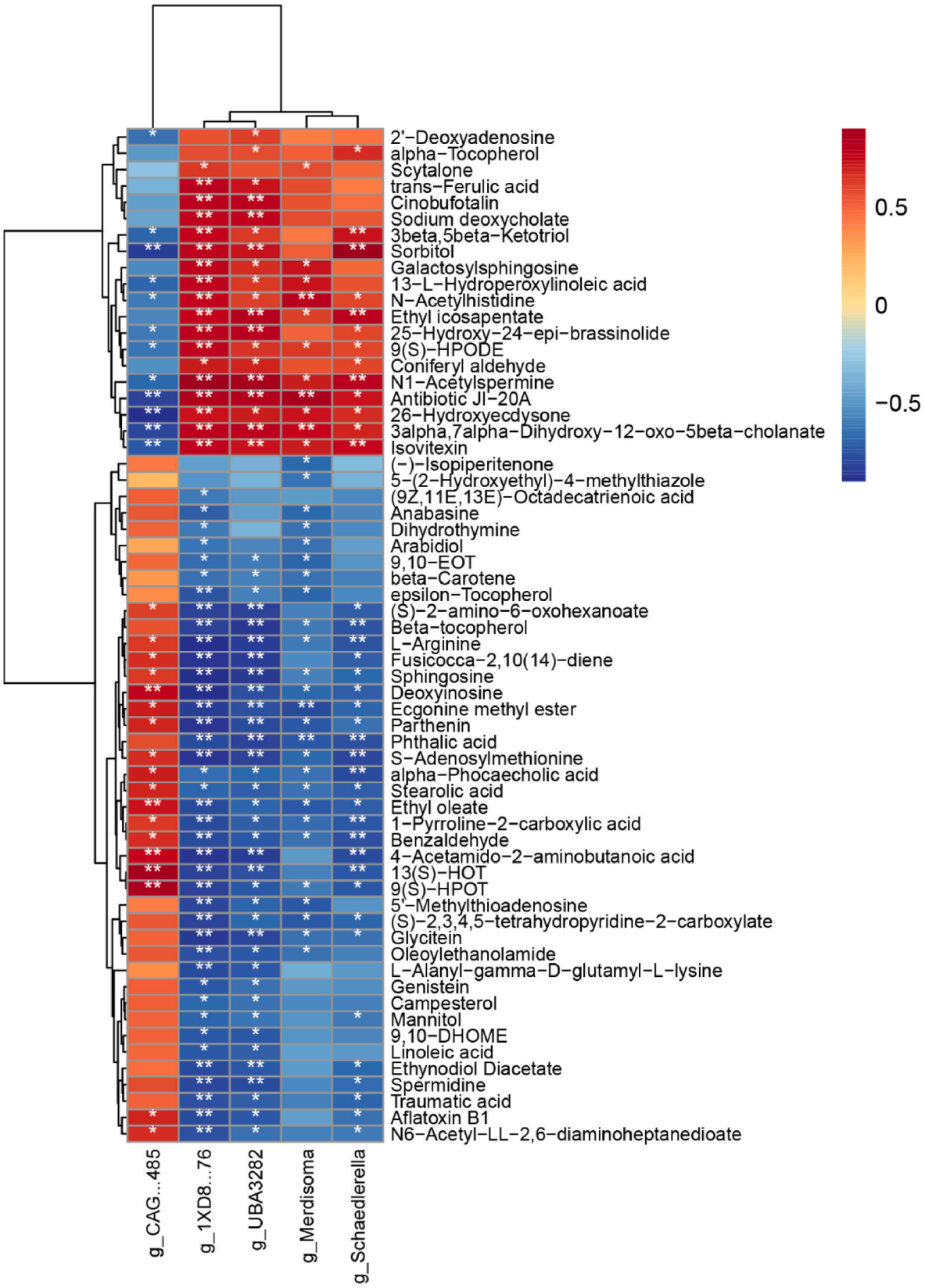

Fig. 9. Gut microbiota modulates metabolite alteration. Heat map of correlation analysis between differential microbes associated with *Areg* upregulation and differential metabolites associated with *Areg* upregulation.

Data and materials availability

The raw data of RNA-seq was uploaded into the NCBI under accession number SRP515357. The raw data of metagenomics was uploaded into the NCBI under accession number SRP515898. The remaining data are available within the Article or Supplementary Data file.

Declaration of competing interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100897.

Data availability

The data that has been used is confidential.

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