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# Dietary delivery of acetate to the colon using acylated starches as a carrier exerts anxiolytic effects in mice

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ABSTRACT

Recently, short-chain fatty acids (SCFA) have been shown to play an important role in mediating the gut–brain interaction and thereby participate in the patho-physiological process of stress-related disorders. In the current study, we examined whether SCFA generated in the lower gut affects host metabolic and behavioral characteristics. To determine this, we used special diets containing acylated starches that can reach the colon without being absorbed in the upper gastrointestinal tract of male mice. The delivery of SCFA to the colon using this method induced a substantial increase in acetate, butyrate, and propionate in the cecum. Moreover, the diets containing acylated starches also decreased microbial diversity in the cecum, concomitant with a significant impact on microbial composition. In marble-burying (MB) tests, the mice that consumed diets containing acetylated starches showed a decrease in anxiety-like behavior compared with the mice that consumed diets containing either butyrylated or propionylated starches. Cecal acetate contents were significantly associated with anxiety-like behaviors when evaluated by elevated plus-maze and MB tests. Collectively, these results indicate that gut acetate elevation of a dietary origin may exert anxiolytic effects on behavioral phenotypes of the host.

## Introduction

Increasing evidence has shown that gut microbes play an important role in the development and regulation of numerous host functions[1-3]. For example, gut microbiota not only has pivotal roles in the maturity of intestinal mucosal immunity [4], it also controls the central nervous system stress response[5] and other behaviors [6–13]. This concept has now been called the "microbiota-gut–brain axis" [14–17], a model indicating refined cross-talks among the microbiota, gut, and brain.

Recently, short-chain fatty acids (SCFA) are implicated to play a key role in mediating the "microbiota-gut–brain axis." Following the ingestion of indigestible carbohydrates, SCFA are produced in the colon of animals through bacterial fermentation, ultimately generating acetate, propionate, and butyrate in a ratio of 3:1:1, respectively [18,19]. In addition to such effects on host metabolism as anti-lipolysis and increased insulin, SCFA also participate in the pathology of neuropsychiatric disorders, via the modulation of glia cells such as microglia [20-22]. Moreover, acetate derived from alcohol contributes to brain histone acetylation and subsequent behavioral changes [23]. This leads to an interesting question—do peripheral sources of acetate, physiologically generated in the colon, affect brain function in a similar fashion?

In general, since oral ingestion of SCFA in foods or beverages is rapidly absorbed in the small intestine, SCFA are unable to reach the large intestine [24]. However, Annison and colleagues [25] reported that special diets containing acylated dietary starches that can be degradated by gut microbiota, not by intestinal amylolysis, induce the increased SCFA in the colon lumen. In the current study, using this dietary manipulation, we investigated the effects of SCFA generated in the lower gut, on host metabolic and behavioral characteristics.

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## Material and methods

### *Animals*

Male BALB/c mice at 5 weeks of age were obtained from KBT Oriental Co Ltd. (Saga, Japan), and were kept in the condition described previously [26]. All animal experiments were reviewed by the Ethics Committee on Animal Experiments of the Graduate School of Medical Sciences, Kyushu University (A27-212–0), and were performed under the control of the Guidelines for Animal Experiments of the Graduate School of Medical Sciences, Kyushu University, and the Law (no. 105) and Notification (no. 6) of the Japanese Government.

### *Preparation of acylated starches*

Acylated starches were produced according to the method reported by Annison and colleagues [25], with a slight modification. In brief, 30 liters of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, USA) was heated to 80 °C in an immersion heater with continuous stirring. For each acylated product, 600 g of maize starch was added to the hot DMSO. The starches were continuously stirred for 1 h to give a clear viscous solution and 110 mL of 1-methylimidazole (Sigma-Aldrich) was added as a catalyst. Anhydride was added to produce a degree of substitution of 0.20 with additions of 115, 180, and 230 mL of acetic, propionic, and butyric anhydride, respectively. After 4 h of incubation, the excess anhydride was decomposed with water; then, the acylated starches were precipitated with ethanol, washed with ethanol, air dried, and milled. The degree of substitution that was determined by release of the SCFA was 0.20, 0.16, and 0.19 for acetylated, propionylated, and butyrylated starch, respectively.

### *Study protocol*

Experimental groups of mice received special diets with acylated starches, whereas the control group received AIN-93 G diet [27]. The special diets consisted of AIN-93 G containing 15% acylated starches (acetylated, propionylated, or butyrylated)instead of 15% cornstarch. Each diet was started at 7 weeks of age and continued for 4 weeks. All mice were processed for behavioral experiments: elevated plus-maze (EPM) tests at 9 weeks of age and marble burying tests at 10 weeks of age. One week later, they were killed by cervical dislocation; then, their cecal materials were collected and frozen on dry ice. They were kept at −80 °C until both bacteriological examination and SCFA analysis, as previously reported [26].

### *Behavioral analyses*

Behavioral tests were conducted between 9:00 a.m. and 5:00 p.m. under low illumination (< 5 lx) according to our previously reported methods [3,9].

The marble burying (MB) test was performed using a transparent polycarbonate box ($L \times B \times H$ = 20.3 cm × 30.0 cm × 26.0 cm, respectively) with a 5-cm sawdust layer covering the floor [9,28]. The MB test is an animal model of anxiety-related or compulsive behavior [29-31]. Twenty clear green glass marbles (diameter = 1.5 cm) were evenly distributed over the sawdust layer. Individual mice were left for 20 min in the box. Thereafter, they were removed from the box. The number of buried marbles, defined as at least two thirds covered, was counted as an indicator of compulsive behavior.

Elevated plus-maze (EPM) tests were conducted, as described in detail elsewhere [32]. Briefly, the apparatus comprised two open (30 × 5 cm) and two closed (30 × 5 × 15 cm) arms that extended from a common central platform (5 × 5 cm). Testing was conducted in a quiet room that was illuminated only by a dim light. The number of open or closed arm entries and the time spent in the open or closed arm were recorded during the test period. Total distance during 20 min was recorded for each mouse and used as a parameter of motor activity. After each trial, the maze was wiped clean with a solution of 70% ethyl alcohol and dried with paper towels [28] before the next mouse was tested. A video camera-based computer tracking system (Limelight, Actimetrics) was used to record each test.

### *SCFA analyses by ion-exclusion high-performance liquid chromatography and 16S ribosomal ribonucleic acid (rRNA) gene sequencing analysis of cecal microbiota*

SCFA concentrations in cecal contents were measured by ion-exclusion high-performance liquid chromatography,as previously described [33].

Deoxyribonucleic acid (DNA) were extracted from the cecal samples by a commercial extraction kit (QuickGene DNA tissue kit; KURABO, Osaka, Japan) [34,35], and were processed for 16S rRNA gene sequencing analysis according to the previously reported method [36]. In brief, after deoxyribonucleic acid (DNA) extraction from the cecal samples by a commercial extraction kit (QuickGene DNA tissue kit; KURABO, Osaka, Japan), the V3–V4 region of the bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) with a TaKaRa Ex Taq 13 HS kit (TaKaRa Bio, Shiga, Japan) and a primer set of Tru357F 14 (5′-CGCTCTTCCGATCTCTGTACGGRAGGCAGCAG-3′) and Tru806R 15 (5′-CGCTCTTCCGATCTGACGGACTACHVGGGTWTCTAAT-3′). The DNA was amplified using the following protocol: 94 °C for 30 s; and 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 5 min. Then, the amplified DNA was verified based on PCR product size using the QIAxcel system (Qiagen, Valencia, CA, USA). Thereafter, PCR products were amplified with a second primer set, which was adapted for the Illumina MiSeq (Illumina, San Diego, CA, USA)) by the following protocol: 94 °C for 30 s; and 8 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 5 min. Subsequently, equal amounts of amplicons from different samples were pooled primer-dimer were removed by gel-extraction with the QIAquick PCR Purification Kit. The pooled libraries were sequenced by Illumina MiSeq with a MiSeq v3 Reagent kit 3 (Illumina, San Diego, CA, USA) in Morinaga Milk Industry Co. Ltd. The obtained sequences were filtered by the bowtie −2 program (ver 2–2.2.4) to remove the reads mapped on PhiX174 sequence. Thereafter, the 3′ region of each read with a PHRED quality score of less than 17 was trimmed. Trimmed reads less than 150 bp in length with an average quality score of less than 25 or those lacking paired reads were also removed. The paired-end reads that passed the above-mentioned quality filters were combined by the fastq-join script in EA-Utils (ver. 1.1.2–537). The sequences were analyzed using the Quantitative Insights into Microbial Ecology (QIIME) software package version 1.8.0. The sequences were assigned to operational taxonomic units (OTUs) by open-reference OTU picking with a 97% pairwise identity threshold and the Greengenes reference database

### *Statistical analysis*

All continuous data are expressed as means ± standard deviations. All analyses of animal experiments were performed using the JMP PRO v.14.2.0 software package for Windows (SAS Institute, Japan).

Changes in cecal SCFA contents were evaluated by Dunnett's post-hoc test after one-way analyses of variance (ANOVAs). Statistical differences in the number of buried marbles between the groups that ingested diets with or without acylated starches were evaluated using the Steel-Dwass test for multiple comparisons. In the EPM test, we used the number of open- or closed-arms entries as a parameter of anxiety-like behavior. Normality of this parameter was verified using the Shapiro Wilk normality test; therefore, the association between cecal SCFA levels and this factor was evaluated using the Pearson's correlation coefficients. In contrast, in the MB test, the association between them was assessed by the Speaman's rank correlation coefficients.

QIIME [37,38] pipeline 1.8 (www.qiime.org) was used to generate

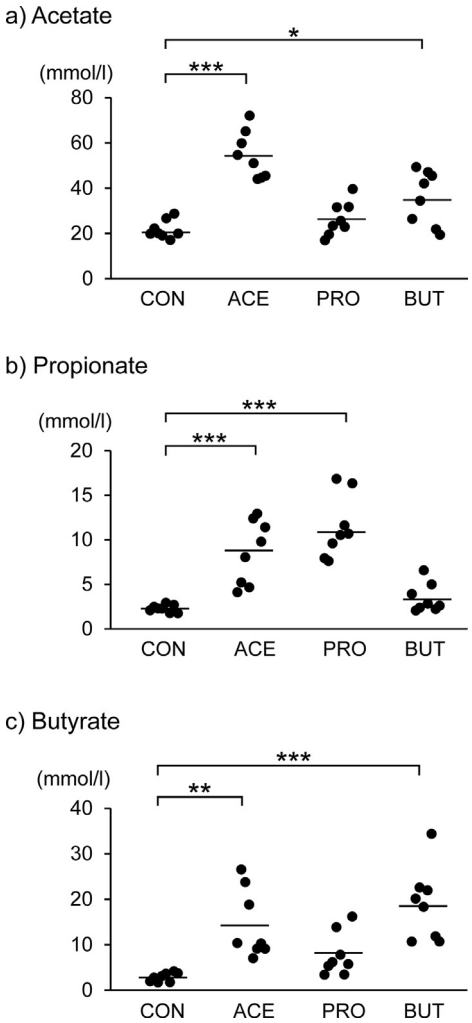


**Fig. 1. Short-chain fatty acids contents after acylated starches.** (**a**) Cecal acetate levels were measured, as described in the methods. Acetate concentrations in the mice ($n$ = 8 per each group) receiving control diet, acetylated, butyrylated, and propionylated starches were 21.7 ± 4.0, 54.6 ± 10.3, 35.7 ± 11.9, and 26.4 ± 7.4, respectively. (**b**) Cecal butyrate concentrations in the mice receiving control diet, acetylated, butyrylated, and propionylated starches were 2.9 ± 1.0, 14.4 ± 7.5, 18.8 ± 8.0, and 7.7 ± 4.8, respectively. (**C**) Cecal propionate concentrations in the mice receiving control diet, acetylated, butyrylated, and propionylated starches were 2.3 ± 0.4, 8.6 ± 3.6, 3.5 ± 1.6, and 11.4 ± 3.5, respectively. ***$p$< .001, **$p$< .01, and *$p$< .05 were considered significantly different from the corresponding control values when evaluated using Dunnett's test. CON, control; ACE, acetate; PRO, propionate; BUT, butyrate.

relative abundance plots and calculate α-diversity metrics (Shannon index, Chao 1, and observed species) and β-diversity parameters of both weighted and unweighted UniFrac metrics. Principal coordinate analysis (PCoA) plots were generated by the unweighted UniFrac method and visualized as a 3D graph using QIIME.Permutational multivariate ANOVA (PERMANOVAs) [39,40] were also conducted to evaluate differences in bacterial composition between the control mice and acetylated-fed mice. They were performed using the Adonis function in the vegan package in R. Differences in relative abundances of genus between all the groups were evaluated using Kruskal-Wallis test followed by Bonferroni correction for multiple testing.

**Table 1**
Cecal SCFAs levels after being fed an acylated diet.

| (mmol/L) | Control | A group | P group | B group |
|---|---|---|---|---|
| PRO | 2.3 ± 0.1 | 7.9 ± 1.2*** | 11.4 ± 1.2*** | 3.5 ± 0.6 |
| BUT | 2.8 ± 0.3 | 15.1 ± 2.9** | 7.7 ± 1.6 | 18.3 ± 3.2*** |
| ACE | 21.6 ± 1.4 | 56.1 ± 3.8*** | 26.3 ± 2.6 | 34.1 ± 4.4* |
| Total SCFA | 26.8 ± 1.7 | 79.1 ± 5.5*** | 45.5 ± 5.2* | 56.0 ± 7.5*** |

Cecal contents from the mice fed control, acetylated (A), propriorylated (P), or butyrylated (B) diets were collected and then their SCFA levels were measured, as described in the methods. SCFA, PRO, BUT, and ACE indicate short-chain fatty acid, propionate, butyrate, and acetate, respectively.
* $P$< .05.
** $P$< .01.
*** $P$< .001.

## Results

### *Successful delivery of SCFA to the gut using acylated starches as a carrier*

We first assessed if specific diets with acylated starches can actually raise SCFA contents in the cecum. As shown in Fig. 1a, dietary treatment with acetylated starches and, to a lesser extent, butyrylated starches, induced a significant increase in cecal acetate levels. Cecal butyrate concentrations were also significantly increased by the ingestion of either butyrylated or acetylated starches (Fig. 1b). Cecal propionate contents were elevated after the diets with not only propionylated but also acetylated starches (Fig. 1c). As summarized in Table 1, total amounts of cecal SCFA were higher in the mice supplemented with either of acylated starches than in the control mice.

### *Acylated diets did not affect body weight gain*

None of the acylated-fed groups showed any significant difference in body weight gain compared with the control group during the observation period (supplementary Fig. 1). Similarly, acetate, propionate, or butyrate concentrations in the cecum did not exhibit any correlation with an increase in body weight; i.e., the difference between the basal and 10-week body weight (data not shown).

### *Diets including acylated starches reduce bacterial diversity*

As shown in Fig. 2a. the rarefaction curves based on Chao 1 index showed decreased diversity in acylated-fed groups compared with the control group. These results were also confirmed by the Shannon index and observed species (Fig. 2b and c). Similarly, PCoA using the unweighted UniFrac distances for each group of mice (Fig. 2d) exhibited a significantly different profile between the control group and the other three groups. This was confirmed by PERMANOVAs that showed a significant difference between the four groups ($f$ = 5.1117, $p$ = .00099).

Regarding relative abundance of microbial composition at genus levels, the Kruskal-Wallistest with Bonferroni's correction exhibited a significant difference in 11 genera between the four groups (Table 2). Among the 11 genera, only genus *Bacteroides* showed a higher relative percentages in the acylated-fed groups than in the control group. In contrast, the relative percentage of the remaining 10 genera were lower in the acylated-fed groups than in the control group; especially, the relative percentage of the unidentified genus that is included in S24-7 family and the most abundant in the control group, exhibited a significant decrease in the acetylated or butyrylated group. The taxonomical distribution at genus levels in all groups is summarized in Supplementary Table 1.

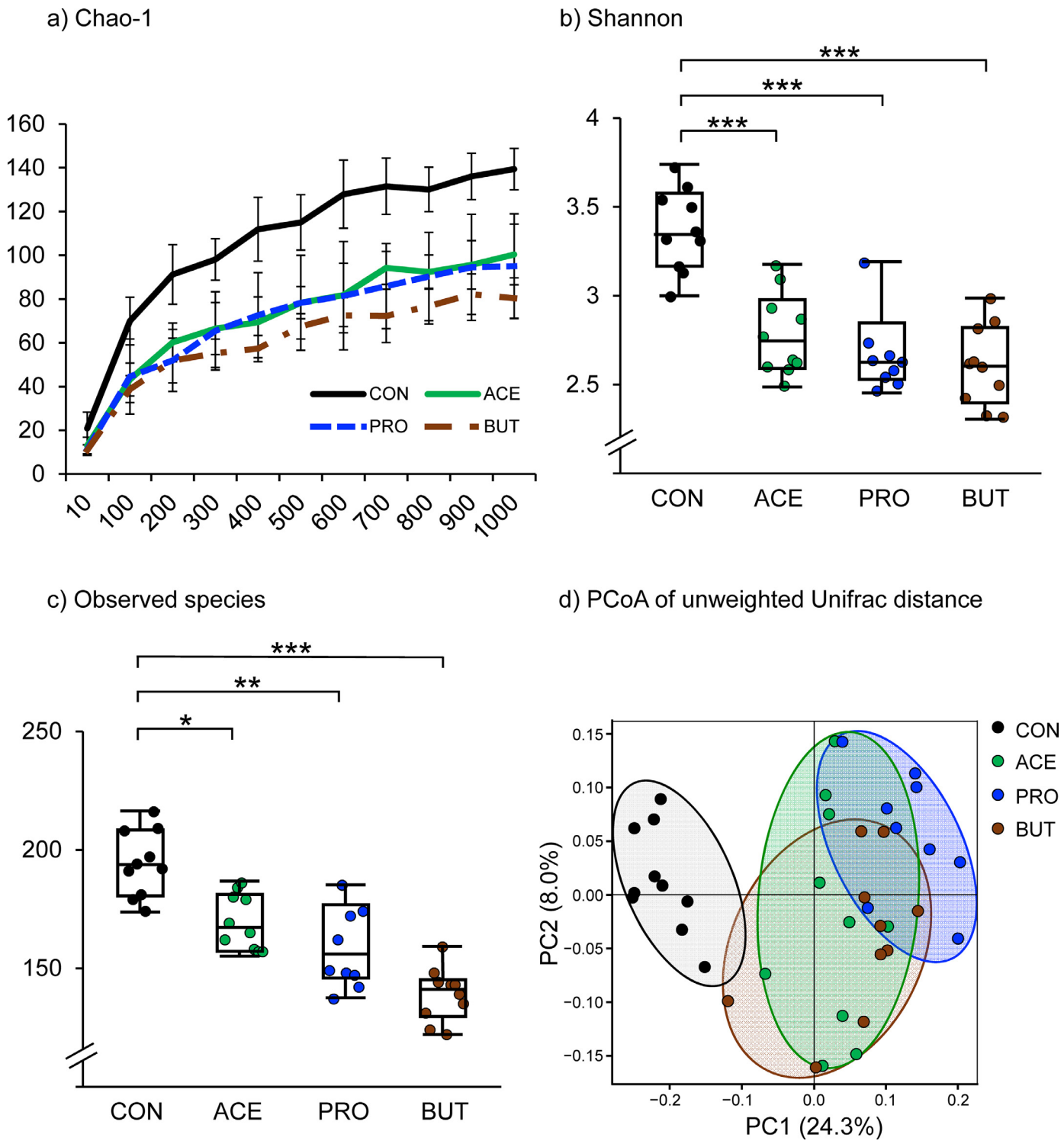


**Fig. 2. Effects of acylated short-chain fatty acids on cecal microbiota. (a)** Chao-1, **(b)** Shannon, and **(c)** observed species values, from either acetylated-fed mice or mice fed a control diet ($n$ = 10 per group), are shown as a parameter of α-diversity. Black, green, blue, and brown lines indicate control, acetylated, propionylated, and butyrlated, respectively. Shannon and observed species data for each group were visualized as box plots with medians (middle lines), first and third quartiles (box boundaries), and minimum and maximum from the box boundaries (whiskers). **(d)** Principal coordinate analysis is shown using the unweighted UniFrac distances for each group of mice ($n$ = 10 per group). Each colored ellipse covers 95% of the samples belonging to a cluster. ***$p$ < .001, **$p$ < .01, and *$p$ < .05 were considered significantly different from the corresponding control values when evaluated using Dunnett's test. CON, control; ACE, acetate; PRO, propionate; BUT, butyrate.

*Cecal acetate levels negatively associate with anxiety-like behaviors*

We next examined the effects of cecal SCFA levels on host behaviors in the groups consuming diets with or without acylated diets. As shown in Fig. 3, in the MB test, the number of buried marbles in the acetylated-supplemented group was lower than that in either the butyrylated- ($Z$ = 2.58, $p$ = .048) or propionylated-supplemented group ($Z$ = 2.96, $p$ = .016). There was no significant difference in the total distance during 20 min between the four groups (control 832 ± 256 cm/20 min, acetylated 803 ± 294 cm/20 min, butyrylated

**Table 2**
Taxonomical distribution at bacterial genus levels with or without acylated diets.

| | CON | ACE | BUT | PRO | KW |
|---|---|---|---|---|---|
| *k_Bacteria;p_Bacteroidetes;c_Bacteroidia; o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides* | 9.52 ± 3.51 | 20.19 ± 6.77* | 27.45 ± 11.14* | 25.64 ± 8.15* | 0.0001 |
| *k_Bacteria;p_Bacteroidetes;c_Bacteroidia; o_Bacteroidales;f_S24-7;g_* | 22.2 ± 7.43 | 5.21 ± 5.19* | 5 ± 8.17* | 14.31 ± 9.14 | 0.0002 |
| *k_Bacteria;p_Deferribacteres;c_Deferribacteres; o_Deferribacterales;f_Deferribacteraceae;g_Mucispirillum* | 1.77 ± 1.65 | 0.42 ± 0.61 | 0.05 ± 0.04* | 0.15 ± 0.12* | 0.0004 |
| *k_Bacteria;p_Firmicutes;c_Clostridia; o_Clostridiales;f_Ruminococcaceae;g_Oscillospira* | 5.2 ± 1.57 | 1.51 ± 0.86* | 1.14 ± 1.28* | 1.42 ± 1.93* | 0.0002 |
| *k_Bacteria;p_Firmicutes;c_Clostridia; o_Clostridiales;f_Ruminococcaceae;g_Butyricicoccus* | 0.21 ± 0.13 | 0.02 ± 0.01* | 0.04 ± 0.11* | 0.08 ± 0.13 | 0.0002 |
| *k_Bacteria;p_Firmicutes;c_Clostridia; o_Clostridiales;f_Dehalobacteriaceae;g_Dehalobacterium* | 0.28 ± 0.1 | 0.09 ± 0.08* | 0.05 ± 0.06* | 0.1 ± 0.11* | 0.0004 |
| *k_Bacteria;p_Firmicutes;c_Clostridia; o_Clostridiales;f_Lachnospiraceae;g_[Ruminococcus]* | 2.61 ± 0.99 | 0.38 ± 0.38* | 0.24 ± 0.16* | 0.36 ± 0.27* | < 0.0001 |
| *k_Bacteria;p_Firmicutes;c_Clostridia; o_Clostridiales;f_Ruminococcaceae;g_Anaerotruncus* | 1.71 ± 0.51 | 0.37 ± 0.67* | 0.1 ± 0.06* | 0.13 ± 0.09* | < 0.0001 |
| *k_Bacteria;p_Firmicutes;c_Clostridia; o_Clostridiales;f_Ruminococcaceae;Other* | 0.77 ± 0.13 | 1.56 ± 3.02 | 0.06 ± 0.05* | 0.11 ± 0.16* | < 0.0001 |
| *k_Bacteria;p_Firmicutes;c_Clostridia; o_Clostridiales;f_Ruminococcaceae;g_Clostridium* | 0.22 ± 0.12 | 0.06 ± 0.07* | 0.01 ± 0.01* | 0.03 ± 0.02* | < 0.0001 |
| *k_Bacteria;p_Firmicutes;c_Clostridia; o_Clostridiales;f_Peptococcaceae;g_* | 0.11 ± 0.06 | 0.01 ± 0.01* | 0 ± 0 | 0.01 ± 0.01* | < 0.0001 |

CON, ACE, BUT, PRO, and KW indicate control, propionate, butyrate, acetate, and Kruskal-Wallis test, respectively. Differences in bacterial distribution at genus level between the four groups were evaluated using the KW followed by the Bonferroni's correction. The total number of identified genera was 57 in this analysis; therefore, p value less than 0.000877 (0.05/57) was considered significant.

* $P < .05$ was significantly different from each control value within a genus when evaluated by Steel test.

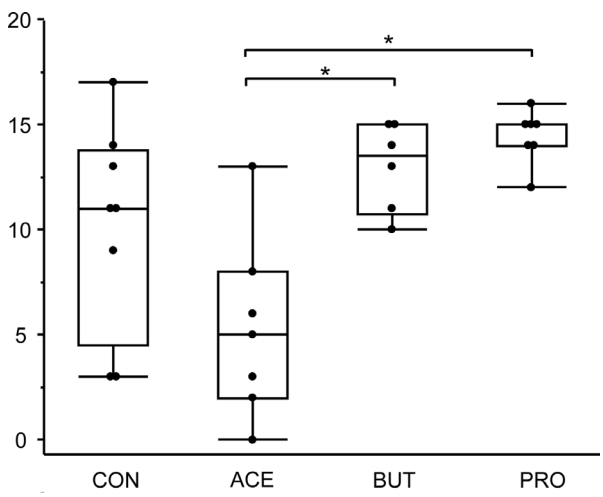


**Fig. 3. Effects of dietary starches on marble burying behavior.** Marble burying behaviors in the mice ($n$ = 6–8 per each group) receiving control (CON) diet, acetylated (ACE), butyrylated (BUT), and propionylated (PRO) starches were evaluated as described in the methods. The vertical axis indicates the number of buried marbles. Each data is shown as box plots with medians (middle lines), first and third quartiles (box boundaries), and minimum and maximum from the box boundaries (whiskers). *$p < .05$ indicates a significant difference between the indicated two groups when evaluated using the Steel-Dwass test.

808 ± 174 cm/20 min, propionylated 940 ± 178 cm/20 min). In correlation analysis between cecal SCFA and behavioral parameters, acetate levels in the cecal lumen were negatively correlated with the number of buried marbles mice (Fig. 4a, Spearman's $\rho = -0.4431$, $p = .0206$). Moreover, in the EPM test, cecal acetate contents were also significantly associated with the open-arm entry number (Fig. 4b, Pearson's $r = 0.3784$, $p = .0471$). In contrast, butyrate and propionate concentrations failed to show a significant association with any anxiety-like behavioral parameters (data not shown).

## Discussion

Targeted delivery of SCFA to the colon using a carrier of starch induced a substantial increase in acetate, butyrate, and propionate in the gut lumen. Supplementation with the diets containing acylated starches decreased microbial diversity in the cecum, concomitant with a significant change in microbial composition. The mice that consumed the diets containing acetylated starches showed reduced number of buried marbles compared with the mice that consumed the diets containing either butyrylated- or propionylated-starches. Cecal acetate contents were negatively associated with anxiety-like behaviors when evaluated by EPM and MB tests. Collectively, these results indicate that elevated acetate in the gut lumen may exert anxiolytic effects on host behavioral phenotypes.

The diets with acetylated starches resulted in a significant amount of acetate and lesser amounts of butyrate and propionate. Boets and coworkers [41] reported that SCFA were interconverted by gut microbes, and that conversion of acetate into butyrate was the most prevalent interconversion by microorganisms. Therefore, these findings indicate that such elevated butyrate and propionate levels after the ingestion of acetylated diets may result from the conversion of acetate into butyrate and propionate.

Cecal acetate levels exerted subsiding effects on anxiety-related behaviors. Consistently, Burokas and colleagues [21] reported that chronic prebiotic treatment increased cecal acetate and propionate concentrations and that such increased acetate levels were negatively associated with host anxiety-like behaviors. Several papers demonstrated that acetate can reduce microglia activation and inflammatory signaling *in vivo*[42] and *in vitro*[43]. Moreover, either acetate supplementation [44] or acetate produced by alcohol [23] was also demonstrated to be involved in brain histone acetylation, thus affecting subsequent gene regulation related to behaviors. This report indicates that alcohol-derived acetyl groups can contribute to ethanol-induced behavioral adaptations. Concurrently, these findings suggest that other sources of acetate, such as those derived from diets or gut microorganisms, may also affect brain function via several mechanisms such as epigenetic modification.

A large number of literatures demonstrate that gut microbiome-

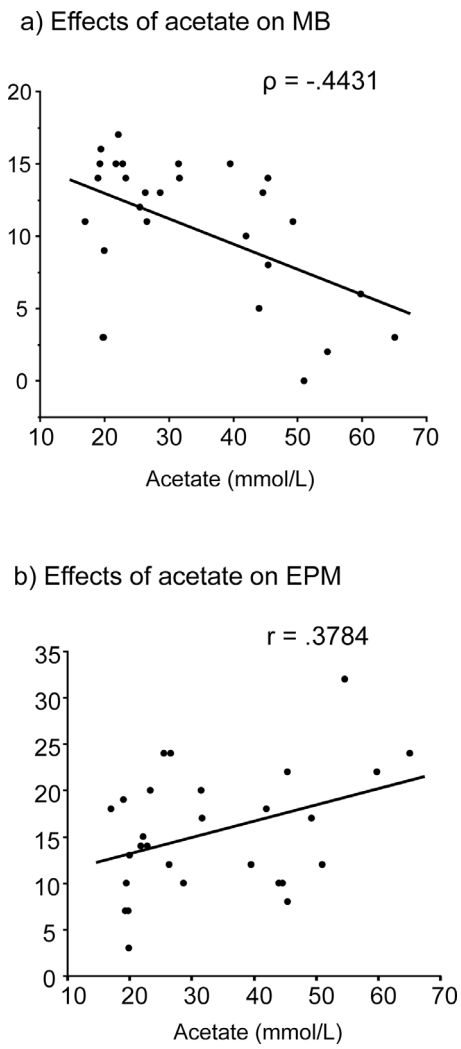


**Fig. 4. Correlation between cecal acetate levels and anxiety-related behaviors.** (a) In the marble burying test (MB), the relationship between acetate concentrations in the cecum and the number of buried marbles in all four groups of mice was evaluated using the Speaman's rank correlation coefficients. The vertical axis indicates the number of buried marbles. (b) In the elevated plus maze test (EPM), the correlation between open-arm entry number and acetate levels in the cecum was evaluated using the Pearson's correlation coefficient. The vertical axis shows the number of open-arm entries.

derived SCFA not only exert multiple beneficial effects on the host's energy metabolism but also have a substantial impact on peripheral immune function via promoting a differentiation of Treg cells [17,45-47]. Moreover, gut microbes also influence the immune system of the central nervous system by regulating microglial cell activation and homeostasis [20,48]. In fact, Erny and co-workers [20] demonstrated that gut microbiota influence the CNS immune system by regulating microglial cell activation and homeostasis. They have shown that SCFA treatment could change microglial cells of germfree mice to mature phenotype in terms of morphology and functionality. In addition to such effects on immune function, vagal and spinal afferent nerves are also involved in the interaction between the microbes-derived SCFA and the brain [5,15,49]. For example, propionate is reported to activate brain-gut neural pathway via acting on FFAR receptor in the portal nerves [50]. The importance of gut-originated SCFA in behavioral and neurological pathologies is further suggested in animal models such as Alzheimer diseases [51], Parkinson diseases [52] and autism spectrum disorder [53].

Microbial diversities, when evaluated using the Chao-1, Shannon index, and observed species, were decreased in groups fed SCFA-acylated diets as compared with the control group. In addition, at the gene levels, several bacteria also showed a significant difference between the groups with or without acylated SCFA. Many factors in the gut environment influence indigenous microbiota [54]; for example, gut pH [55,56], bile acids [57,58], gut motility [59], mucosal immunity [60], and so on. Many of the host's dietary nutrients, such as oligosaccharides and simple sugars, impact pH as they promote acid production via fermentation [61,62]. Therefore, it is likely that SCFA may render colonic luminal pH lower and substantially affect the composition of gut microbes. In fact, in a paper using acylated starches [25], pH in the cecum was lower in SCFA-groups than in the control group. The relative abundance of S24-7, an uncultured family of *Bacteroidetes* phylum, exhibited a significant decrease in the acylated diet groups than in the control group. The S24-7 family is highly detected in the digestive tracts of mammals and is recognized as an abundant component of gut microbiota [63,64]; however, available information is still limited about its functional characteristics.The present results suggest that this family may be highly sensitive to acidic pH. On the contrary, the relative abundance of some *Bacteroides* species were higher in the acylated diet groups than in the control group. In general, *Bacteroides* species grow poorly at low pH [65]; contrastingly, *Bacteroides uniformis* is resistant to mild acidic pH [54]. Therefore, these findings suggest that some *Bacteroides* species,like *Bacteroides uniformis*, can grow even at low pH. Alternatively, SCFA are known to modulate gastrointestinal motility [66], which may consequently affect the composition of gut microorganisms. It is also possible that gut microbes may derive energy from converting metabolized SCFA, i.e. cross-feeding, and in turn, end up more abundant in response to supplementing SCFA [67]. Further researches are still needed to clarify the mechanism whereby SCFA can change microbial profiles in the gut.

As an alternate method to deliver SCFA to the colon, inulin-propionate ester is reported to induce a sufficient amount of propionate in the colon. In fact, in some clinical studies [68,69], it was demonstrated that propionate chemically bound by an ester bond to inulin successfully reached the colon, and then propionate was locally released from its chemical compound. Therefore, not only acylated starches used in the current experiment, but also chemicals containing inulin as a carrier are considered to be a useful tool when delivering a large amount of SCFA to the colon.

This study had some limitations. First, the number of animals used in the experiment was relatively small. Moreover, the current experiment was conducted using male mice only. Therefore, a new project is now in progress to confirm the current results using a larger number of both sexes of mice. Second, behavioral phenotypes and their association with SCFA were not systematically investigated in the current study; therefore, in future studies, more comprehensive evaluation using updated analytical approaches is needed to elucidate overall pictures of gut-derived SCFA-induced behavioral modulation. Finally, the precise mechanisms whereby SCFA derived from the gut affects anxiety-like behaviors were not addressed in this study; therefore, it is critically important to clarify this in future studies.

In conclusion, this study showed that increased acetate in the gut may exert anti-anxiety effects on hosts' behavioral characteristics. These results may ultimately contribute to a novel therapeutic option for treating anxiety-related disorders.

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## Declaration of Competing Interest

The authors declare no conflicts of interest associated with this manuscript.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2020.113004.

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