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Positive effect on cognitive ability of Lactobacillus acidophilus EG004 in healthy mouse and fecal microbiome analysis using full-length 16S-23S rRNA metagenome sequencing

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Prof. Heebal Kim
Seoul National University
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Re: Spectrum01815-21 (Positive effect on cognitive ability of Lactobacillus acidophilus EG004 in healthy mouse and fecal microbiome analysis using full-length 16S-23S rRNA metagenome sequencing)

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Reviewer comments:

Reviewer #3 (Public repository details (Required)):

The metagenome sequencing data (16S-23S rRNA) should be submitted to GenBank if it is not submitted yet.

Reviewer #3 (Comments for the Author):

Manuscript: Spectrum01815-21 Positive effect on cognitive ability of Lactobacillus acidophilus EG004 in healthy mouse and fecal microbiome analysis using full-length 16S-23S rRNA metagenome sequencing

The aim of the paper is to study effect on cognitive ability of Lactobacillus acidophilus EG004 in healthy mouse and fecal microbiome analysis using full-length 16S-23S rRNA metagenome sequencing.
In the manuscript, the authors studied a bacterial strain Lactobacillus acidophilus EG004 with a positive effect on cognitive ability using a healthy animal model. The authors experimentally verified improved cognitive ability by cognitive behavioral tests. The authors performed full 16S-23S rRNA sequencing and provided gut microbiome composition at a species level. The provided microbiome composition consisted of candidate microbial groups as a biomarker that shows positive effects on cognitive ability. Therefore, their study suggests a new perspective for probiotic strain use applicable for medicine.
The uniqueness of the text is 90% by AntyPlagiarism.net.
The manuscript is written well. English is proper, well understandable.

Reviewer has some comments:

Line 74 - most of researches were - should be - most of the researches was.
Line 73 - many researches - should be - many pieces of research.
Line 82 - industrialization process - should be - industrialization processes.
Line 105 - for the sentence - Autism, Alzheimer's disease, and Parkinson's disease (7-9) - add additional citation (Danilenko et al., 2021) and add to the References - Danilenko, V.N., Devyatkin, A.V., Marsova, M.V., Shibilova, M.U., Ilyasov, R.A., Shmyrev, V.I., 2021b. Common inflammatory mechanisms in COVID-19 and Parkinson's diseases: the role of microbiome and probiotics in their prevention. Journal of Inflammation Research 14, (In press). doi: 10.2147/JIR.S333887.
Line 108 -to the sentence - the neural pathways of the brain-gut axis (10). - add additional citation (Fetissov et al., 2019). and add to the References - Fetissov, S.O., Averina, O.V., Danilenko, V.N., 2019. Neuropeptides in the microbiota-brain axis and feeding behavior in autism spectrum disorder. Nutrition 61, 43-48. doi: 10.1016/j.nut.2018.10.030.
Line 112 - Second, the second suggestion - should be - Second, the suggestion
Line 113 - microbiome affect brain - should be - microbiome affects brain.
Line 113 - metabolic pathway - should be - metabolic pathways.
Line 127 - remove one dot.
Line 153 - The averages daily - should be - The averages of daily.
Line 168 - In the comparison of - should be - The comparison of.
Line 194 - light room - should be - lightroom.
Line 195 - remove italics of the word - group.
Line 226 - comparison - should be - comparative.
Line 236 - familiae - should be - families.
Line 275 - whole genome - should be - whole-genome.
Line 308 - recognition test and passive avoidance task - should be - recognition tests and passive avoidance tasks.
Line 321 - were - should be - was.
Line 343 - factor - should be - factors.
Line 350 - purpose - should be - purposes.
Line 370 - these evidences - should be - this evidence.
Line 398 - negative effect - should be - negative effects.
Line 408 - to provide - should be - provide.
Line 413 - These analyses were not covered to identification of a biological factor caused - should be - These analyses were not covered in the identification of a biological factor that caused.
Line 416 - probiotics ingestion - should be - probiotic ingestion.
Line 444 - by - should be - at.
Line 442 - with - should be - at.
Line 453 - add space after dot.
Line 457 - from probiotics intake - should be - after probiotic intake.
Line 459 - room condition - should be - room conditions.
Line 472 - rodent's habit - should be - rodents' habits.
Line 478 - entered - should be - that entered.
Line 486 - preference - should be - preferences.
Line 516 - After 1 minute for adaptation - should be - After 1 minute of adaptation.
Line 531 - time taken - should be - time is taken.
Line 554 - correction - should be - corrections.
Line 789 - statistic - should be - statistics.
The metagenome sequencing data (16S-23S rRNA) should be submitted to GenBank.
Please check English by professional translator one more times.
In further authors should study details of biological factors and molecular mechanisms that caused improved cognitive ability in mice after treatment with L. acidophilus EG004 strain.

No other comments.
A minor revision is required.

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Running title

Positive effect on cognitive ability of *Lactobacillus acidophilus* EG004

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Abstract

The concept of the ‘Gut-brain axis’ has risen. Many types of research demonstrated the effect and mechanism of the GBA. Although many studies have been reported, most of the studies are focused on neurodegenerative disease and it is still not clear what type of bacterial strains have positive effects on the brain. Therefore, we designed an experiment to discover a strain that positively affects cognitive ability using healthy mice. The experimental group consisted of a control group and three probiotic consumption groups, *Lactobacillus acidophilus*, *Lacticaseibacillus paracasei*, and *Lacticaseibacillus rhamnosus*, which are verified to have beneficial effects for host health as the gut microbiome. Cognitive ability was measured by 4 cognitive-behavioral tests and the group fed on *L. acidophilus* showed the most improved cognitive ability. To provide an understanding of the altered microbial composition effect on the brain, we performed full 16S-23S rRNA sequencing using Nanopore, and OTUs were identified at a species level. In the group fed on *L. acidophilus*, the intestinal bacterial ratio of Firmicutes and Proteobacteria phyla increased and the bacterial proportions of 16 species were significantly different from those of the control group. We estimated that the positive results on the cognitive behavioral tests were due to the increased proportion of *L. acidophilus* EG004 strain in the subjects’ intestines since the strain is capable of producing butyrate and therefore modulating neurotransmitters and neurotrophic factors. We expect that our new strain expands the industrial field of *L. acidophilus* and helps understand the mechanism of the brain-gut axis.

Importance

In recent, the concept of 'gut-brain axis' has risen that microbes in the GI tract affect brain by

modulating signal molecules. Although many researches were reported in a short period, a signaling mechanism and effect of a specific bacterial strain are still unclear. Besides, since most of researches were focused on neurodegenerative disease, the study with a healthy animal model is still insufficient. In this study, we provide a bacterial strain (*Lactobacillus acidophilus* EG004) with a positive effect on cognitive ability using a healthy animal model. We experimentally verified improved cognitive ability by cognitive behavioral tests. We performed full 16S-23S rRNA sequencing using nanopore MinION, and provided gut microbiome composition at a species level. The provided microbiome composition consisted of candidate microbial groups as a biomarker that shows positive effects on cognitive ability. Therefore, our study suggests a new perspective for probiotic strain use applicable for various industrialization process.

Keywords

Lactobacillus acidophilus, gut microbiome, gut-brain axis, cognitive ability, Nanopore sequencing

Introduction

The human body is a complex community that habituates various bacteria. Among the bacterial communities in the human body, the gastrointestinal tract is the best bacterial community that has the most abundant and various bacteria (1). In 2006, having been released research that obesity is associated with bacterial composition in the gut, a study for gut microbiome began in earnest (2). The gut microbiome is defined as the collective genomes of microorganisms that live in the gastrointestinal tract. Functions of the gut microbiome have been reported such as nutrient metabolism and regulation of the immune system for the host (3). Microbial composition in the gut is altered by environmental factors like age, diet, stress, and lifestyle, and the change in microbial composition can induce physical changes in the host (4). In recent, the gut microbiome's effects on the brain have been proved and the concept of the brain-gut axis has risen to the surface (5). The brain-gut axis is a complex system involving the enteric nervous system and central nervous system including the brain and spinal cord, and it works with bidirectional communication between the central and the enteric nervous system (6). Although the brain is located apart from the gut, the gut microbiome can affect the brain by stimulating the enteric nervous system and vagus nerve. Thus, dysbiosis of the gut microbiome often causes brain diseases. The recent experimental results described that gut microbiome dysbiosis was observed in patients with Autism, Alzheimer's disease, and Parkinson's disease (7-9). At the same time, studies on the mechanisms to understand the brain-gut axis have been conducted. First, it was suggested that the microbial-derived metabolites are the main components acting on the neural pathways of the brain-gut axis (10). The most well-studied substances are short-chain fatty acids (SCFA) such as acetate, propionate, and butyrate, which are produced in the process of decomposing non-digestible fibers and carbohydrates (11). It promotes indirect signaling to

the brain by modulation and induction of neurotransmitter and neurotrophic factors like γ-aminobutyric acid (GABA) and Brain-derived neurotrophic factor (BNDF). Second, the second suggestion was that the gut microbiome affect brain function by regulating metabolic pathway (12). Previous research reported that the level of tryptophan metabolites including serotonin and indolepyruvate was altered by the gut microbiome. These metabolites have roles in the functioning of the gut-brain axis such as signaling and anti-oxidant. Third, the gut microbiome may affect the brain by immune pathway (13). Interferon (IFN), Tumor necrosis factor (TNF), and Interleukin are well-known immune factors. According to recent studies, the amount of the immune factors is regulated by the intestinal microflora. These immune factors affect brain function by stimulating and activating the hypothalamic-pituitary-adrenal axis. Finally, it was suggested that gut microbes directly influence the brain by altering the fatty acid composition of the brain (14). Several studies have been reported on the correlation between intestinal microbes and the brain, but the specific mechanism of the brain-gut axis is still not clear.

Probiotics are defined as bacteria that have positive effects on the host body (15). Probiotics have been widely used as a health supplement since it has various beneficial functions to host's health with high adhesion property to the intestine and low side effect. Most probiotics include bacteria genera that are gram-positive, facultative anaerobic and rod-shaped.. *Lacticaseibacillus rhamnosus* (*Lcb. rhamnosus*) is one of the longest-studied probiotic species, and many strains such as LGG and GR-1 belonging to this genus are commercially available. It is well known that *Lcb. rhamnosus* has positive effects on diarrhea, acute gastroenteritis, and atopic dermatitis (16-18). Recently, its neurobehavioral effects such as anxiety and depression relief have been reported (19). *Lacticaseibacillus paracasei* (*Lcb. paracasei*) is one of the representative probiotic species, and it has been studied to be effective in treating ulcerative colitis and allergic rhinitis

(20, 21). In a recent study, an effect on age-related cognitive decline and a stress relief effect was reported with several strains of this species (22). *Lactobacillus acidophilus* (*L. acidophilus*) is another representative probiotic strain. This strain lowers cholesterol levels and has beneficial health effects such as antibacterial effects against harmful bacteria like *Streptococcus mutans* and *Salmonella typhimurium* (23, 24).

In this study, we aimed to present a new strain that has an enhancing effect on cognitive ability through the brain-gut axis and provide an additional understanding of the brain-gut axis. Three probiotic strains, *L. acidophilus*, *Lcb. paracasei*, and *Lcb. rhamnosus*, which have previously demonstrated beneficial effects to the host as one of the gut-microbiome strains, were used to confirm their positive effects on cognitive ability. Full 16S and 23S rRNA sequencing was performed to annotate the gut microbiome at a species level for downstream analysis. We expect our results to provide an understanding of the role of the gut microbiome.

Results

Bacterial and animal treatments

Three probiotic strains, *L. acidophilus* EG004, *Lcb. paracasei* EG005, and *Lcb. rhamnosus* EG006, have been identified by the molecular method. These strains were clustered with available *L. acidophilus*, *Lcb. paracasei*, and *Lcb. rhamnosus* strains, respectively, in a phylogenetic tree of 16S rRNA gene (Figure S1). Probiotic strains were consumed by mice for 8 weeks with assessments of cognitive ability (Figure 1). The averages daily water intake per subject were similar between groups (Figure 2A). Daily probiotic intakes were maintained constantly and the average amount of *L. acidophilus* group, *Lcb. paracasei* group, and *Lcb. rhamnosus* group were calculated as (7.82E09 ± 1.95E09), (4.37E10±5.17E09), and (3.74E10±3.98E09) CFUs (Figure 2B). To identify the additional effect of probiotics, the body weights of mice were measured every week (Figure 2C and S2). Patterns of weight gain in the 4 groups were similar for 8 weeks. The mean body weight gains of the control group showed the highest value, which was 9.08 g. *Lcb. paracasei* group showed a significant difference from the control group with P-value under 0.05 in the second measurement, but the difference was immediately recovered. Similar to weekly weight change, statistical significance was not found in accumulated weight between experimental groups for 8 weeks.

Cognitive behavioral tests

Spontaneous alternation test was conducted to assess spatial learning and short-term memory. Although the average number of the total entries to each arm in *Lcb. paracasei* group

was slightly low, the difference between groups was not found (Figure 3A). In the comparison of the mouse ratio showed spontaneous alternation for the first 3 entries, *L. acidophilus* group showed the highest value as 75.0%. (Table S1). In spontaneous alternation, the average values of probiotics-fed groups were higher than the value of the vehicle-fed group (Figure 3B). Among the 4 experimental groups, *L. acidophilus* group showed the highest alternation ratio. Wilcoxon rank-sum test was performed to identify statistical significance, but there was no statistical difference between the experimental groups and control group.

Novel object recognition (NOR) test was performed to evaluate long-term and explicit memory using 4 different features (Figure 3C, 3D, and Table S1). *L. acidophilus* group exhibited the highest average ratio of mouse that touched the novel object before the familiar object, whereas *Lcb. rhamnosus* group showed the lowest value under the control group. At discrimination ratio comparison, the three probiotics-fed groups showed higher average values than the control, and *L. acidophilus* group showed the highest values. To identify if there is a significant difference, Wilcoxon rank-sum test was performed. When compared to the vehicle-fed group, *L. acidophilus* and *Lcb. Paracasei* groups displayed statistically significant differences with the adjusted P-value of 0.037. To identify animal behavior detail, the number of objects touch and the total time of object observation in each group were compared. In a comparison of object touch, statistical differences were significant in *L. acidophilus* and *Lcb. Paracasei* groups with P-values of 0.031 and 0.042, respectively. Also, *L. acidophilus* group had a significant difference between the time taken to observe the familiar object and the novel object.

Passive avoidance task was conducted to measure long-term and implicit memory. Step-through latency was used to compare the mean difference between the experimental groups.

Most of the subjects were transferred into a darkroom for a minute on day 1 (Figure 3E). Only 3 animals took over 100 seconds to get into the darkroom. The difference between the experimental group and the control was not found on day 1. When compared to the latency time on day 1, the average latency time increased on day 2, and unexpectedly, 26 animals stayed in the light room for over 300 seconds (Figure 3F). *Lcb. rhamnosus* group presented the highest average latency time, followed by *L. acidophilus* group while the control group showed the lowest average (Table S1). The Mann-Whitney U test was conducted to check the mean difference, the P-values of *L. acidophilus* and *Lcb. rhamnosus* groups were less than 0.05 compared to the control group. The adjusted P values of both groups were 0.040.

To assess spatial learning and long-term memory, forced alternation was conducted. Memory was evaluated by forced alternation (%), the number of arms that the mouse entered, and the percentage of mice in a group that entered the novel arm as their first entry. While the total number of the entries into each arm was diverse, there was no significant difference between the experimental groups and control (Figure 3G). *L. acidophilus* group scored the highest ratio of mice entered the novel arm as their first entry (Table S1). Forced alternation values of *L. acidophilus* and *Lcb. rhamnosus* groups were higher than the value of the control group (Figure 3H). Forced alternation of *Lcb. rhamnosus* group and the control group had a significant difference with the adjusted P-value of 0.038.

Full 16S-23S rRNA sequencing and biological diversity

Metagenome sequencing was performed with *L. acidophilus* and control groups, which showed the most improvement in cognitive ability. We compared the microbial composition of

both groups. Gut microbial component information annotated at a species level was completely constructed by sequencing the entire 16S-23S rRNA of the mouse stool (Table 1). Averagely, 323870.0±84085.5 reads were generated from 10 stool samples. The total number of identified OTU was 252401.6±56284.7 in *L. acidophilus* group and 259945.6±78526.0 in the control group. The produced OTUs were annotated as a total of 528.4±90.4 species in *L. acidophilus* group and 539.8±55.4 species in the control group. To check the sufficiency of the sequencing depth for the analysis, a rarefaction curve was created (Figure 4A).

Alpha diversity was calculated to compare species richness within a group (Figure 4B). In the comparison of the two groups, no significant difference was found in Chao1 Shannon indexes. Beta diversity was measured to compare the diversity of the microbial community between the two groups (Figure 4C and D). It was confirmed that both beta diversity evaluations (Bray-Curtis and Unifrac distance) had significant differences.

Microbial composition

In the comparison analysis of microbial compositions, taxonomies with significantly different ratios were found between *L. acidophilus* group and the control group. At the phylum level, Bacteroidota accounted for the highest proportion in both groups, followed by Firmicutes (Figure 4E). Significant differences between the two groups were found in 2 of the 12 phyla (Firmicutes, Proteobacteria), all of which were high in *L. acidophilus* group. At the class level, Bacteroidia showed the highest proportion in both groups. Also, the proportion of Bacilli and Gammaproteobacteria classes were increased in *L. acidophilus* group when compared to the control group (Figure S3). At the order level, Bacteroidales showed the highest percentage in

both groups, and Lactobacillales and Enterobacterales orders were found to exhibit higher proportions in *L. acidophilus* group. At the family level, *Muribaculaceae* showed the highest proportion in both groups. It was found that 2 familiae (*Lactobacillaceae* and *Enterobacteriaceae*) showed increased proportions in *L. acidophilus* group, while a decreased percentage was observed in one family (*Ruminococcaceae*). In the Genus comparison, *Muribaculum* genus showed the highest ratio in the two groups, and 12 genera showed differences between groups. Three genera showed an increased proportion in the experimental group, whereas 9 genera showed higher mean values in the control group. The genus increased in *L. acidophilus* group were *Lactobacillus*, *Staphylococcus_A*, and *Escherichia*, whereas the genera decreased in *L. acidophilus* group were *Bacteroides_F*, *Desulfotomaculum*, *Lachnobacterium*, *Bittarella*, *Agathobacter*, *Roseburia*, *Bariatricus*, and *Lachnospirarea*. At the Species level, *Muribaculum intestinale* was found to account for the largest proportion, with over 50% in both groups. Following *M. intestinale*, the species *Lactobacillus acidophilus*, *Lactobacillus johnsonii*, *Lactobacillus_B murinus*, and *Lactobacillus_H reuteri* were found with a high proportion in *L. acidophilus* group, while *Lactobacillus_B murinus*, *Bacteroides_B vulgatus*, *Faecalibaculum rodentium*, and *Kineothrix alysoides* species showed a high proportion in the control group. No unique bacterial species were found in either of the two groups. Seventeen species showed differences between groups, and it was confirmed that the proportions of *L. acidophilus* and *E. flexneri* were increased in *L. acidophilus* group (Figure 4F).

Functional profiling and correlation analysis

Functional profiling was performed at the KEGG level 3 to estimate the effect of the

differential composition of intestinal microbes on the mice (Figure 5). By calculating the LDA score, it was confirmed that the two groups showed significantly different patterns in 9 categories. All nine categories were predicted to be more activated in *L. acidophilus* group. The Phosphotransferase system (PTS) scored the highest, followed by *Staphylococcus aureus* infection, Synthesis and degradation of ketone bodies.

To further estimate the influence of the altered gut microbiota, Spearman's correlation analysis of cognitive-behavioral abilities and bacterial OTUs, and fermentation products were performed (Figure 6). *L. acidophilus* and *E. flexneri* showed a positive correlation with all assessments of cognitive abilities, while the other 14 OTUs presented a negative correlation. In particular, step-through latency at Day 2 and Step latency difference for 2 days of the PAT results showed a significant negative correlation with the *Gemella massiliensis* ($r = -0.8379$, $p = 0.03248$ and $r = -0.8182$, $p = 0.0376$) and *Desulfotomaculum nigrificans* ($r = -0.8781$, $p = 0.01914$ and $r = -0.8450$, $p = 0.03225$).

To provide evidence to indirectly infer the mechanism of action of the gut microbiome, the concentration of SCFA in the microbial culture was measured (Table S2). Lactic acid and acetic acid were found in three microbial cultures. Lactic acid was identified in the highest concentration in *Lcb. paracasei* EG005, and acetic acid was included in the highest concentration in *L. acidophilus* EG004 culture. Propionate and butyrate were not within detectable ranges.

Comparative analysis of genetic contents in bacterial whole genome sequences

To identify its safety and functionality, several genetic factors were detected. Fourteen genomic islands, two prophage regions, one CRISPR region, and three bacteriocins were found

in the genome of *L. acidophilus* EG004. In *Lcb. paracasei* EG005, 29 genomic islands, 7 prophage regions, 3 CRISPR regions, and 2 bacteriocins were detected. In the case of *Lcb. rhamnosus* EG006, 23 genomic islands, 8 prophage regions, 3 CRISPR regions, and 1 bacteriocin were found in the genome. To estimate a genetic factor related to cognitive ability, protein annotation was conducted (Figure 7A). Protein metabolism, Carbohydrates, Amino acids and derivatives showed high proportions, but there was a difference in order by bacterial strains. Protein metabolism had the highest proportion in *L. acidophilus* EG004 and carbohydrates presented the highest proportion in *Lcb. paracasei* EG005 and *Lcb. rhamnosus* EG006. In a subcategory comparison of predicted functional sequences, a difference of genetic contents was found (Figure 7B). CDSs related to Fatty acids were found in the genomes of *Lcb. paracasei* EG005 and *Lcb. rhamnosus* EG006. Genes of 3 subcategories (Aromatic amino acids and derivatives, Alanine, serine, and glycine, and Proline and 4-hydroxyproline) were detected in *Lcb. rhamnosus* EG006, while genes of 3 other categories in Amino Acids in Derivatives were contained in only *L. acidophilus* EG004.

Discussion

As interest in Gut-Brain Axis has increased, many types of research in this criterion have been published. However, it is still unclear about the integral mechanism and which strain has a positive or negative effect. Therefore, we aimed to develop a new strain that has a positive effect on the host's cognition, and we found 3 strains that caused positive effects in 4 different cognitive tests (Figure 3). *Lcb. paracasei* group showed improved cognitive ability in the novel object recognition test. A previous study indicated that this bacterium prevents age-related cognitive decline and improves cognitive ability (22). Other strain, *Lcb. rhamnosus*, displayed improved cognitive ability in passive avoidance task and forced alternation test. Several studies demonstrated that *Lcb. rhamnosus* consumption could increase cognitive ability (25, 26). Similar to previous studies, we experimentally confirmed that *Lcb. paracasei* and *Lcb. rhamnosus* could enhance cognitive function. On the other hand, although it is indicated that *L. acidophilus* strain has a neuroprotective effect against traumatic brain injury, there was no experimental research related to its cognitive ability (27, 28). In our study, we identified that *L. acidophilus* group presented the highest classical measured values as well as incidental measured values in novel object recognition test and passive avoidance task. This indicates that *L. acidophilus* is capable of improving cognitive ability comparable to that of previously reported strains. Our results will help further broaden the industrial field of *L. acidophilus*. In addition, although probiotic consumptions were carried out as the same method, three experimental groups showed improved cognitive ability in different tests. It implies that different probiotic strains affect cognitive ability by different mechanisms.

To understand the effect of the gut microbiome on the brain as our secondary goal, we performed gut microbiome analysis of *L. acidophilus* group, which showed the best cognitive improvement, along with the control group, The difference of species richness was not found in the comparison of alpha diversity, whereas the difference was found in the comparison of beta diversity (Figure 4B, 4C, and 4D). It represents that the number of OTUs constituting the two gut microbial communities is similar, but the composition of the OTUs is different. In the comparison of the two communities, significant differences were observed at all taxonomic levels except for the bacteria kingdom, which were mostly *L. acidophilus.* Naturally, *L. acidophilus* group was confirmed to show a significant increase in *L. acidophilus* abundance and ultimately show a high ratio of *L. acidophilus.* This indicates that a large amount of *L. acidophilus* is capable of safely reaching the intestines without being affected by digestive juices such as gastric acid and pancreatic enzymes.

We estimated that the positive effect on cognitive ability due to the increased proportion of *L. acidophilus* in the intestines was based on two rationales: modulation of neurotransmitters and neurotrophic factors and production of SCFAs. First, *L. acidophilus* modulates several types of neurotransmitters in the intestine. Microbial-derived intermediates, which affect the brain through gut epithelial and blood-brain barriers, are such as GABA (γ-aminobutyric acid), glutamate, dopamine, noradrenaline, serotonin (5-Hydroxytryptamine; 5-HT), and Brain-derived neurotrophic factor (BNDF). These neurotransmitters are synthesized from various amino acids. GABA and glutamate are produced from the gut microbiome such as *Bifidobacterium* and *Lactobacillus* (29). Glutamate has a role as a neurotransmitter by itself, and it is used at GABA synthesis (30). Dopamine and Noradrenaline are synthesized from specific amino acids such as tyrosine and phenylalanine (31). L-Tryptophan is a well-known precursor of serotonin (32).

Therefore, altered amino acid composition by the gut microbiome seems to affect the host's neurotransmitter synthesis. In the comparison of the functional protein genes, *L. acidophilus* EG004 showed a higher composition of the gene related to amino acid metabolism, than *Lcb. paracasei* EG005 and *Lcb. rhamnosus* EG006 showed (Figure 7A). Changes in intestinal amino acid composition caused by ingested *L. acidophilus* may have led to differences in cognitive ability. It has been proven that *L. acidophilus* consumption produces and up-regulates neurotransmitter and neurotrophic factor including GABA and serotonin (33-36). Thus, it is estimated that increased *L. acidophilus* EG004 in the gut modulates neurotransmitters and affects the animal's nerve system. Second, SCFAs, fermentation products of *L. acidophilus*, positively apply to brain function. For example, acetate, one of the short-chain fatty acids (SCFAs), promotes the activation of the parasympathetic nervous system (37). Also, it is indicated that acetate improved cognitive ability and neurogenesis in the hippocampus with increasing BDNF and IGF-1 levels as a glatiramer acetate form (38). Likewise, butyrate, a famous HDAC inhibitor, has been used for a pharmacological purpose since lower global histone acetylation is a common phenomenon observed in many neurodegenerative diseases (39). Its therapeutic effect on neurodegenerative diseases including Parkinson's disease was verified, showing enhancement of neurotrophic factors and improvement in learning and memorizing (40). However, SCFAs are not produced until non-digestible carbohydrates reach the small intestine to be broken down by microbial metabolism, so it is not fully produced by the human digestive enzymes without specific microbes. *L. acidophilus* is a representative species that produces SCFAs through non-digestive carbohydrates, and it can be assumed that the intake of *L. acidophilus* EG004 caused the increase in SCFAs of the experimental mice's gut. The result of SCFA measurement in bacterial culture raises the possibility of this assumption (Table S2). Although it is different from

the metabolism in the gut since the SCFAs were measured in the medium to which glucose is the main energy source, it indirectly estimates its SCFA-producing ability. The result of functional profiling in our study also upholds this (Figure 5B). In the analysis of functional profiling, activation of genes of synthesis and degradation of ketone bodies was predicted by comparing it with control. The ketone body is one of the main fuels of the brain like lactate and butyrate, which is the main product of *L. acidophilus*, and is also capable of replacing glucose as an alternative fuel. Similar to butyrate mentioned earlier, ketone bodies modulate the brain with anti-oxidant reaction, energy supply, regulation of deacetylation activity, and regulation of the immune system. In recent studies, it is indicated that the increase of ketone body's concentration induces an alleviation effect on brain diseases such as epilepsy, Alzheimer's disease, and Parkinson's disease as well as memory improvement (41-43). Based on these evidences, ingested *L. acidophilus* EG004 in our experimental group seems to have produced SCFAs and modulated neurotransmitters, and *L. acidophilus*-derived metabolite would have raised cognitive ability. Although we did not measure microbial-derived metabolites, previous researches demonstrated that probiotic consumption leads to an increase of microbial-derived metabolites in the intestines.

Among detected species with the ratio difference, several species were indicated as important factors in the research of brain disease. *Adlercreutzia equolifaciens* is equol (phytoestrogen) producing bacteria, which obstructs microglial function. In previous studies, a higher ratio of *A. equolifaciens* was found in the gut of patients with Alzheimer's disease and Autism spectrum disorder (44, 45). In other studies, *Roseburia hominis* and *Bacteroides_F pectinophilus* were detected with a higher ratio in the patients with Alzheimer's disease than the normal persons (46, 47). When comparing gut microbiome between the Parkinson's disease group and normal group, *Soleaferrea massiliensis* was more frequently discovered in the patient

group (48). Interestingly, those strains that showed a high ratio from the previous studies of brain disease patients were found to show a lower ratio in *L. acidophilus* group when compared to the control group (Figure 4F). Decreased bacterial ratio related to brain diseases seems to positively affect cognitive ability and we believe that it is due to *L. acidophilus* consumption. As antibacterial activity is the essential property of probiotics, such activity of *L. acidophilus* against harmful and pathogenic bacteria has been reported. In our previous study, we proved that *L. acidophilus* EG004 is capable of demonstrating the antimicrobial activity (49). Therefore, we suggest that the antibacterial activity of *L. acidophilus* EG004 was the potential reason for cognitive ability enhancement.

In functional profiling analysis, we offered explainable factors for the microbial effect on the brain. Three KEGG categories were related to toxic chemical degradation: Dioxin degradation, Xylene degradation, and Caprolactam degradation (Figure 5B). Dioxin, a neurotoxin, can raise autism and neurodegenerative disease (50, 51). Xylene inhibits normal protein synthesis of neuronal function and induces instability in the neuronal membrane. When it is inhaled, psychological deficits can be caused (52, 53). These chemicals are noxious to the brain, so activation of these chemical degradations would have diminished negative effect in *L. acidophilus* group. Besides, two KEGG categories related to the immune system were found. One of them is *Staphylococcus aureus* infection, which is known to cause brain abscess. Since there have been many studies demonstrating that *L. acidophilus* has antimicrobial activity against *S. aureus*, activation of this category is thought to be due to an increase in the amount of *L. acidophilus*. The function of renal cell carcinoma was predicted in the experimental group. As it involves not only tumor suppressor genes such as VHL, GH, and BHD, but also oncogenes such

as MET and PRCC-TFE3, it seems to be necessary to confirm the exact mechanism and side effects.

The purpose of this study was to develop a new strain that can improve cognitive ability and to provide an underlying biological mechanism affecting the brain by the gut microbiome. It is necessary to measure metabolite changes in order to provide an understanding of the mechanism of altered cognitive ability. However, altered metabolite from animal body was not fully identified. To overcome this limitation, we conducted the metagenome analysis, correlation analysis between cognitive ability and gut microbiome, measurement of SCFA producing ability, and whole-genome comparison analysis. These analyses were not covered to identification of a biological factor caused improved cognitive ability, but presented a group of genes and mechanisms that can infer the process. Although we did not provide direct evidence of phenotype changes caused by probiotics ingestion, we hope that our findings will help infer the process of the brain-gut axis.

Materials and Methods

Animals

4-week-old male C57BL/6 mice (n = 48, average weight 26g) were gained from YoungBio (Seongnam, Korea). All mice were housed in a group of four per cage under standard controlled laboratory conditions (temperature of 20±5℃, humidity of 55~60%) on a 12-h light/dark cycle (light on at 7:00 a.m.). Each group was constituted of 12 mice, and it was nurtured by distributing 4 mice to 3 cages. Twelve cages were located at random. All animals received *ad libitum* access to food. All animal experiments were performed following protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University, and the permission number is SNU-190607-4-3.

Bacterial treatment

The bacterial strains were isolated from fermented dairy foods. When identifying the brain-gut axis effect, the important factors to be considered were viability and adherence capacity. Therefore, we selected the species that are known to have adherence capacity in the GI tract, as well as the potential for gut-brain axis effect. To identify species of each strain, 16S rRNA genes were sequenced by Macrogen Inc. (Seoul, Korea) with 27F and 1492R primers. Obtained sequences were compared with sequences in the NCBI database using BLAST. The experiment was constituted with 4 groups; 3 experimental groups were fed on autoclaved tap water mixed with *L. acidophilus* EG004, *Lcb. paracasei* EG005, and *Lcb. rhamnosus* EG006, and a control group was fed on sterilized tap water. Each group consisted of 12 mice. Bacteria to delivery were

freshly cultivated every day. Probiotic colonies were sub-cultured into 5ml MRS broth for 8 hours. After the sub-culture, 3 probiotic strains were inoculated in 500 ml MRS broth for 16 hours. Cultivated cells were spun down by centrifugation with 4,000 rpm for 10 min. The supernatant was removed, and the pellet was suspended by 0.85 % NaCl solution. Re-suspended cells were centrifuged by 4,000 rpm for 10 min to remove medium ingredients. The washing process was conducted twice. Washed cells were dissolved into autoclaved tap water. The final cell concentration of vehicles was about 1.0E9 CFU/ml. To estimate the probiotics amount per day per subject, daily water intake and probiotic concentration in vehicles were recorded. Cell viability of probiotics was measured by serial dilution and spreading in MRS agar plate. The probiotics amount per day per subject was calculated as an average of daily water intake per subject, by multiplying the average of daily probiotic concentration.

Animal treatment

The animal experiment was designed to minimize animal stress.All animal treatment was described in Figure 1 by timeline. Four weeks old mice were allowed to habituate freely for acclimatization for 1 week. After a week, tap water and water mixed with probiotics were delivered every day. Water intake was monitored every day and body weight was measured every week. Evaluations of cognitive ability were conducted after 4 weeks from probiotics intake. Behavioral tests were conducted at least 2 days after the weight-measurement day to minimize the stress effect. Animals were carried to a behavioral test room to assimilate room condition and were allowed to relax for 6 hours before any behavioral test. In order to reduce the variance of feeding time, the experimental order of the mice was distributed evenly. All apparatus and

objects for the behavioral tests were cleaned with 70 % ethanol and dried after every trial to remove odors and any clues. The mice were sacrificed at the end of 13 weeks after the evaluations of the cognitive behavior. Preliminary experiments were conducted to obtain appropriate experimental values under our experimental environmental conditions. The three to five experimental conditions referring to published results were tested in our laboratory, and the experimental conditions showing a value similar to the average value of the previous studies were determined.

Y maze (Spontaneous alternation; SA)

Short-term spatial memory was assessed with a Y maze apparatus. SA was used to measure rodent's habit to explore a new environment. The Y maze consisted of 3 identical arms that cross each other with 120° (JEUNGDO Bio & Plant Co., Ltd., Korea). Mice are laid in the middle of the Y maze facing a corner, not an arm. Each animal was allowed to freely navigate all three arms for 5 minutes and the animal's entries to any arm were recorded. An arm entry was determined as any instance when the whole body of the mouse entered the arm and navigated at least 70% of the space. The spatial memory was evaluated by spontaneous alternation, the number of arm entries, and the ratio of mice per group entered spontaneous alternation during the first three entries. Spontaneous alternation was calculated as shown below.

$$\text{Spontaneous alternation [\%]} = \frac{\text{Number of spontaneous alternation}}{\text{Total number of arm entries} - 2} \times 100$$

Novel object recognition test (NOR)

Based on the concept that mice tend to prefer a new object over a familiar one, a novel object recognition test (NOR test) was performed in an open field (40×40×40 cm (W×D×H), JEUNGDO Bio & Plant Co., Ltd., Korea). Two objects for this test were selected showing similar preference through the preference test. The test consisted of Sample trial (T1; 10 min), Interval time (IT; 60 min), and Novel object trial (T2; 5 min). In T1, 2 identical objects were located at 1/3 and 2/3 diagonal of the open field, respectively. The animal was laid facing the wall with the same distance to two objects, and was allowed to explore objects for 10 min. After exploration, the mouse came back to the cage and had a rest. In T2, objects were positioned at the same position as T1, but one of the objects was changed to a novel object. To measure the time taken to interact with objects, all experiment processes were recorded, and the exploration time was measured by Movavi software with 3 decimal places. It was recognized as significant only when the mouse approached facing the objects within 2.5 cm. Cases that the mouse climbed objects and individuals with exploration time less than 2 seconds were excluded. The results were presented as a discrimination ratio, the number of object touches, and the ratio of mouse that touched the novel object first before it touched the familiar object. The discrimination ratio was defined as the below equation.

$$\text{Discrimination ratio [\%]} = \frac{\text{Novel object interaction time}}{\text{Novel object interaction time + Familar object interaction time}} \times 100$$

Passive avoidance task (PAT)

The passive avoidance task is designed to evaluate inhibitory avoidance memory according to rodent habit that a mouse prefers dark environment naturally. Shuttle box (41×21×30 cm (W×D×H), JEUNGDO Bio & Plant Co., Ltd., Korea) is an apparatus made for the passive

avoidance task and consists of a bright chamber and a dark chamber which are separated by a sliding door. The floor of the chambers is made of stainless-steel grids to flow current. The test was conducted for 2 days; Acquisition (Day 1) and Test (Day 2). On day 1, a subject was put in the bright chamber facing the wall across the closed sliding door. After the mouse explored the bright chamber for 1 minute, and the moment the mouse was away from the door for over 100 mm, facing the wall not the door, the door was opened so that the mouse could freely enter and move around the dark chamber. Latency time was measured until the mouse entered the dark chamber completely. The door was closed when the animal entered the dark compartment wholly including its tail, and 0.25 mA electric shock was provided to the paws by steel grid for 3 seconds. To memorize the situation, the mouse was kept in the dark chamber for 30 seconds after the shock and returned to the home cage for 24 hours. On day 2, the mouse was laid again into the bright chamber. After 1 minute for adaptation, the sliding door was opened when the mouse faced the wall like day 1. Latency time was measured again until the mouse entered the dark chamber. If the animal rather stayed in the bright chamber for more than 300 seconds (which was the cut-off time), the experiment was completed. All experimental processes were recorded and the time was measured by the Movavi program with 3 decimal places.

Y maze (Forced alternation; FA)

Forced alternation was assessed with the same Y maze as described above. This test consisted of 3 phases; Training trial (T1; 5 min), Interval time (IT; 60 min), and Test trial (T2; 5 min). A mouse was placed at a starting arm of Y maze facing the wall. The subject freely explored the maze during T1, while an entry was blocked with white expanded polystyrene. After

the learning trial, the mouse was returned to the home cage and rested for 1 hour. In T2, the mouse was again placed into the starting arm without the plate blocking the novel entry, and explored all three arms. All movements of mice were recorded through video. Forced alternation was evaluated by the ratio of time spent in the novel arm compared to the whole experimental time, time taken to first enter the novel arm, and the percentage of mice per group that entered the novel arm as their first entry. The case that the mouse passed at 2/3 of the arms was admitted as a valid entrance. An individual that showed no navigation of the maze or that had entered the arms less than 5 times was excluded.

Feces collection and cognitive ability evolution

After all cognitive assessments had been completed, 2-3 stool samples were taken from each experimental subject. Sterilized stainless-steel tweezers were used for fecal picking, tweezers were washed with 70% alcohol and dried sufficiently before collecting new samples. The fresh samples were immediately enclosed into a 1.5ml Eppendorf tube and were put on ice. Then, it was stored at -80 degrees Celsius until used for 16S rRNA sequencing.

In order to determine the group that showed the best increase in cognitive ability, a score was assigned to the cognitive ability evaluation item. The items used for evaluation are spontaneous alternation, group ratio of SA, discrimination ratio, group ratio of NOR, step latency at day 2, forced alternation, and group ratio of FA (Table S2). Scores were given in ascending order of ranking (1-4 points), and the group with the highest total was selected as the group with the highest cognitive ability increase.

Statistics

Data were analyzed by R studio. Ineligible data were cut based on the requirements mentioned above. Data normality was assessed using the Shapiro-Wilks test and homogeneity of variance was assessed using Levene's test. Wilcoxon rank-sum test and independence t-test was used to evaluate statistical significance between experimental groups. P-values were adjusted by the FDR method for multiple testing correction. Statistical significance was set as P-value under 0.05. All data are expressed as mean ± SEM.

Full 16S-23S rRNA sequencing

To characterize the microbial community associated with measured cognitive assessment, metagenome sequencing of the 16S-23S rRNA gene was carried out by Oxford Nanopore MinION. Metagenome sequencing was performed for the control group and *L. acidophilus* group, which showed a significant difference from the control in the cognitive ability evaluation. Among the 12 stored stool samples of each group, 5 samples with sufficient amount for sequencing were selected. For library construction, gDNA was extracted from fecal samples using AccuPrep® Stool DNA extraction Kit (Bioneer, Daejeon, South Korea). To identify the quality of extracted gDNA, A260/A280 and A260/A230 absorbance were used with 0.7 % agarose gel electrophoresis. After performing quality control, selected samples were used for the library construction. Stool samples were lysed and bacterial cells were disrupted by Zirconia/Silica Beads and proteinase K. The sequencing library was prepared by 16S-26S rRNA PCR amplification with Nanopore Ligation Kit (SQK-LSK109, Nanopore, Oxford, UK) following the manufacturer's instructions. Purification and quality checks were conducted using

agencourt AMPure XP cleanup (Beckman Coulter, CA, USA), Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen, Ireland), and 0.7% agarose gel. The PCR products were diluted and end-repaired using NEBNext FFPE Repair Mix (New England BioLabs, Ipswich, USA). The amplicon was Nick-repaired using NEBNext End repair/dA-tailing Module (New England BioLabs), prior to adapter ligation by NEBNext Quick Ligation Module (New England BioLabs). The sequencing library was loaded on primed Flongle flow cell according to Nanopore protocol. Sequencing was performed by MinION MK1b. Sequencing data was acquired by MinKNOW software (19.12.5) without live base-calling.

Metagenome analysis

Raw data were obtained as fast5 files. Base-calling was carried out by Guppy 4.0.11 with 2,000 chunk size and 4 base callers (54). Porechop version 3 was executed for trimming adapter sequences (https://github.com/rrwick/Porechop). To annotate bacterial taxonomy, trimmed sequences were aligned with reference data from GTDB using Minimap2 (55). In Operational Taxonomic Unit (OTU) identification, only results with more than 2,500 matching bases and more than 3,500 bases including gaps in mapping were used. To normalize abundance data, the TMM (The trimmed mean of M-values) method was used by the edgeR package of R software (56). To characterize each group, biological diversity was calculated through the physeq package of R software (57). A rarefaction curve was constructed to check the saturation of genome sequencing. To compare species richness, alpha diversity was calculated as chao1 and Shannon indexes. To compare between groups, beta diversity was calculated using Bray-Curtis dissimilarity and Unifrac distance. P-value was calculated by the Adonis test. For detection of

unequal features, Wilcoxon rank-sum test was performed in each taxonomic level with 0.95 confidence level. To compare functional profile, PICRUSt2 was performed (58). Correlation between cognitive ability and bacterial OTUs was inferred by Spearman's rank correlation analysis. P values were adjusted by FDR method.

SCFA identification in bacterial culture

To identify the amount of short-chain fatty acids (SCFAs), high-performance liquid chromatography (HPLC) was performed using Ultimate3000 (Thermo Dionex, USA) and Aminex 87H column (300x10mm, Bio-Rad, USA). Bacterial cultures of EG004, EG005, and EG006 were inoculated for 24 hours. After cultivation, the samples were filtered with 0.45 μm of a membrane filter. The filtered sample of 10μL was injected into the HPLC.

Whole-genome sequencing and assembly of EG005 and EG006

To identify probiotic safety and potential secondary metabolite producing ability, whole-genome sequencing of *Lcb. paracasei* EG005 and *Lcb. rhamnosus* EG006 was performed. For library construction, DNA was extracted from cultured bacterial cells. After performing quality control, gDNA was used for the library construction. Bacterial cells were lysed by lysozyme for gram-positive bacteria, and removed RNA and protein to isolate DNA. Quality control for gDNA was conducted by 260/280, 260/230 absorbance with 0.8% agarose gel. Genomic DNA was fragmented to a target length of 20Kb using g-Tube (Covaris, MA, USA) and Short DNA fragments <5 kb are depleted by SRE (Circulomics, MD, USA). The fragments were End-prepared, Nick-Repaired, and then ligated with Nanopore adapter. After every enzyme reaction,

615 the DNA samples were purified using AMPure XP beads (Beckman Coulter, CA, USA) and QC
616 with Quant-iT™ PicoGreen™ dsDNA Assay Kit. The sequencing library was loaded on primed
617 Flongle flow cell according to Nanopore protocol. Sequencing was performed on a MinION by
618 MinKNOW software.

619 Base-calling from raw data was conducted by Guppy Basecaller v4.0.15 with filtering with
620 an average basecall Phred quality score. Adapter sequences were trimmed by PoreChop v0.2.4.
621 Genome assembly was conducted by Canu. Assembled contigs were polished by Nanopolish and
622 racon, and pilon. Circlator circularized each contig and detect replication origin. Assembled
623 contig was assessed by BUSCO 3.0.2. The complete sequence of *L. acidophilus* EG004 that is
624 deposited in the NCBI database with accession number PRJNA657145 was used.

625

626 **Comparative analysis of bacterial genome sequences**

627 To check safety and functionality as probiotics, genetic factors were identified by whole-
628 genome sequences. Virulence factor and prophage gene were detected by VirulenceFinder 2.0
629 and PHASTER, respectively. IslandViewer4 identified genomic island and crisprfinder searched
630 CRISPR region. Bacteriocin detection was conducted by BAGLE4. To compare functional gene
631 contents, protein prediction was performed by the RAST server. Predicted protein sequences
632 were classified by the SEED system. Categorized protein sequences showed as the proportion in
633 the total predicted sequences.

634

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Tables

Table 1. Metagenomic sequencing statistic of *L. acidophilus group* and control

	The number of samples	Total number of reads	Estimated base (Mb)	N50	Total number of counts	Total number of OTUs
LA[a]	5	312,384±31,887	1,434±143	4,872±90	252401.6±25,171	528.4±40
W[b]	5	335,356±45,814	1,485.6±215	4,748±40	259945.6±35,117	539.8±25
Total	10	323,870±37,604	1,459.8±173	4810±72	256173.6±28,860	534.1±32

[a]: *L. acidophilus* group, [b]: control group. There was no significant difference between groups. All values were presented as average ± standard error of the mean. Fecal samples compiled after 8 weeks of probiotic ingestion were used for metagenome sequencing.

Figure legends

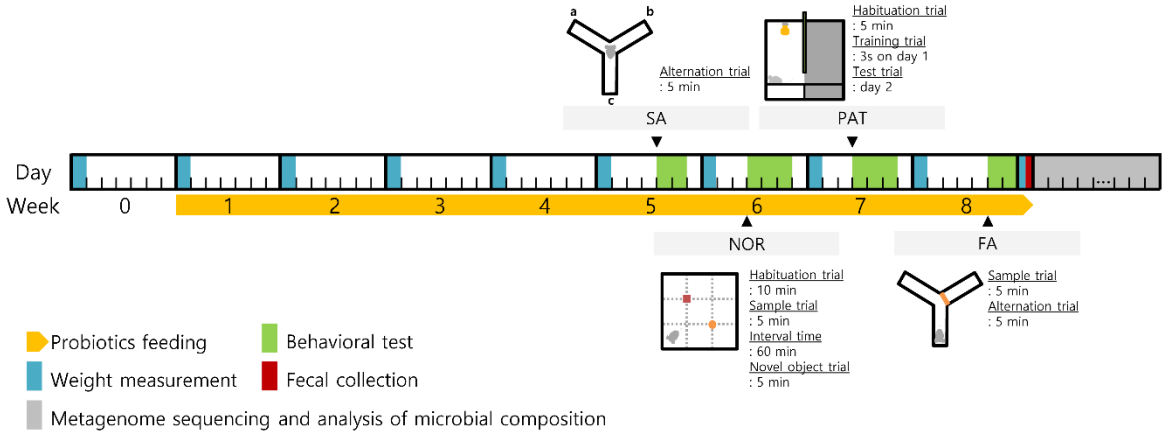
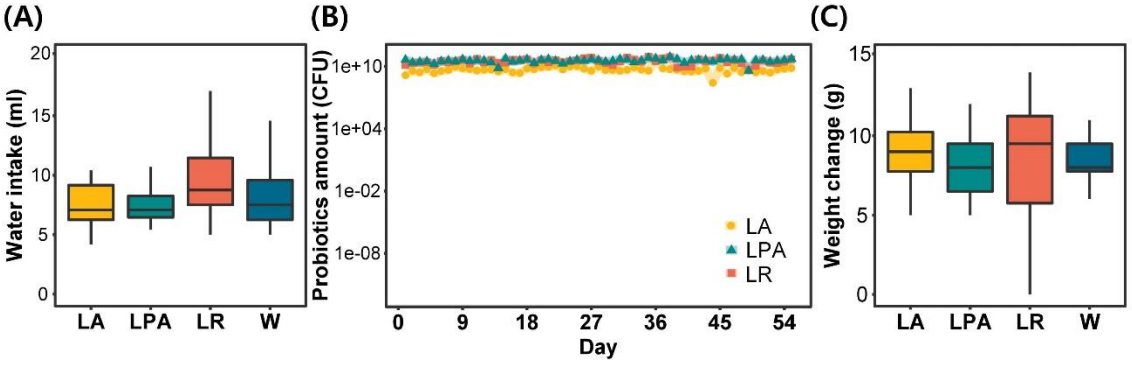


Figure 1. Schematic diagram of the study to discover a new probiotic strain with improved cognitive ability

The diagram displays the experimental schedule by day and week for identifying probiotic strain with improved cognitive ability. Cognitive ability was measured once a week by four behavioral tests. The diagram of each experiment shows the first position of the animal.

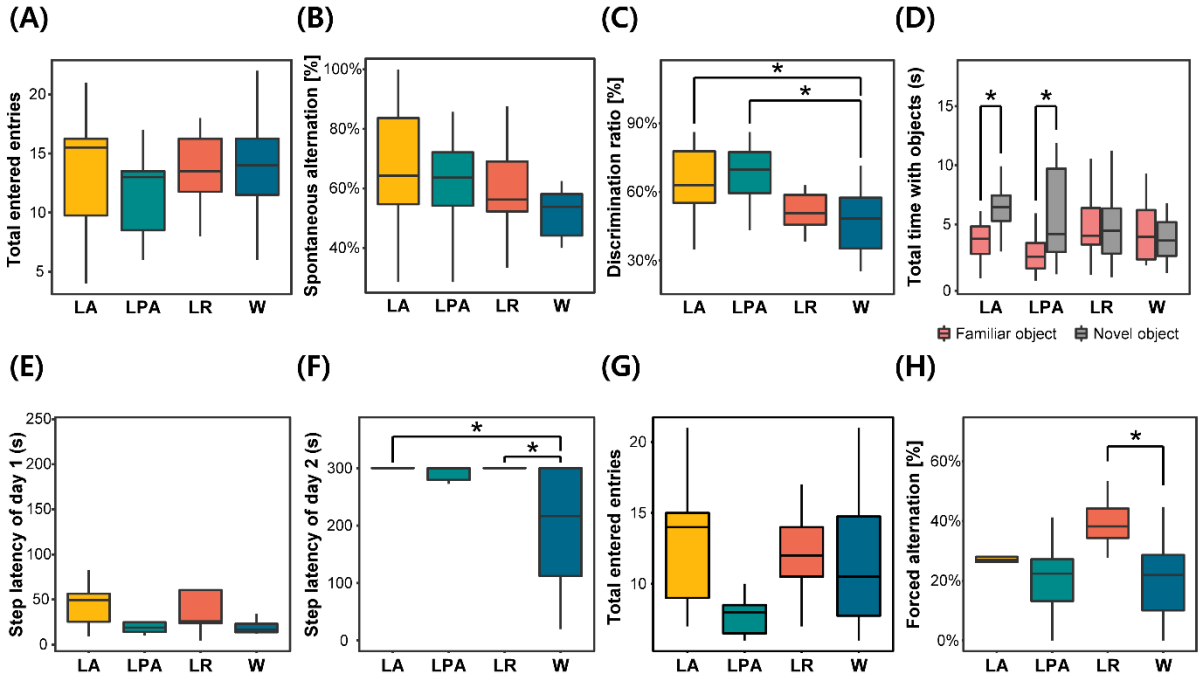


802

803 **Figure 2. Measurement of additional effect after probiotic consumption**

804 Experimental groups are expressed in abbreviations. LA: *L. acidophilus* group, LPA: *Lcb.*
805 *Paracasei* group, LR: *Lcb. Rhamnosus group*, and W: tap water-fed group (control). **(A)** The
806 average daily water intake. All groups showed a similar average. **(B)** The change of daily intaken
807 probiotic amount by timeline. *L. acidophilus* was ingested in smaller amounts compared to the
808 other two strains. **(C)** The average body weight change for 8 weeks. All groups showed similar
809 averages.

810



811

812 **Figure 3. Results of cognitive behavioral tests**

813 Experimental groups are expressed in abbreviations. LA: *L. acidophilus* group, LPA: *Lcb.*
814 *Paracasei* group, LR: *Lcb. rhamnosus* group, and W: the group fed on tap water (control). (**A)**
815 Total arm entries during spontaneous alternation test. **(B)** Spontaneous alternation. This is the
816 representative value of spontaneous alternation test. **(C)** Discrimination ratio. It is the
817 representative value of the novel object recognition test. **(D)** Comparison of the total time to
818 observe two objects. **(E)** Step-through latency of day 1. **(F)** Step-through latency of day 2. This is
819 the representative result of the passive avoidance task. **(G)** Total arm entries during forced
820 alternation test. **(H)** Forced alternation. This result is a representative value of forced alternation.
821 All comparison of average between experimental groups was measured by Wilcoxon rank-sum
822 test. Significant difference is presented with symbol (Adjusted P-value* < 0.05).

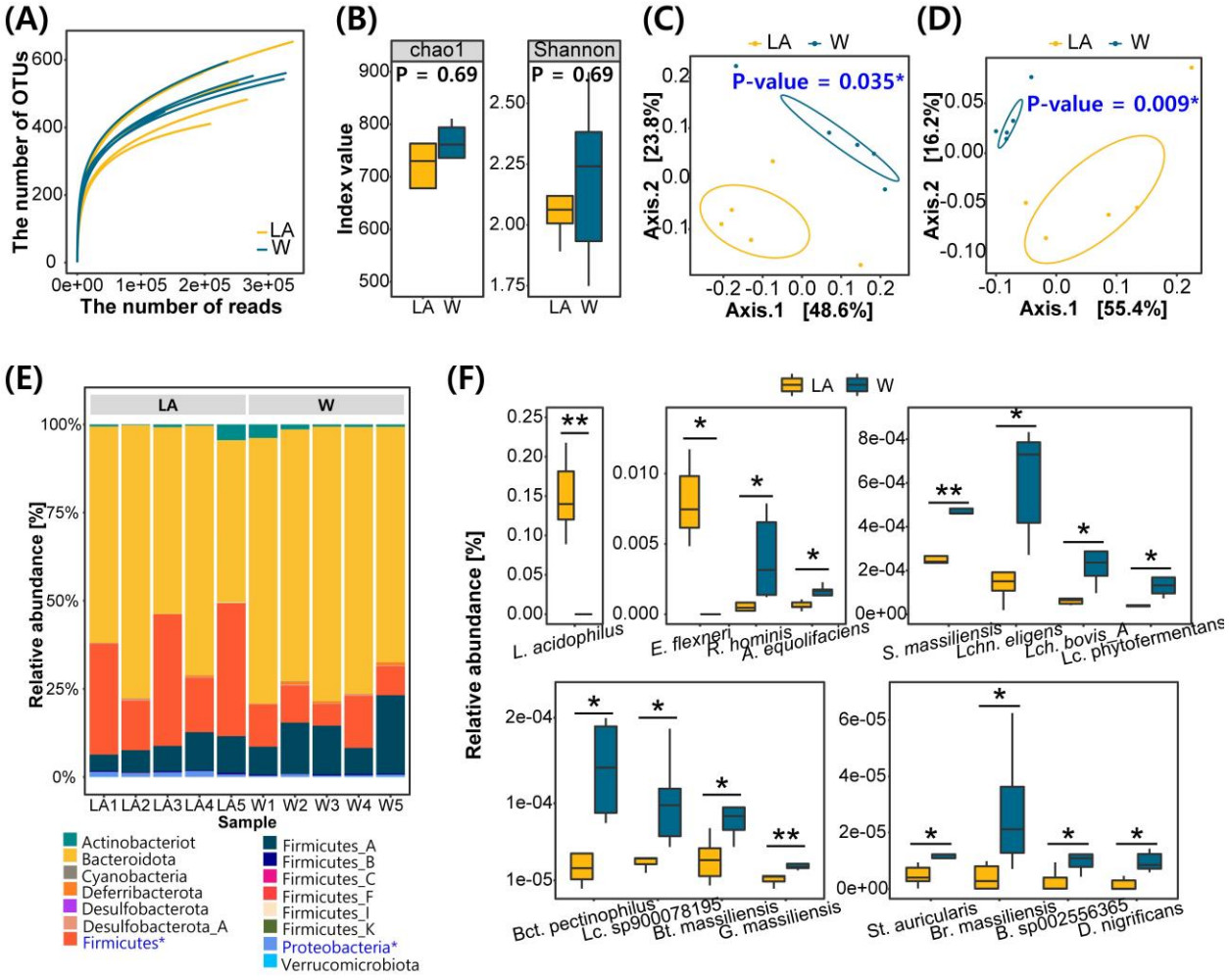
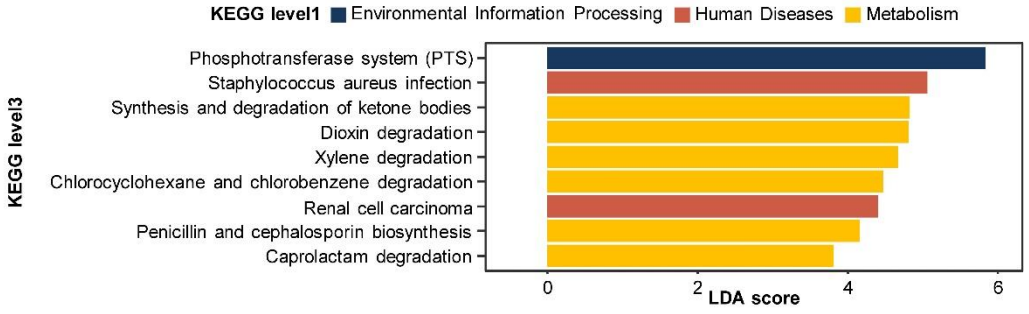


Figure 4. Results of metagenomics sequencing

Experimental groups are expressed in abbreviations. LA: *L. acidophilus group* and W: the group fed on tap water (control). **(A)** Rarefaction curve of metagenome sequencing. **(B)** Alpha-diversity of the *L. acidophilus* group and control. **(C)** Beta-diversity using Bray-Cutis distance between the *L. acidophilus* group and control. **(D)** Beta-diversity using Unifrac distance between both groups. **(E)** Comparison of microbial composition at the phylum level. The blue-colored phylum with the (*) symbol showed a significant difference compared to the two experimental groups. **(F)** Comparison of microbial composition at the species level. L. acidophilus: *Lactobacillus*

acidophilus, E. flexneri: *Escherichia flexneri*, R. hominis: *Roseburia hominis*, A. equolifaciens: *Adlercreutzia equolifaciens*, S. massiliensis: *Soleaferrea massiliensis*, Lchn. Eligens: *Lachnospira eligens*, Lch. Bovis_A: *Lachnobacterium bovis_A*, Lc. Phytofermentans: *Lachnoclostridium phytofermentans*, Bct. Pectinophilus: *Bacteroides_F pectinophilus*, Lc. Sp900078195: *Lachnoclostridium sp900078195*, Bt. Massiliensis: *Bittarella massiliensis*, G. massiliensis: *Gemella massiliensis*, St. auricularis: *Staphylococcus auricularis*, Br. Massiliensis: *Bariatricus massiliensis*, B. sp002556365: *Bacillus_AW sp002556365*, D. nigrificans: *Desulfotomaculum nigrificans.* All comparisons of average between experimental groups were measured by independence t-test. Significant difference is presented with symbol (Adjusted P-value* < 0.05, P-value** < 0.01).



843

844 **Figure 5. Results of functional profiling**

845 Predictive functional profiling of microbiome. All predicted functions have a positive LDA score

846 for the *L. acidophilus*. group

847

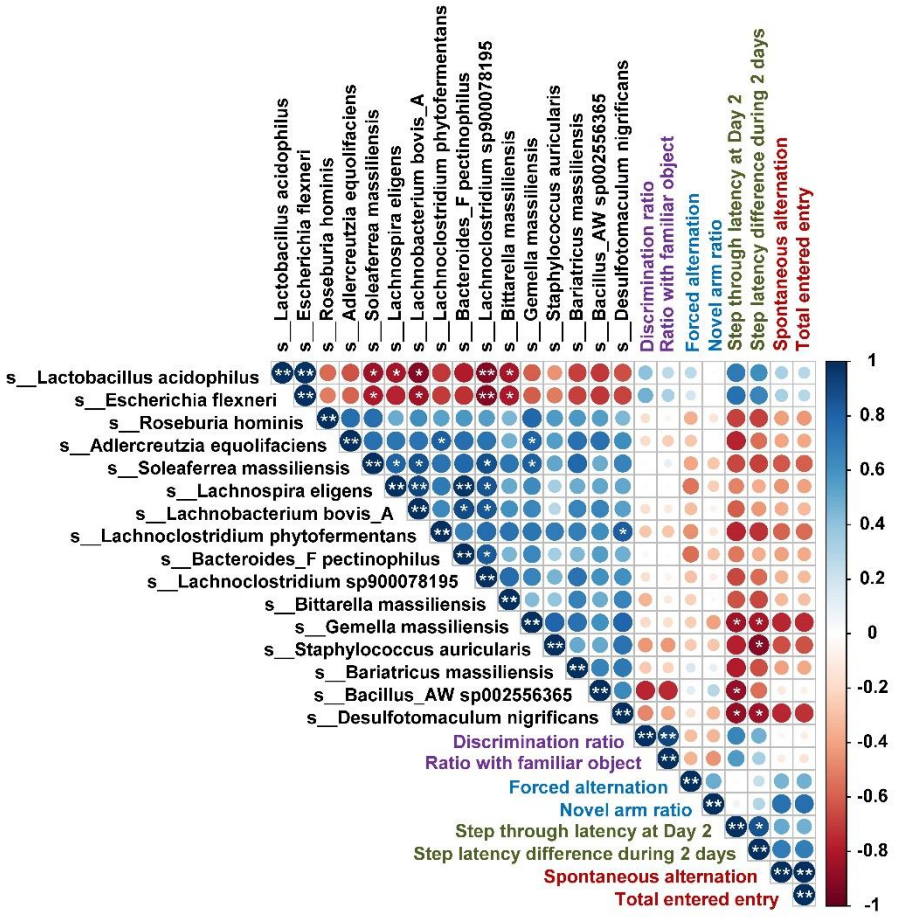
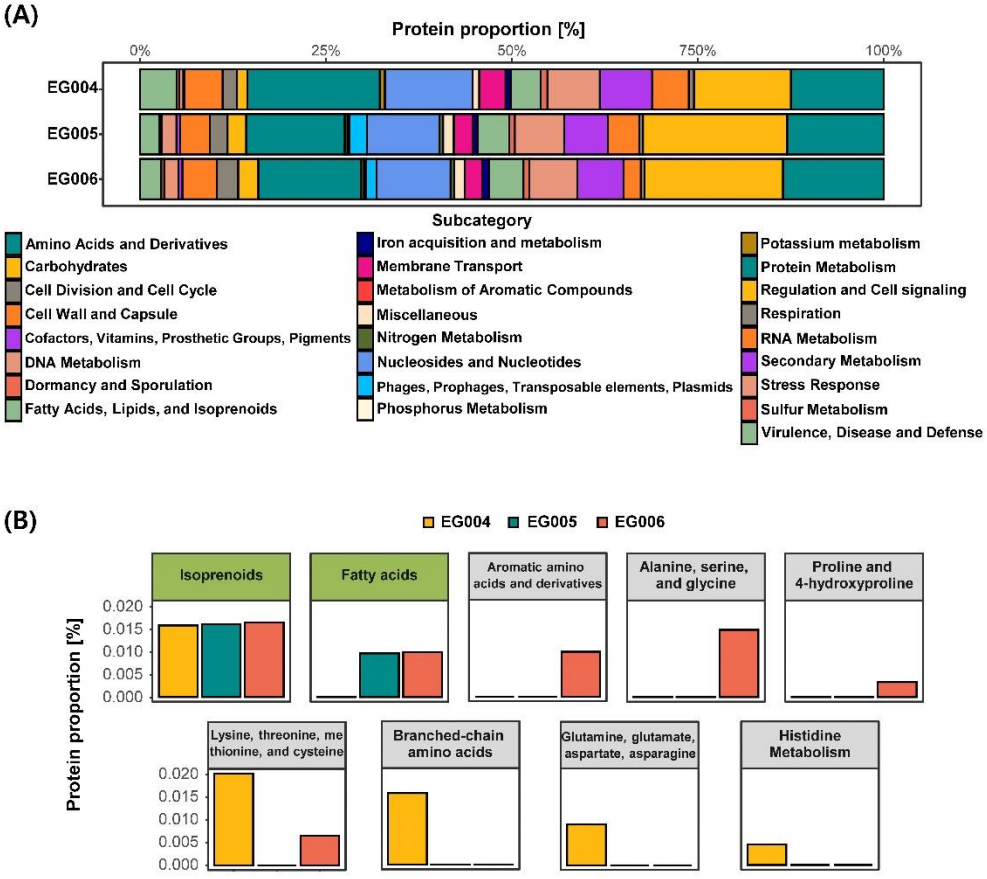


Figure 6. Spearman's rank correlation analysis

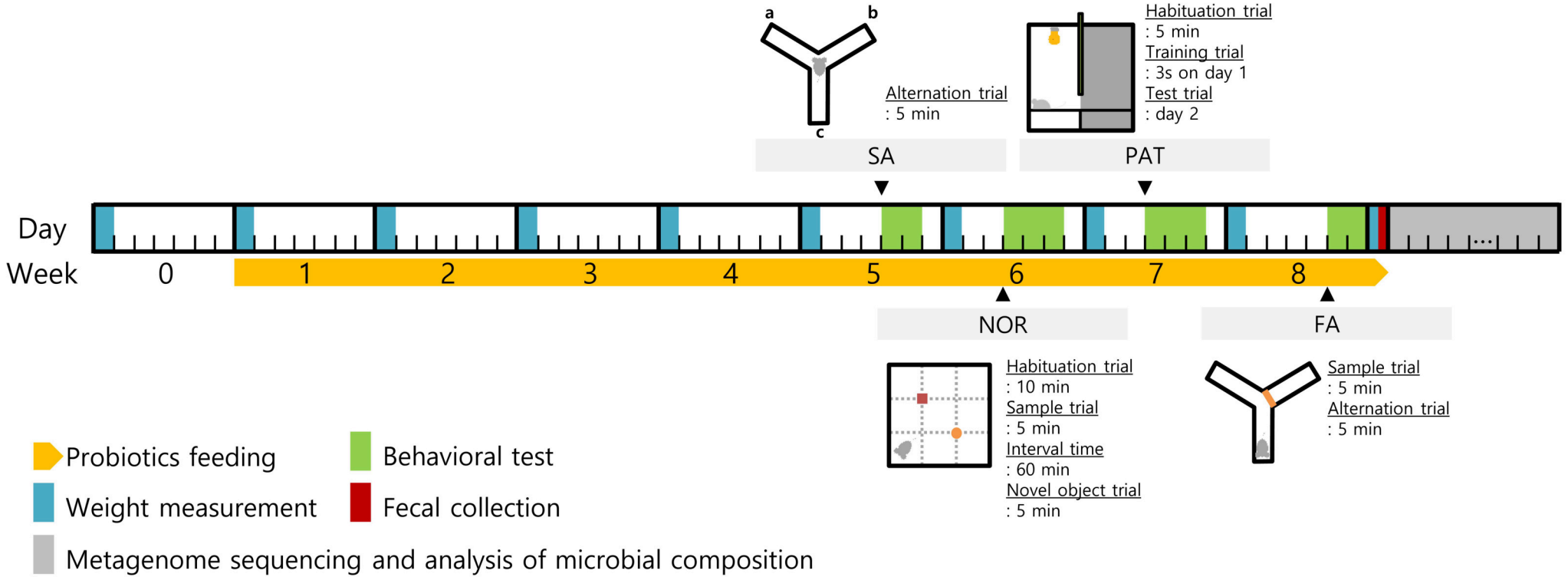
Correlation analysis was conducted to detect association among bacterial OTUs, measured cognitive abilities, and fermentation products. The color intensity and circle size show the strength of the correlation. Red color represents a negative correlation, and blue color is a positive correlation. Only circles with adjusted P-value under 0.01 are illustrated in the matrix. Results of cognitive ability evaluation were classified by 4 colors: NOR (purple), FA (blue), PAT (deep green), and SA (brown). Significant P values indicated by the symbol * (<0.05) and ** (<0.01).

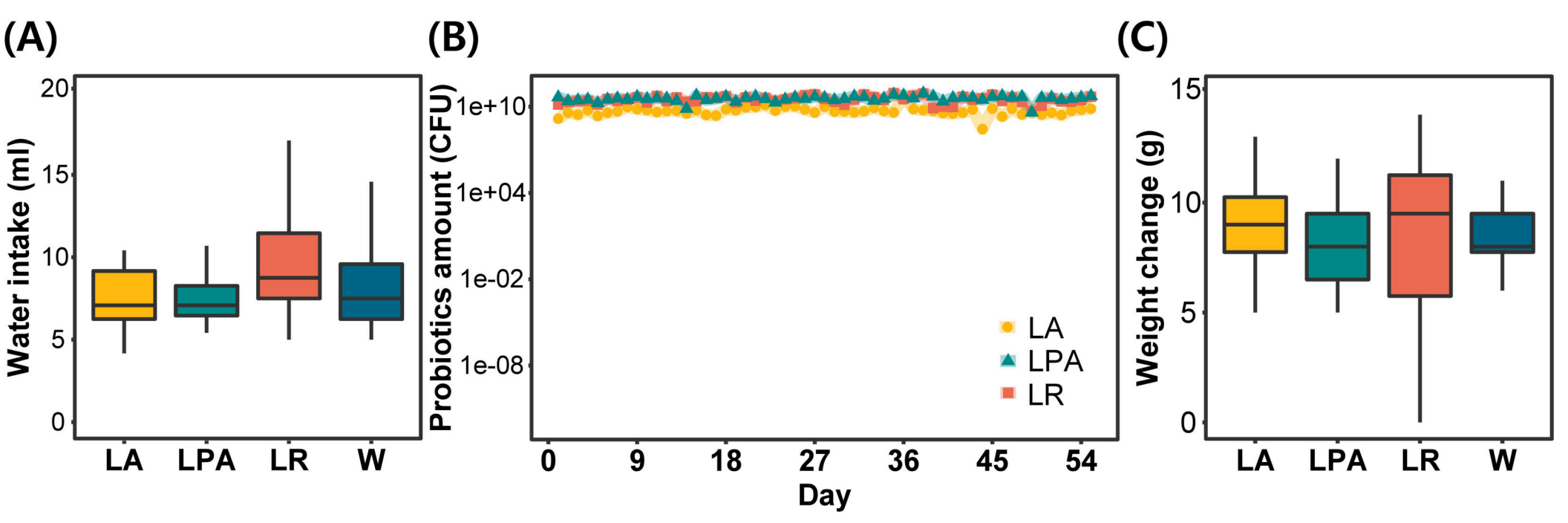


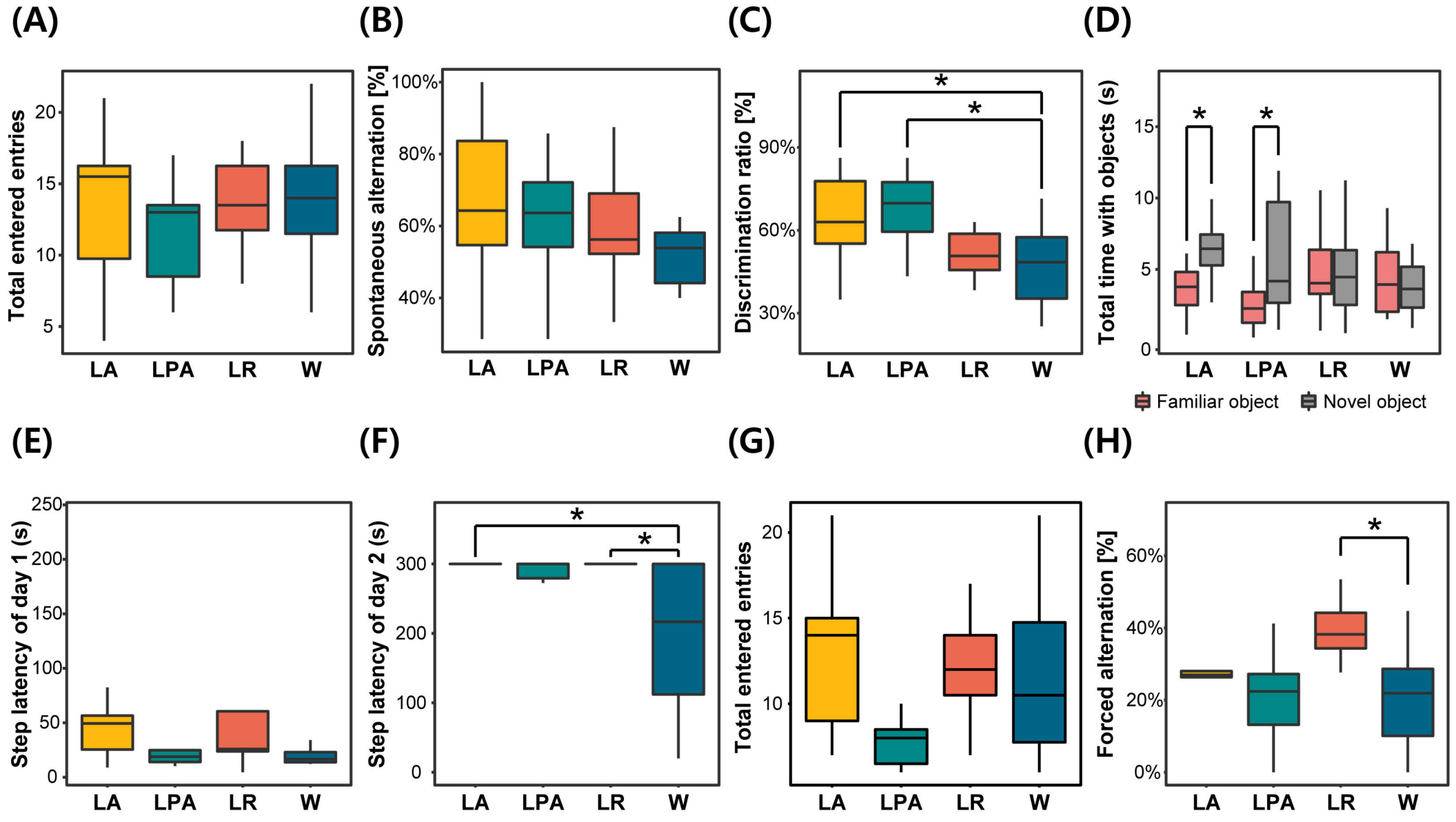
858

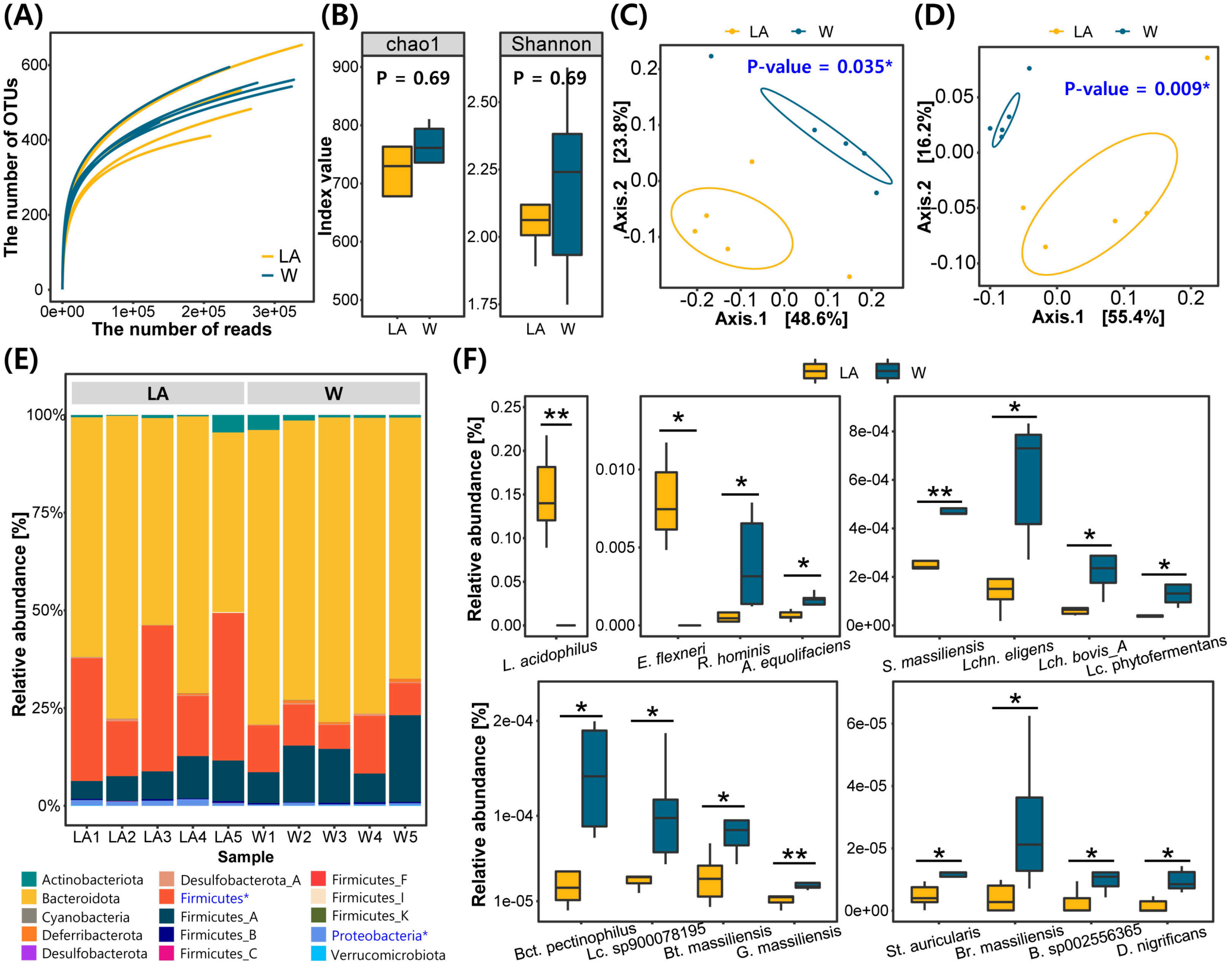
859 **Figure 7. Genomic comparison of 3 probiotic strains**

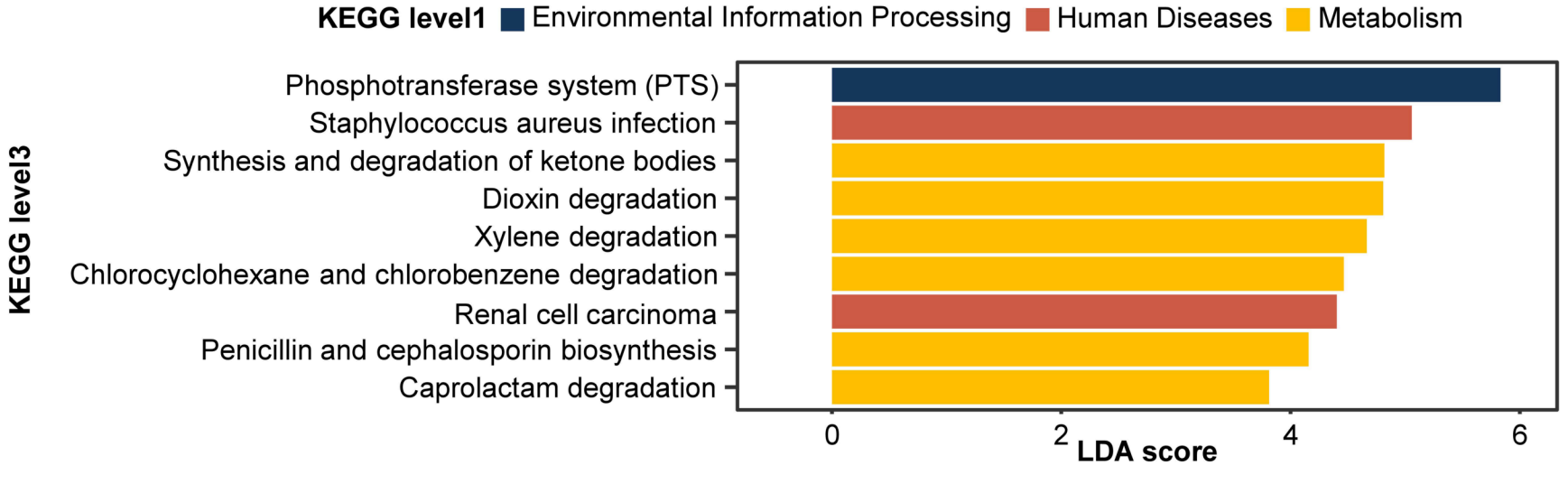
860 (A) Functional classification of protein coding sequences. All predicted protein sequences were
861 classified by categories by SEED system. (B) Subcategories in [Fatty Acids, Lipids, and
862 Isoprenolds] and [Amino Acids and Derivatives]. [Fatty Acids, Lipids, and Isoprenolds]
863 subcategory showed yellow-green colored head and [Amino Acids and Derivatives] category
864 presented light gray colored head.

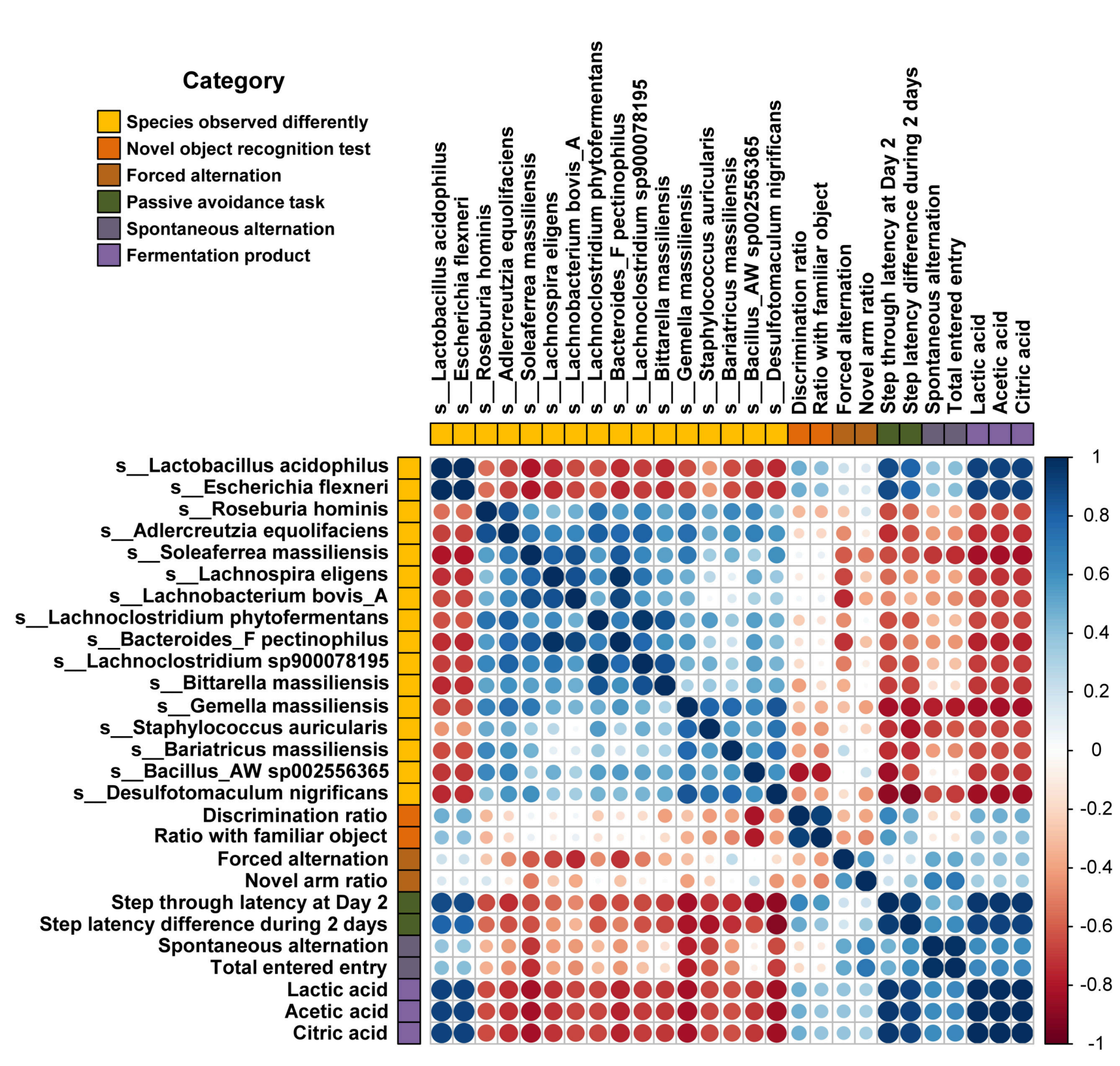

a
b
c
Alternation trial
: 5 min
Habituation trial
: 5 min
Training trial
: 3s on day 1
Test trial
: day 2
SA
PAT
Day
Week
0
1
2
3
4
5
6
7
8
NOR
FA
Habituation trial
: 10 min
Sample trial
: 5 min
Interval time
: 60 min
Novel object trial
: 5 min
Sample trial
: 5 min
Alternation trial
: 5 min
Probiotics feeding
Behavioral test
Weight measurement
Fecal collection
Metagenome sequencing and analysis of microbial composition

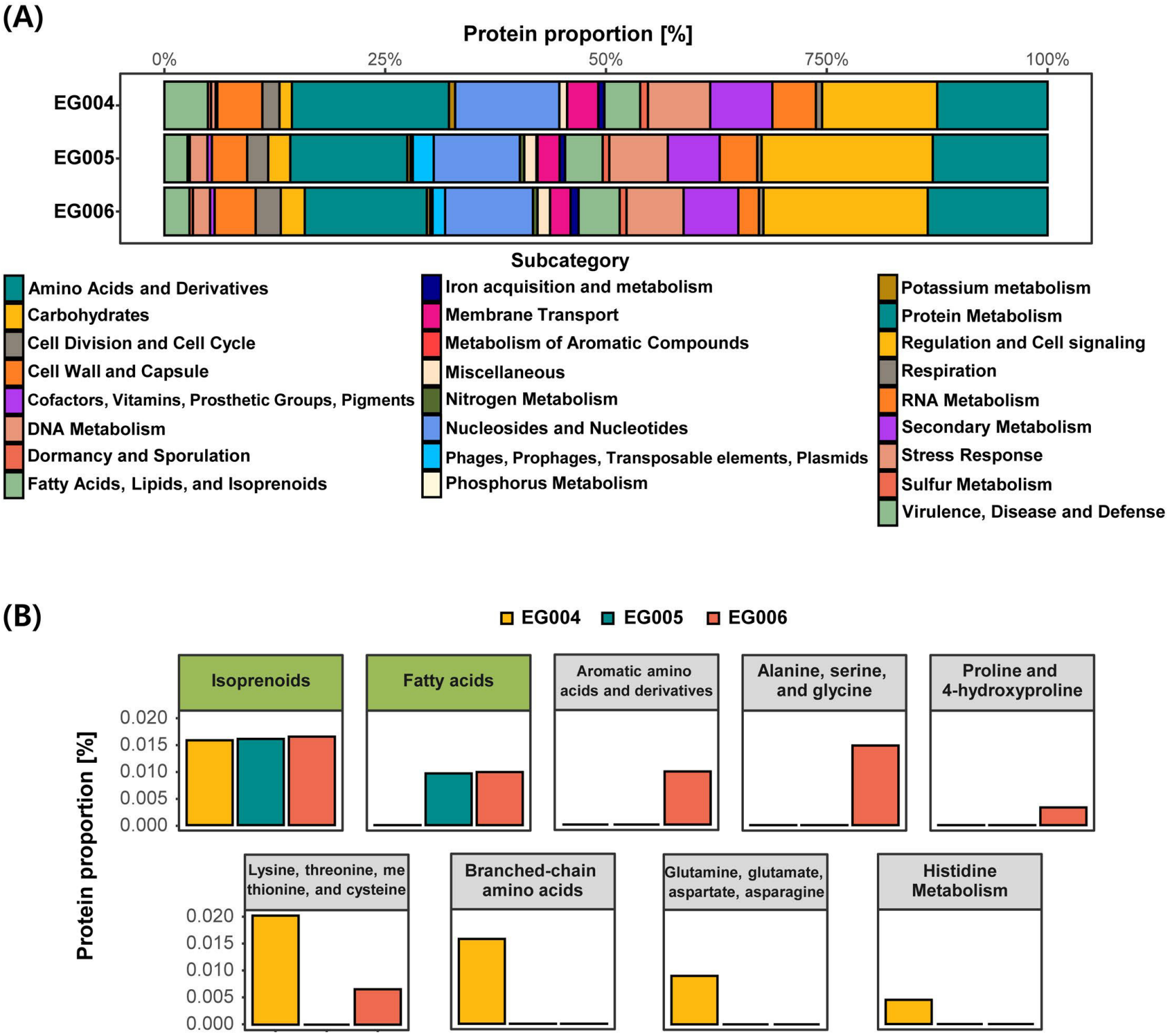

(A)
Water intake (ml)
20
15
10
5
0
LA
LPA
LR
W
(B)
Probiotics amount (CFU)
1e+10
1e+04
1e-02
1e-08
LA
LPA
LR
0
9
18
27
36
45
54
Day
(C)
Weight change (g)
15
10
5
0
LA
LPA
LR
W


(A)
Total entered entries
20
15
10
5
LA
LPA
LR
W
(B)
Spontaneous alternation [%]
100%
80%
60%
40%
LA
LPA
LR
W
(C)
Discrimination ratio [%]
90%
60%
30%
*
*
LA
LPA
LR
W
(D)
Total time with objects (s)
15
10
5
0
*
*
LA
LPA
LR
W
Familiar object
Novel object
(E)
Step latency of day 1 (s)
250
200
150
100
50
0
LA
LPA
LR
W
(F)
Step latency of day 2 (s)
300
200
100
0
*
*
LA
LPA
LR
W
(G)
Total entered entries
20
15
10
LA
LPA
LR
W
(H)
Forced alternation [%]
60%
40%
20%
0%
*
LA
LPA
LR
W


(A)
The number of OTUs
The number of reads
LA
W
(B)
chao1
Shannon
P = 0.69
P = 0.69
Index value
LA W
(C)
LA W
P-value = 0.035*
Axis.2 [23.8%]
Axis.1 [48.6%]
(D)
LA W
P-value = 0.009*
Axis.2 [16.2%]
Axis.1 [55.4%]
(E)
LA
W
Relative abundance [%]
Sample
LA1 LA2 LA3 LA4 LA5 W1 W2 W3 W4 W5
Actinobacteriota
Bacteroidota
Cyanobacteria
Deferribacterota
Desulfobacterota
Desulfobacterota_A
Firmicutes*
Firmicutes_A
Firmicutes_B
Firmicutes_C
Firmicutes_F
Firmicutes_I
Firmicutes_K
Proteobacteria*
Verrucomicrobiota
(F)
LA W
Relative abundance [%]
L. acidophilus
E. flexneri
R. hominis
A. equolifaciens
S. massiliensis
Lchn. eligens
Lch. bovis_A
Lc. phytofermentans
Bct. pectinophilus
Lc. sp900078195
Bt. massiliensis
G. massiliensis
St. auricularis
Br. massiliensis
B. sp002556365
D. nigrificans


KEGG level1
Environmental Information Processing
Human Diseases
Metabolism
KEGG level3
Phosphotransferase system (PTS)
Staphylococcus aureus infection
Synthesis and degradation of ketone bodies
Dioxin degradation
Xylene degradation
Chlorocyclohexane and chlorobenzene degradation
Renal cell carcinoma
Penicillin and cephalosporin biosynthesis
Caprolactam degradation
0
2
4
6
LDA score


Category
Species observed differently
Novel object recognition test
Forced alternation
Passive avoidance task
Spontaneous alternation
Fermentation product
s__Lactobacillus acidophilus
s__Escherichia flexneri
s__Roseburia hominis
s__Adlercreutzia equolifaciens
s__Soleaferrea massiliensis
s__Lachnospira eligens
s__Lachnobacterium bovis_A
s__Lachnoclostridium phytofermentans
s__Bacteroides_F pectinophilus
s__Lachnoclostridium sp900078195
s__Bittarella massiliensis
s__Gemella massiliensis
s__Staphylococcus auricularis
s__Bariatricus massiliensis
s__Bacillus_AW sp002556365
s__Desulfotomaculum nigrificans
Discrimination ratio
Ratio with familiar object
Forced alternation
Novel arm ratio
Step through latency at Day 2
Step latency difference during 2 days
Spontaneous alternation
Total entered entry
Lactic acid
Acetic acid
Citric acid
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1


(A)
Protein proportion [%]
0%
25%
50%
750%
100%
EG004
EG005
EG006
Subcategory
Amino Acids and Derivatives
Carbohydrates
Cell Division and Cell Cycle
Cell Wall and Capsule
Cofactors, Vitamins, Prosthetic Groups, Pigments
DNA Metabolism
Dormancy and Sporulation
Fatty Acids, Lipids, and Isoprenoids
Iron acquisition and metabolism
Membrane Transport
Metabolism of Aromatic Compounds
Miscellaneous
Nitrogen Metabolism
Nucleosides and Nucleotides
Phages, Prophages, Transposable elements, Plasmids
Phosphorus Metabolism
Potassium metabolism
Protein Metabolism
Regulation and Cell signaling
Respiration
RNA Metabolism
Secondary Metabolism
Stress Response
Sulfur Metabolism
Virulence, Disease and Defense
(B)
EG004
EG005
EG006
Protein proportion [%]
Isoprenoids
Fatty acids
Aromatic amino acids and derivatives
Alanine, serine, and glycine
Proline and 4-hydroxyproline
Lysine, threonine, methionine, and cysteine
Branched-chain amino acids
Glutamine, glutamate, aspartate, asparagine
Histidine Metabolism
0.020
0.015
0.010
0.005
0.000

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Republic of Korea

Brief Rebuttal to the remarks of the reviewer3

Comment #1 of the reviewer3: The metagenome sequencing data (16S-23S rRNA) should be submitted to GenBank if it is not submitted yet.

Amendment for comment #1
We thank the reviewer for raising this issue. As the reviewer's comment, we have finished uploading the metagenome sequencing data and whole-genome sequence data of *Lcb. paracasei* EG005 and *Lcb. rhamnosus* EG006. For readers and other researchers' access to data, we added a 'Data availability' session with NCBI accession numbers (***Line 636-640, page 31-32***). Circularized genomes of the three probiotics were added with annotation information in the supplementary information (***Supplementary_data, page 5-7***). We expect that this will give more credit to our research and provide a new application to other researchers.

Comment #2 of the reviewer3: The aim of the paper is to study effect on cognitive ability of Lactobacillus acidophilus EG004 in healthy mouse and fecal microbiome analysis using full-length 16S-23S rRNA metagenome sequencing.

In the manuscript, the authors studied a bacterial strain Lactobacillus acidophilus EG004 with a positive effect on cognitive ability using a healthy animal model. The authors experimentally verified improved cognitive ability by cognitive behavioral tests. The authors performed full 16S-23S rRNA sequencing and provided gut microbiome composition at a species level. The provided microbiome composition consisted of candidate microbial groups as a biomarker that shows positive effects on cognitive ability. Therefore, their study suggests a new perspective for probiotic strain use applicable for medicine.

The uniqueness of the text is 90% by AntyPlagiarism.net.

The manuscript is written well. English is proper, well understandable.

Reviewer has some comments:

- Line 74 - most of researches were - should be - most of the researches was.
- Line 73 - many researches - should be - many pieces of research.
- Line 82 - industrialization process - should be - industrialization processes.
- Line 105 - for the sentence - Autism, Alzheimer's disease, and Parkinson's disease (7-9) - add additional citation (Danilenko et al., 2021) and add to the References - Danilenko, V.N., Devyatkin, A.V., Marsova, M.V., Shibilova, M.U., Ilyasov, R.A., Shmyrev, V.I., 2021b. Common inflammatory mechanisms in COVID-19 and Parkinson's diseases: the role of microbiome and probiotics in their prevention. Journal of Inflammation Research 14, (In press). doi: 10.2147/JIR.S333887.
- Line 108 -to the sentence - the neural pathways of the brain-gut axis (10). - add additional citation (Fetissov et al., 2019). and

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add to the References - Fetissov, S.O., Averina, O.V., Danilenko, V.N., 2019. Neuropeptides in the microbiota-brain axis and feeding behavior in autism spectrum disorder. Nutrition 61, 43-48. doi: 10.1016/j.nut.2018.10.030.

- Line 112 - Second, the second suggestion - should be - Second, the suggestion
- Line 113 - microbiome affect brain - should be - microbiome affects brain.
- Line 113 - metabolic pathway - should be - metabolic pathways.
- Line 127 - remove one dot.
- Line 153 - The averages daily - should be - The averages of daily.
- Line 168 - In the comparison of - should be - The comparison of.
- Line 194 - light room - should be - lightroom.
- Line 195 - remove italics of the word - group.
- Line 226 - comparison - should be - comparative.
- Line 236 - familiae - should be - families.
- Line 275 - whole genome - should be - whole-genome.
- Line 308 - recognition test and passive avoidance task - should be - recognition tests and passive avoidance tasks.
- Line 321 - were - should be - was.
- Line 343 - factor - should be - factors.
- Line 350 - purpose - should be - purposes.
- Line 370 - these evidences - should be - this evidence.
- Line 398 - negative effect - should be - negative effects.
- Line 408 - to provide - should be - provide.
- Line 413 - These analyses were not covered to identification of a biological factor caused - should be - These analyses were not covered in the identification of a biological factor that caused.
- Line 416 - probiotics ingestion - should be - probiotic ingestion.
- Line 444 - by - should be - at.
- Line 442 - with - should be - at.
- Line 453 - add space after dot.
- Line 457 - from probiotics intake - should be - after probiotic intake.
- Line 459 - room condition - should be - room conditions.
- Line 472 - rodent's habit - should be - rodents' habits.
- Line 478 - entered - should be - that entered.
- Line 486 - preference - should be - preferences.
- Line 516 - After 1 minute for adaptation - should be - After 1 minute of adaptation.
- Line 531 - time taken - should be - time is taken.
- Line 554 - correction - should be - corrections.
- Line 789 - statistic - should be - statistics.

Please check English by professional translator one more times.

In further authors should study details of biological factors and molecular mechanisms that caused improved cognitive ability in mice after treatment with L. acidophilus EG004 strain.

Amendment for comment #2

Thank you for reading carefully and giving us kind advice. This is the kindest comment we've ever received. Based on the reviewer's comments, we revised the manuscript. However, the paper the reviewer recommended was not found because the paper was not published yet. So, we added another paper that indicated the relationship between the gut microbiome and Parkinson's disease (Danilenko VN, Stavrovskaya AV, Voronkov DN, Gushchina AS, Marsova MV, Yamshchikova NG, Ol'shansky AS, Ivanov M, Ivanov M, Illarioshkin SNJAoC, Neurology E. 2020. The use of a pharmabiotic based on the Lactobacillus fermentum U-21 strain to modulate the neurodegenerative process in an experimental model of Parkinson disease). We expect it to help the readers understand our contents. To deliver accurately, grammatical errors have been corrected throughout the entire manuscript again. As the reviewer mentioned, we are designing a further

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study including measurement of metabolite changes to understand the biological mechanism accurately. We hope to report positive results again in the near future.

November 29, 2021

Prof. Heebal Kim
Seoul National University
Seoul
Korea (South), Republic of

Re: Spectrum01815-21R1 (Positive effect on cognitive ability of Lactobacillus acidophilus EG004 in healthy mouse and fecal microbiome analysis using full-length 16S-23S rRNA metagenome sequencing)

Dear Prof. Heebal Kim:

Thank you for submitting your manuscript to Microbiology Spectrum. As you will see your paper is very close to acceptance. Please modify the manuscript along the lines recommended by Reviewer 3 (see below). As these revisions are quite minor, I expect that you should be able to turn in the revised paper in less than 30 days, if not sooner. If your manuscript was reviewed, you will find the reviewers' comments below.

When submitting the revised version of your paper, please provide (1) point-by-point responses to the issues I raised in your cover letter, and (2) a PDF file that indicates the changes from the original submission (by highlighting or underlining the changes) as file type "Marked Up Manuscript - For Review Only". Please use this link to submit your revised manuscript. Detailed instructions on submitting your revised paper are below.

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Sincerely,

Jan Claesen

Editor, Microbiology Spectrum

Reviewer comments:

Reviewer #3 (Comments for the Author):

Reviewer comments

Manuscript: Spectrum01815-21R1 Positive effect on cognitive ability of Lactobacillus acidophilus EG004 in healthy mouse and fecal microbiome analysis using full-length 16S-23S rRNA metagenome sequencing

The manuscript was improved but I have some questions:

Line 54 - Cognition is one of the functions of the brain. The authors should write in the Manuscript the idea that they study bacterial strain that has positive effects on brain function, which can be recognized through changes in cognitive processes.
Line 68 - In the annotation, you do not say a word about strains EG005 and EG006. Why? Also, add into the discussion part more information about comparison and differences in the action of these three strains. Explain the reasons for these differences.
Line 130 - will be better if you use the word - healing effects
Line 150 - what kind of molecular method? add the explanation into the text.
Line 390 - you wrote - that the antibacterial activity of L. acidophilus EG004 was the potential reason for cognitive ability enhancement. - how it is possible? Why do you assume this?
Line 407 - Line 54 - Cognition is one of the functions of the brain. The authors should write in the Manuscript the idea that they study bacterial strain that has positive effects on brain function, which can be recognized through changes in cognitive processes.

Line 421 - why male?
Line 605 - Why is EG004 do not present here?
Line 623 - Add here the information from Data availability - The complete sequences of Lcb. paracasei EG005 and Lcb. rhamnosus EG006 is available in the NCBI database with accession numbers, SAMN23227569 and SAMN23227570, respectively. The metagenomic sequences are available in the NCBI database under the accession number PRJNA781018.
Please answer my question and add information to the Manuscript.

I added the PDF file with highlighted comments.
No other comments.
A minor revision is required.

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• Manuscript: A .DOC version of the revised manuscript
• Figures: Editable, high-resolution, individual figure files are required at revision, TIFF or EPS files are preferred

For complete guidelines on revision requirements, please see the journal Submission and Review Process requirements at https://journals.asm.org/journal/Spectrum/submission-review-process. **Submissions of a paper that does not conform to Microbiology Spectrum guidelines will delay acceptance of your manuscript.** "

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Title

Positive effect on cognitive ability of *Lactobacillus acidophilus* EG004 in healthy mouse and fecal microbiome analysis using full-length 16S-23S rRNA metagenome sequencing

Running title

Positive effect on cognitive ability of *L. acidophilus*

Authors

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Word count

Abstract; 249 words

Text; 4,245 words (excluding materials and methods) and 6,952 words (including materials and methods)

Abstract

The concept of the ‘Gut-brain axis’ has risen. Many types of research demonstrated the effect and mechanism of the GBA. Although many studies have been reported, most of the studies are focused on neurodegenerative disease and it is still not clear what type of bacterial strains have positive effects on the brain. Therefore, we designed an experiment to discover a strain that positively affects cognitive ability using healthy mice. The experimental group consisted of a control group and three probiotic consumption groups, *Lactobacillus acidophilus*, *Lacticaseibacillus paracasei*, and *Lacticaseibacillus rhamnosus*, which are verified to have beneficial effects for host health as the gut microbiome. Cognitive ability was measured by 4 cognitive-behavioral tests and the group fed on *L. acidophilus* showed the most improved cognitive ability. To provide an understanding of the altered microbial composition effect on the brain, we performed full 16S-23S rRNA sequencing using Nanopore, and OTUs were identified at a species level. In the group fed on *L. acidophilus*, the intestinal bacterial ratio of Firmicutes and Proteobacteria phyla increased and the bacterial proportions of 16 species were significantly different from those of the control group. We estimated that the positive results on the cognitive behavioral tests were due to the increased proportion of *L. acidophilus* EG004 strain in the subjects’ intestines since the strain is capable of producing butyrate and therefore modulating neurotransmitters and neurotrophic factors. We expect that our new strain expands the industrial field of *L. acidophilus* and helps understand the mechanism of the brain-gut axis.

Importance

In recent, the concept of 'gut-brain axis' has risen that microbes in the GI tract affect brain by

modulating signal molecules. Although many pieces of research were reported in a short period, a signaling mechanism and effect of a specific bacterial strain are still unclear. Besides, since most of the researches was focused on neurodegenerative disease, the study with a healthy animal model is still insufficient. In this study, we provide a bacterial strain (*Lactobacillus acidophilus* EG004) with a positive effect on cognitive ability using a healthy animal model. We experimentally verified improved cognitive ability by cognitive behavioral tests. We performed full 16S-23S rRNA sequencing using Nanopore MinION, and provided gut microbiome composition at a species level. The provided microbiome composition consisted of candidate microbial groups as a biomarker that shows positive effects on cognitive ability. Therefore, our study suggests a new perspective for probiotic strain use applicable for various industrialization processes.

Keywords

Lactobacillus acidophilus, gut microbiome, gut-brain axis, cognitive ability, Nanopore sequencing

Introduction

The human body is a complex community that habituates various bacteria. Among the bacterial communities in the human body, the gastrointestinal tract is the best bacterial community that has the most abundant and various bacteria (1). In 2006, having been released research that obesity is associated with bacterial composition in the gut, a study for gut microbiome began in earnest (2). The gut microbiome is defined as the collective genomes of microorganisms that live in the gastrointestinal tract. Functions of the gut microbiome have been reported such as nutrient metabolism and regulation of the immune system for the host (3). Microbial composition in the gut is altered by environmental factors like age, diet, stress, and lifestyle, and the change in microbial composition can induce physical changes in the host (4). In recent, the gut microbiome's effects on the brain have been proved and the concept of the brain-gut axis has risen to the surface (5). The brain-gut axis is a complex system involving the enteric nervous system and central nervous system including the brain and spinal cord, and it works with bidirectional communication between the central and the enteric nervous system (6). Although the brain is located apart from the gut, the gut microbiome can affect the brain by stimulating the enteric nervous system and vagus nerve. Thus, dysbiosis of the gut microbiome often causes brain diseases. The recent experimental results described that gut microbiome dysbiosis was observed in patients with Autism, Alzheimer's disease, and Parkinson's disease (7-10). At the same time, studies on the mechanisms to understand the brain-gut axis have been conducted. First, it was suggested that the microbial-derived metabolites are the main components acting on the neural pathways of the brain-gut axis (11, 12). The most well-studied substances are short-chain fatty acids (SCFA) such as acetate, propionate, and butyrate, which are produced in the process of decomposing non-digestible fibers and carbohydrates (13). It promotes indirect

signaling to the brain by modulation and induction of neurotransmitter and neurotrophic factors like γ-aminobutyric acid (GABA) and Brain-derived neurotrophic factor (BNDF). Second, the suggestion was that the gut microbiome affects brain function by regulating metabolic pathways (14). Previous research reported that the level of tryptophan metabolites including serotonin and indolepyruvate was altered by the gut microbiome. These metabolites have roles in the functioning of the gut-brain axis such as signaling and anti-oxidant. Third, the gut microbiome may affect the brain by immune pathway (15). Interferon (IFN), Tumor necrosis factor (TNF), and Interleukin are well-known immune factors. According to recent studies, the amount of the immune factors is regulated by the intestinal microflora. These immune factors affect brain function by stimulating and activating the hypothalamic-pituitary-adrenal axis. Finally, it was suggested that gut microbes directly influence the brain by altering the fatty acid composition of the brain (16). Several studies have been reported on the correlation between intestinal microbes and the brain, but the specific mechanism of the brain-gut axis is still not clear.

Probiotics are defined as bacteria that have positive effects on the host body (17). Probiotics have been widely used as a health supplement since it has various beneficial functions to host's health with high adhesion property to the intestine and low side effect. Most probiotics include bacteria genera that are gram-positive, facultative anaerobic and rod-shaped. *Lacticaseibacillus rhamnosus* (*Lcb. rhamnosus*) is one of the longest-studied probiotic species, and many strains such as LGG and GR-1 belonging to this genus are commercially available. It is well known that *Lcb. rhamnosus* has positive effects on diarrhea, acute gastroenteritis, and atopic dermatitis (18-20). Recently, its neurobehavioral effects such as anxiety and depression relief have been reported (21). *Lacticaseibacillus paracasei* (*Lcb. paracasei*) is one of the representative probiotic species, and it has been studied to be effective in treating ulcerative colitis and allergic rhinitis

(22, 23). In a recent study, an effect on age-related cognitive decline and a stress relief effect was reported with several strains of this species (24). *Lactobacillus acidophilus* (*L. acidophilus*) is another representative probiotic strain. This strain lowers cholesterol levels and has beneficial health effects such as antibacterial effects against harmful bacteria like *Streptococcus mutans* and *Salmonella typhimurium* (25, 26).

In this study, we aimed to present a new strain that has an enhancing effect on cognitive ability through the brain-gut axis and provide an additional understanding of the brain-gut axis. Three probiotic strains, *L. acidophilus*, *Lcb. paracasei*, and *Lcb. rhamnosus*, which have previously demonstrated beneficial effects to the host as one of the gut-microbiome strains, were used to confirm their positive effects on cognitive ability. Full 16S and 23S rRNA sequencing was performed to annotate the gut microbiome at a species level for downstream analysis. We expect our results to provide an understanding of the role of the gut microbiome.

Results

Bacterial and animal treatments

Three probiotic strains, *L. acidophilus* EG004, *Lcb. paracasei* EG005, and *Lcb. rhamnosus* EG006, have been identified by the molecular method. These strains were clustered with available *L. acidophilus*, *Lcb. paracasei*, and *Lcb. rhamnosus* strains, respectively, in a phylogenetic tree of 16S rRNA gene (Figure S1). Probiotic strains were consumed by mice for 8 weeks with assessments of cognitive ability (Figure 1). The averages of daily water intake per subject were similar between groups (Figure 2A). Daily probiotic intakes were maintained constantly and the average amount of *L. acidophilus* group, *Lcb. paracasei* group, and *Lcb. rhamnosus* group were calculated as (7.82E09 ± 1.95E09), (4.37E10±5.17E09), and (3.74E10±3.98E09) CFUs (Figure 2B). To identify the additional effect of probiotics, the body weights of mice were measured every week (Figure 2C and S2). Patterns of weight gain in the 4 groups were similar for 8 weeks. The mean body weight gains of the control group showed the highest value, which was 9.08 g. *Lcb. paracasei* group showed a significant difference from the control group with P-value under 0.05 in the second measurement, but the difference was immediately recovered. Similar to weekly weight change, statistical significance was not found in accumulated weight between experimental groups for 8 weeks.

Cognitive behavioral tests

Spontaneous alternation test was conducted to assess spatial learning and short-term memory. Although the average number of the total entries to each arm in *Lcb. paracasei* group

was slightly low, the difference between groups was not found (Figure 3A). The comparison of the mouse ratio showed spontaneous alternation for the first 3 entries, *L. acidophilus* group showed the highest value as 75.0%. (Table S1). In spontaneous alternation, the average values of probiotics-fed groups were higher than the value of the vehicle-fed group (Figure 3B). Among the 4 experimental groups, *L. acidophilus* group showed the highest alternation ratio. Wilcoxon rank-sum test was performed to identify statistical significance, but there was no statistical difference between the experimental groups and control group.

Novel object recognition (NOR) test was performed to evaluate long-term and explicit memory using 4 different features (Figure 3C, 3D, and Table S1). *L. acidophilus* group exhibited the highest average ratio of mouse that touched the novel object before the familiar object, whereas *Lcb. rhamnosus* group showed the lowest value under the control group. At discrimination ratio comparison, the three probiotics-fed groups showed higher average values than the control, and *L. acidophilus* group showed the highest values. To identify if there is a significant difference, Wilcoxon rank-sum test was performed. When compared to the vehicle-fed group, *L. acidophilus* and *Lcb. paracasei* groups displayed statistically significant differences with the adjusted P-value of 0.037. To identify animal behavior detail, the number of objects touch and the total time of object observation in each group were compared. In a comparison of object touch, statistical differences were significant in *L. acidophilus* and *Lcb. paracasei* groups with P-values of 0.031 and 0.042, respectively. Also, *L. acidophilus* group had a significant difference between the time taken to observe the familiar object and the novel object.

Passive avoidance task was conducted to measure long-term and implicit memory. Step-through latency was used to compare the mean difference between the experimental groups.

Most of the subjects were transferred into a darkroom for a minute on day 1 (Figure 3E). Only 3 animals took over 100 seconds to get into the darkroom. The difference between the experimental group and the control was not found on day 1. When compared to the latency time on day 1, the average latency time increased on day 2, and unexpectedly, 26 animals stayed in the lightroom for over 300 seconds (Figure 3F). *Lcb. rhamnosus* group presented the highest average latency time, followed by *L. acidophilus* group while the control group showed the lowest average (Table S1). The Mann-Whitney U test was conducted to check the mean difference, the P-values of *L. acidophilus* and *Lcb. rhamnosus* groups were less than 0.05 compared to the control group. The adjusted P values of both groups were 0.040.

To assess spatial learning and long-term memory, forced alternation was conducted. Memory was evaluated by forced alternation (%), the number of arms that the mouse entered, and the percentage of mice in a group that entered the novel arm as their first entry. While the total number of the entries into each arm was diverse, there was no significant difference between the experimental groups and control (Figure 3G). *L. acidophilus* group scored the highest ratio of mice entered the novel arm as their first entry (Table S1). Forced alternation values of *L. acidophilus* and *Lcb. rhamnosus* groups were higher than the value of the control group (Figure 3H). Forced alternation of *Lcb. rhamnosus* group and the control group had a significant difference with the adjusted P-value of 0.038.

Full 16S-23S rRNA sequencing and biological diversity

Metagenome sequencing was performed with *L. acidophilus* and control groups, which showed the most improvement in cognitive ability. We compared the microbial composition of

both groups. Gut microbial component information annotated at a species level was completely constructed by sequencing the entire 16S-23S rRNA of the mouse stool (Table 1). Averagely, 323870.0±84085.5 reads were generated from 10 stool samples. The total number of identified OTU was 252401.6±56284.7 in *L. acidophilus* group and 259945.6±78526.0 in the control group. The produced OTUs were annotated as a total of 528.4±90.4 species in *L. acidophilus* group and 539.8±55.4 species in the control group. To check the sufficiency of the sequencing depth for the analysis, a rarefaction curve was created (Figure 4A).

Alpha diversity was calculated to compare species richness within a group (Figure 4B). In the comparison of the two groups, no significant difference was found in Chao1 Shannon indexes. Beta diversity was measured to compare the diversity of the microbial community between the two groups (Figure 4C and D). It was confirmed that both beta diversity evaluations (Bray-Curtis and Unifrac distance) had significant differences.

Microbial composition

In the comparative analysis of microbial compositions, taxonomies with significantly different ratios were found between *L. acidophilus* group and the control group. At the phylum level, Bacteroidota accounted for the highest proportion in both groups, followed by Firmicutes (Figure 4E). Significant differences between the two groups were found in 2 of the 12 phyla (Firmicutes, Proteobacteria), all of which were high in *L. acidophilus* group. At the class level, Bacteroidia showed the highest proportion in both groups. Also, the proportion of Bacilli and Gammaproteobacteria classes were increased in *L. acidophilus* group when compared to the control group (Figure S3). At the order level, Bacteroidales showed the highest percentage in

both groups, and Lactobacillales and Enterobacterales orders were found to exhibit higher proportions in *L. acidophilus* group. At the family level, *Muribaculaceae* showed the highest proportion in both groups. It was found that 2 families (*Lactobacillaceae* and *Enterobacteriaceae*) showed increased proportions in *L. acidophilus* group, while a decreased percentage was observed in one family (*Ruminococcaceae*). In the Genus comparison, *Muribaculum* genus showed the highest ratio in the two groups, and 12 genera showed differences between groups. Three genera showed an increased proportion in the experimental group, whereas 9 genera showed higher mean values in the control group. The genus increased in *L. acidophilus* group were *Lactobacillus*, *Staphylococcus_A*, and *Escherichia*, whereas the genera decreased in *L. acidophilus* group were *Bacteroides_F*, *Desulfotomaculum*, *Lachnobacterium*, *Bittarella*, *Agathobacter*, *Roseburia*, *Bariatricus*, and *Lachnospirarea*. At the Species level, *Muribaculum intestinale* was found to account for the largest proportion, with over 50% in both groups. Following *M. intestinale*, the species *Lactobacillus acidophilus*, *Lactobacillus johnsonii*, *Lactobacillus_B murinus*, and *Lactobacillus_H reuteri* were found with a high proportion in *L. acidophilus* group, while *Lactobacillus_B murinus*, *Bacteroides_B vulgatus*, *Faecalibaculum rodentium*, and *Kineothrix alysoides* species showed a high proportion in the control group. No unique bacterial species were found in either of the two groups. Seventeen species showed differences between groups, and it was confirmed that the proportions of *L. acidophilus* and *E. flexneri* were increased in *L. acidophilus* group (Figure 4F).

Functional profiling and correlation analysis

Functional profiling was performed at the KEGG level 3 to estimate the effect of the

differential composition of intestinal microbes on the mice (Figure 5). By calculating the LDA score, it was confirmed that the two groups showed significantly different patterns in 9 categories. All nine categories were predicted to be more activated in *L. acidophilus* group. The Phosphotransferase system (PTS) scored the highest, followed by *Staphylococcus aureus* infection, Synthesis and degradation of ketone bodies.

To further estimate the influence of the altered gut microbiota, Spearman's correlation analysis of cognitive-behavioral abilities and bacterial OTUs, and fermentation products were performed (Figure 6). *L. acidophilus* and *E. flexneri* showed a positive correlation with all assessments of cognitive abilities, while the other 14 OTUs presented a negative correlation. In particular, step-through latency at Day 2 and Step latency difference for 2 days of the PAT results showed a significant negative correlation with the *Gemella massiliensis* ($r = -0.8379$, $p = 0.03248$ and $r = -0.8182$, $p = 0.0376$) and *Desulfotomaculum nigrificans* ($r = -0.8781$, $p = 0.01914$ and $r = -0.8450$, $p = 0.03225$).

To provide evidence to indirectly infer the mechanism of action of the gut microbiome, the concentration of SCFA in the microbial culture was measured (Table S2). Lactic acid and acetic acid were found in three microbial cultures. Lactic acid was identified in the highest concentration in *Lcb. paracasei* EG005, and acetic acid was included in the highest concentration in *L. acidophilus* EG004 culture. Propionate and butyrate were not within detectable ranges.

Comparative analysis of genetic contents in bacterial whole-genome sequences

To identify its safety and functionality, several genetic factors were detected. Fourteen genomic islands, two prophage regions, one CRISPR region, and three bacteriocins were found

in the genome of *L. acidophilus* EG004. In *Lcb. paracasei* EG005, 29 genomic islands, 7 prophage regions, 3 CRISPR regions, and 2 bacteriocins were detected (Figure S4-S6). In the case of *Lcb. rhamnosus* EG006, 23 genomic islands, 8 prophage regions, 3 CRISPR regions, and 1 bacteriocin were found in the genome. To estimate a genetic factor related to cognitive ability, protein annotation was conducted (Figure 7A). Protein metabolism, Carbohydrates, Amino acids and derivatives showed high proportions, but there was a difference in order by bacterial strains. Protein metabolism had the highest proportion in *L. acidophilus* EG004 and carbohydrates presented the highest proportion in *Lcb. paracasei* EG005 and *Lcb. rhamnosus* EG006. In a subcategory comparison of predicted functional sequences, a difference of genetic contents was found (Figure 7B). CDSs related to Fatty acids were found in the genomes of *Lcb. paracasei* EG005 and *Lcb. rhamnosus* EG006. Genes of 3 subcategories (Aromatic amino acids and derivatives, Alanine, serine, and glycine, and Proline and 4-hydroxyproline) were detected in *Lcb. rhamnosus* EG006, while genes of 3 other categories in Amino Acids in Derivatives were contained in only *L. acidophilus* EG004.

Discussion

As interest in Gut-Brain Axis has increased, many types of research in this criterion have been published. However, it is still unclear about the integral mechanism and which strain has a positive or negative effect. Therefore, we aimed to develop a new strain that has a positive effect on the host's cognition, and we found 3 strains that caused positive effects in 4 different cognitive tests (Figure 3). *Lcb. paracasei* group showed improved cognitive ability in the novel object recognition test. A previous study indicated that this bacterium prevents age-related cognitive decline and improves cognitive ability (24). Other strain, *Lcb. rhamnosus*, displayed improved cognitive ability in passive avoidance task and forced alternation test. Several studies demonstrated that *Lcb. rhamnosus* consumption could increase cognitive ability (27, 28). Similar to previous studies, we experimentally confirmed that *Lcb. paracasei* and *Lcb. rhamnosus* could enhance cognitive function. On the other hand, although it is indicated that *L. acidophilus* strain has a neuroprotective effect against traumatic brain injury, there was no experimental research related to its cognitive ability (29, 30). In our study, we identified that *L. acidophilus* group presented the highest classical measured values as well as incidental measured values in novel object recognition tests and passive avoidance tasks. This indicates that *L. acidophilus* is capable of improving cognitive ability comparable to that of previously reported strains. Our results will help further broaden the industrial field of *L. acidophilus*. In addition, although probiotic consumptions were carried out as the same method, three experimental groups showed improved cognitive ability in different tests. It implies that different probiotic strains affect cognitive ability by different mechanisms.

To understand the effect of the gut microbiome on the brain as our secondary goal, we performed gut microbiome analysis of *L. acidophilus* group, which showed the best cognitive improvement, along with the control group, The difference of species richness was not found in the comparison of alpha diversity, whereas the difference was found in the comparison of beta diversity (Figure 4B, 4C, and 4D). It represents that the number of OTUs constituting the two gut microbial communities is similar, but the composition of the OTUs is different. In the comparison of the two communities, significant differences were observed at all taxonomic levels except for the bacteria kingdom, which was mostly *L. acidophilus.* Naturally, *L. acidophilus* group was confirmed to show a significant increase in *L. acidophilus* abundance and ultimately show a high ratio of *L. acidophilus.* This indicates that a large amount of *L. acidophilus* is capable of safely reaching the intestines without being affected by digestive juices such as gastric acid and pancreatic enzymes.

We estimated that the positive effect on cognitive ability due to the increased proportion of *L. acidophilus* in the intestines was based on two rationales: modulation of neurotransmitters and neurotrophic factors and production of SCFAs. First, *L. acidophilus* modulates several types of neurotransmitters in the intestine. Microbial-derived intermediates, which affect the brain through gut epithelial and blood-brain barriers, are such as GABA (γ-aminobutyric acid), glutamate, dopamine, noradrenaline, serotonin (5-Hydroxytryptamine; 5-HT), and Brain-derived neurotrophic factor (BNDF). These neurotransmitters are synthesized from various amino acids. GABA and glutamate are produced from the gut microbiome such as *Bifidobacterium* and *Lactobacillus* (31). Glutamate has a role as a neurotransmitter by itself, and it is used at GABA synthesis (32). Dopamine and Noradrenaline are synthesized from specific amino acids such as tyrosine and phenylalanine (33). L-Tryptophan is a well-known precursor of serotonin (34).

Therefore, altered amino acid composition by the gut microbiome seems to affect the host's neurotransmitter synthesis. In the comparison of the functional protein genes, *L. acidophilus* EG004 showed a higher composition of the gene related to amino acid metabolism, than *Lcb. paracasei* EG005 and *Lcb. rhamnosus* EG006 showed (Figure 7A). Changes in intestinal amino acid composition caused by ingested *L. acidophilus* may have led to differences in cognitive ability. It has been proven that *L. acidophilus* consumption produces and up-regulates neurotransmitter and neurotrophic factors including GABA and serotonin (35-38). Thus, it is estimated that increased *L. acidophilus* EG004 in the gut modulates neurotransmitters and affects the animal's nerve system. Second, SCFAs, fermentation products of *L. acidophilus*, positively apply to brain function. For example, acetate, one of the short-chain fatty acids (SCFAs), promotes the activation of the parasympathetic nervous system (39). Also, it is indicated that acetate improved cognitive ability and neurogenesis in the hippocampus with increasing BDNF and IGF-1 levels as a glatiramer acetate form (40). Likewise, butyrate, a famous HDAC inhibitor, has been used for pharmacological purposes since lower global histone acetylation is a common phenomenon observed in many neurodegenerative diseases (41). Its therapeutic effect on neurodegenerative diseases including Parkinson's disease was verified, showing enhancement of neurotrophic factors and improvement in learning and memorizing (42). However, SCFAs are not produced until non-digestible carbohydrates reach the small intestine to be broken down by microbial metabolism, so it is not fully produced by the human digestive enzymes without specific microbes. *L. acidophilus* is a representative species that produces SCFAs through non-digestive carbohydrates, and it can be assumed that the intake of *L. acidophilus* EG004 caused the increase in SCFAs of the experimental mice's gut. The result of SCFA measurement in bacterial culture raises the possibility of this assumption (Table S2). Although it is different from

the metabolism in the gut since the SCFAs were measured in the medium to which glucose is the main energy source, it indirectly estimates its SCFA-producing ability. The result of functional profiling in our study also upholds this (Figure 5B). In the analysis of functional profiling, activation of genes of synthesis and degradation of ketone bodies was predicted by comparing it with control. The ketone body is one of the main fuels of the brain like lactate and butyrate, which is the main product of *L. acidophilus*, and is also capable of replacing glucose as an alternative fuel. Similar to butyrate mentioned earlier, ketone bodies modulate the brain with anti-oxidant reaction, energy supply, regulation of deacetylation activity, and regulation of the immune system. In recent studies, it is indicated that the increase of ketone body's concentration induces an alleviation effect on brain diseases such as epilepsy, Alzheimer's disease, and Parkinson's disease as well as memory improvement (43-45). Based on this evidence, ingested *L. acidophilus* EG004 in our experimental group seems to have produced SCFAs and modulated neurotransmitters, and *L. acidophilus*-derived metabolite would have raised cognitive ability. Although we did not measure microbial-derived metabolites, previous researches demonstrated that probiotic consumption leads to an increase of microbial-derived metabolites in the intestines.

Among detected species with the ratio difference, several species were indicated as important factors in the research of brain disease. *Adlercreutzia equolifaciens* is equol (phytoestrogen) producing bacteria, which obstructs microglial function. In previous studies, a higher ratio of *A. equolifaciens* was found in the gut of patients with Alzheimer's disease and Autism spectrum disorder (46, 47). In other studies, *Roseburia hominis* and *Bacteroides_F pectinophilus* were detected with a higher ratio in the patients with Alzheimer's disease than the normal persons (48, 49). When comparing gut microbiome between the Parkinson's disease group and normal group, *Soleaferrea massiliensis* was more frequently discovered in the patient

group (50). Interestingly, those strains that showed a high ratio from the previous studies of brain disease patients were found to show a lower ratio in *L. acidophilus* group when compared to the control group (Figure 4F). Decreased bacterial ratio related to brain diseases seems to positively affect cognitive ability and we believe that it is due to *L. acidophilus* consumption. As antibacterial activity is the essential property of probiotics, such activity of *L. acidophilus* against harmful and pathogenic bacteria has been reported. In our previous study, we proved that *L. acidophilus* EG004 is capable of demonstrating the antimicrobial activity (51). Therefore, we suggest that the antibacterial activity of *L. acidophilus* EG004 was the potential reason for cognitive ability enhancement.

In functional profiling analysis, we offered explainable factors for the microbial effect on the brain. Three KEGG categories were related to toxic chemical degradation: Dioxin degradation, Xylene degradation, and Caprolactam degradation (Figure 5B). Dioxin, a neurotoxin, can raise autism and neurodegenerative disease (52, 53). Xylene inhibits normal protein synthesis of neuronal function and induces instability in the neuronal membrane. When it is inhaled, psychological deficits can be caused (54, 55). These chemicals are noxious to the brain, so activation of these chemical degradations would have diminished negative effects in *L. acidophilus* group. Besides, two KEGG categories related to the immune system were found. One of them is *Staphylococcus aureus* infection, which is known to cause brain abscess. Since there have been many studies demonstrating that *L. acidophilus* has antimicrobial activity against *S. aureus*, activation of this category is thought to be due to an increase in the amount of *L. acidophilus*. The function of renal cell carcinoma was predicted in the experimental group. As it involves not only tumor suppressor genes such as VHL, GH, and BHD, but also oncogenes such

as MET and PRCC-TFE3, it seems to be necessary to confirm the exact mechanism and side effects.

The purpose of this study was to develop a new strain that can improve cognitive ability and provide an underlying biological mechanism affecting the brain by the gut microbiome. It is necessary to measure metabolite changes in order to provide an understanding of the mechanism of altered cognitive ability. However, altered metabolite from animal body was not fully identified. To overcome this limitation, we conducted the metagenome analysis, correlation analysis between cognitive ability and gut microbiome, measurement of SCFA producing ability, and whole-genome comparison analysis. These analyses were not covered in the identification of a biological factor that caused improved cognitive ability, but presented a group of genes and mechanisms that can infer the process. Although we did not provide direct evidence of phenotype changes caused by probiotic ingestion, we hope that our findings will help infer the process of the brain-gut axis.

Materials and Methods

Animals

4-week-old male C57BL/6 mice (n = 48, average weight 26g) were gained from YoungBio (Seongnam, Korea). All mice were housed in a group of four per cage under standard controlled laboratory conditions (temperature of 20±5℃, humidity of 55~60%) on a 12-h light/dark cycle (light on at 7:00 a.m.). Each group was constituted of 12 mice, and it was nurtured by distributing 4 mice to 3 cages. Twelve cages were located at random. All animals received *ad libitum* access to food. All animal experiments were performed following protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University, and the permission number is SNU-190607-4-3.

Bacterial treatment

The bacterial strains were isolated from fermented dairy foods. When identifying the brain-gut axis effect, the important factors to be considered were viability and adherence capacity. Therefore, we selected the species that are known to have adherence capacity in the GI tract, as well as the potential for gut-brain axis effect. To identify species of each strain, 16S rRNA genes were sequenced by Macrogen Inc. (Seoul, Korea) with 27F and 1492R primers. Obtained sequences were compared with sequences in the NCBI database using BLAST. The experiment was constituted with 4 groups; 3 experimental groups were fed on autoclaved tap water mixed with *L. acidophilus* EG004, *Lcb. paracasei* EG005, and *Lcb. rhamnosus* EG006, and a control group was fed on sterilized tap water. Each group consisted of 12 mice. Bacteria to delivery were

freshly cultivated every day. Probiotic colonies were sub-cultured into 5ml MRS broth for 8 hours. After the sub-culture, 3 probiotic strains were inoculated in 500 ml MRS broth for 16 hours. Cultivated cells were spun down by centrifugation at 4,000 rpm for 10 min. The supernatant was removed, and the pellet was suspended by 0.85 % NaCl solution. Re-suspended cells were centrifuged at 4,000 rpm for 10 min to remove medium ingredients. The washing process was conducted twice. Washed cells were dissolved into autoclaved tap water. The final cell concentration of vehicles was about 1.0E9 CFU/ml. To estimate the probiotics amount per day per subject, daily water intake and probiotic concentration in vehicles were recorded. Cell viability of probiotics was measured by serial dilution and spreading in MRS agar plate. The probiotics amount per day per subject was calculated as an average of daily water intake per subject, by multiplying the average of daily probiotic concentration.

Animal treatment

The animal experiment was designed to minimize animal stress. All animal treatment was described in Figure 1 by timeline. Four weeks old mice were allowed to habituate freely for acclimatization for 1 week. After a week, tap water and water mixed with probiotics were delivered every day. Water intake was monitored every day and body weight was measured every week. Evaluations of cognitive ability were conducted after 4 weeks after probiotic intake. Behavioral tests were conducted at least 2 days after the weight-measurement day to minimize the stress effect. Animals were carried to a behavioral test room to assimilate room conditions and were allowed to relax for 6 hours before any behavioral test. In order to reduce the variance of feeding time, the experimental order of the mice was distributed evenly. All apparatus and

objects for the behavioral tests were cleaned with 70 % ethanol and dried after every trial to remove odors and any clues. The mice were sacrificed at the end of 13 weeks after the evaluations of the cognitive behavior. Preliminary experiments were conducted to obtain appropriate experimental values under our experimental environmental conditions. The three to five experimental conditions referring to published results were tested in our laboratory, and the experimental conditions showing a value similar to the average value of the previous studies were determined.

Y maze (Spontaneous alternation; SA)

Short-term spatial memory was assessed with a Y maze apparatus. SA was used to measure rodents' habit to explore a new environment. The Y maze consisted of 3 identical arms that cross each other with 120° (JEUNGDO Bio & Plant Co., Ltd., Korea). Mice are laid in the middle of the Y maze facing a corner, not an arm. Each animal was allowed to freely navigate all three arms for 5 minutes and the animal's entries to any arm were recorded. An arm entry was determined as any instance when the whole body of the mouse entered the arm and navigated at least 70% of the space. The spatial memory was evaluated by spontaneous alternation, the number of arm entries, and the ratio of mice per group that entered spontaneous alternation during the first three entries. Spontaneous alternation was calculated as shown below.

$$\text{Spontaneous alternation [\%]} = \frac{\text{Number of spontaneous alternation}}{\text{Total number of arm entries} - 2} \times 100$$

Novel object recognition test (NOR)

Based on the concept that mice tend to prefer a new object over a familiar one, a novel object recognition test (NOR test) was performed in an open field (40×40×40 cm (W×D×H), JEUNGDO Bio & Plant Co., Ltd., Korea). Two objects for this test were selected showing similar preferences through the preference test. The test consisted of Sample trial (T1; 10 min), Interval time (IT; 60 min), and Novel object trial (T2; 5 min). In T1, 2 identical objects were located at 1/3 and 2/3 diagonal of the open field, respectively. The animal was laid facing the wall with the same distance to two objects, and was allowed to explore objects for 10 min. After exploration, the mouse came back to the cage and had a rest. In T2, objects were positioned at the same position as T1, but one of the objects was changed to a novel object. To measure the time taken to interact with objects, all experiment processes were recorded, and the exploration time was measured by Movavi software with 3 decimal places. It was recognized as significant only when the mouse approached facing the objects within 2.5 cm. Cases that the mouse climbed objects and individuals with exploration time less than 2 seconds were excluded. The results were presented as a discrimination ratio, the number of object touches, and the ratio of mouse that touched the novel object first before it touched the familiar object. The discrimination ratio was defined as the below equation.

$$\text{Discrimination ratio [\%]} = \frac{\text{Novel object interaction time}}{\text{Novel object interaction time + Familar object interaction time}} \times 100$$

Passive avoidance task (PAT)

The passive avoidance task is designed to evaluate inhibitory avoidance memory according to rodent habit that a mouse prefers dark environment naturally. Shuttle box (41×21×30 cm (W×D×H), JEUNGDO Bio & Plant Co., Ltd., Korea) is an apparatus made for the passive

avoidance task and consists of a bright chamber and a dark chamber which are separated by a sliding door. The floor of the chambers is made of stainless-steel grids to flow current. The test was conducted for 2 days; Acquisition (Day 1) and Test (Day 2). On day 1, a subject was put in the bright chamber facing the wall across the closed sliding door. After the mouse explored the bright chamber for 1 minute, and the moment the mouse was away from the door for over 100 mm, facing the wall not the door, the door was opened so that the mouse could freely enter and move around the dark chamber. Latency time was measured until the mouse entered the dark chamber completely. The door was closed when the animal entered the dark compartment wholly including its tail, and 0.25 mA electric shock was provided to the paws by steel grid for 3 seconds. To memorize the situation, the mouse was kept in the dark chamber for 30 seconds after the shock and returned to the home cage for 24 hours. On day 2, the mouse was laid again into the bright chamber. After 1 minute of adaptation, the sliding door was opened when the mouse faced the wall like day 1. Latency time was measured again until the mouse entered the dark chamber. If the animal rather stayed in the bright chamber for more than 300 seconds (which was the cut-off time), the experiment was completed. All experimental processes were recorded and the time was measured by the Movavi program with 3 decimal places.

Y maze (Forced alternation; FA)

Forced alternation was assessed with the same Y maze as described above. This test consisted of 3 phases; Training trial (T1; 5 min), Interval time (IT; 60 min), and Test trial (T2; 5 min). A mouse was placed at a starting arm of Y maze facing the wall. The subject freely explored the maze during T1, while an entry was blocked with white expanded polystyrene. After

the learning trial, the mouse was returned to the home cage and rested for 1 hour. In T2, the mouse was again placed into the starting arm without the plate blocking the novel entry, and explored all three arms. All movements of mice were recorded through video. Forced alternation was evaluated by the ratio of time spent in the novel arm compared to the whole experimental time, time is taken to first enter the novel arm, and the percentage of mice per group that entered the novel arm as their first entry. The case that the mouse passed at 2/3 of the arms was admitted as a valid entrance. An individual that showed no navigation of the maze or that had entered the arms less than 5 times was excluded.

Feces collection and cognitive ability evolution

After all cognitive assessments had been completed, 2-3 stool samples were taken from each experimental subject. Sterilized stainless-steel tweezers were used for fecal picking, tweezers were washed with 70% alcohol and dried sufficiently before collecting new samples. The fresh samples were immediately enclosed into a 1.5ml Eppendorf tube and were put on ice. Then, it was stored at -80 degrees Celsius until used for 16S rRNA sequencing.

In order to determine the group that showed the best increase in cognitive ability, a score was assigned to the cognitive ability evaluation item. The items used for evaluation are spontaneous alternation, group ratio of SA, discrimination ratio, group ratio of NOR, step latency at day 2, forced alternation, and group ratio of FA (Table S2). Scores were given in ascending order of ranking (1-4 points), and the group with the highest total was selected as the group with the highest cognitive ability increase.

Statistics

Data were analyzed by R studio. Ineligible data were cut based on the requirements mentioned above. Data normality was assessed using the Shapiro-Wilks test and homogeneity of variance was assessed using Levene's test. Wilcoxon rank-sum test and independence t-test was used to evaluate statistical significance between experimental groups. P-values were adjusted by the FDR method for multiple testing corrections. Statistical significance was set as P-value under 0.05. All data are expressed as mean ± SEM.

Full 16S-23S rRNA sequencing

To characterize the microbial community associated with measured cognitive assessment, metagenome sequencing of the 16S-23S rRNA gene was carried out by Oxford Nanopore MinION. Metagenome sequencing was performed for the control group and *L. acidophilus* group, which showed a significant difference from the control in the cognitive ability evaluation. Among the 12 stored stool samples of each group, 5 samples with sufficient amount for sequencing were selected. For library construction, gDNA was extracted from fecal samples using AccuPrep® Stool DNA extraction Kit (Bioneer, Daejeon, South Korea). To identify the quality of extracted gDNA, A260/A280 and A260/A230 absorbance were used with 0.7 % agarose gel electrophoresis. After performing quality control, selected samples were used for the library construction. Stool samples were lysed and bacterial cells were disrupted by Zirconia/Silica Beads and proteinase K. The sequencing library was prepared by 16S-26S rRNA PCR amplification with Nanopore Ligation Kit (SQK-LSK109, Nanopore, Oxford, UK) following the manufacturer's instructions. Purification and quality checks were conducted using

agencourt AMPure XP cleanup (Beckman Coulter, CA, USA), Quant-iT^{TM} PicoGreen^{TM} dsDNA Assay Kit (Invitrogen, Ireland), and 0.7% agarose gel. The PCR products were diluted and end-repaired using NEBNext FFPE Repair Mix (New England BioLabs, Ipswich, USA). The amplicon was Nick-repaired using NEBNext End repair/dA-tailing Module (New England BioLabs), prior to adapter ligation by NEBNext Quick Ligation Module (New England BioLabs). The sequencing library was loaded on primed Flongle flow cell according to Nanopore protocol. Sequencing was performed by MinION MK1b. Sequencing data was acquired by MinKNOW software (19.12.5) without live base-calling.

Metagenome analysis

Raw data were obtained as fast5 files. Base-calling was carried out by Guppy 4.0.11 with 2,000 chunk size and 4 base callers (56). Porechop version 3 was executed for trimming adapter sequences (https://github.com/rrwick/Porechop). To annotate bacterial taxonomy, trimmed sequences were aligned with MIrROR (http://mirror.egnome.co.kr/) using Minimap2 (57). In Operational Taxonomic Unit (OTU) identification, only results with more than 2,500 matching bases and more than 3,500 bases including gaps in mapping were used. To normalize abundance data, the TMM (The trimmed mean of M-values) method was used by the edgeR package of R software (58). To characterize each group, biological diversity was calculated through the physeq package of R software (59). A rarefaction curve was constructed to check the saturation of genome sequencing. To compare species richness, alpha diversity was calculated as chao1 and Shannon indexes. To compare between groups, beta diversity was calculated using Bray-Curtis dissimilarity and Unifrac distance. P-value was calculated by the Adonis test. For detection of

unequal features, Wilcoxon rank-sum test was performed in each taxonomic level with 0.95 confidence level. To compare functional profile, PICRUSt2 was performed (60). Correlation between cognitive ability and bacterial OTUs was inferred by Spearman's rank correlation analysis. P values were adjusted by FDR method.

SCFA identification in bacterial culture

To identify the amount of short-chain fatty acids (SCFAs), high-performance liquid chromatography (HPLC) was performed using Ultimate3000 (Thermo Dionex, USA) and Aminex 87H column (300x10mm, Bio-Rad, USA). Bacterial cultures of EG004, EG005, and EG006 were inoculated for 24 hours. After cultivation, the samples were filtered with 0.45 μm of a membrane filter. The filtered sample of 10μL was injected into the HPLC.

Whole-genome sequencing and assembly of EG005 and EG006

To identify probiotic safety and potential secondary metabolite producing ability, whole-genome sequencing of *Lcb. paracasei* EG005 and *Lcb. rhamnosus* EG006 was performed. For library construction, DNA was extracted from cultured bacterial cells. After performing quality control, gDNA was used for the library construction. Bacterial cells were lysed by lysozyme for gram-positive bacteria, and removed RNA and protein to isolate DNA. Quality control for gDNA was conducted by 260/280, 260/230 absorbance with 0.8% agarose gel. Genomic DNA was fragmented to a target length of 20Kb using g-Tube (Covaris, MA, USA) and Short DNA fragments <5 kb are depleted by SRE (Circulomics, MD, USA). The fragments were End-prepared, Nick-Repaired, and then ligated with Nanopore adapter. After every enzyme reaction,

615 the DNA samples were purified using AMPure XP beads (Beckman Coulter, CA, USA) and QC
616 with Quant-iT™ PicoGreen™ dsDNA Assay Kit. The sequencing library was loaded on primed
617 Flongle flow cell according to Nanopore protocol. Sequencing was performed on a MinION by
618 MinKNOW software.
619 Base-calling from raw data was conducted by Guppy Basecaller v4.0.15 with filtering with
620 an average basecall Phred quality score. Adapter sequences were trimmed by PoreChop v0.2.4.
621 Genome assembly was conducted by Canu. Assembled contigs were polished by Nanopolish and
622 racon, and pilon. Circlator circularized each contig and detect replication origin. Assembled
623 contig was assessed by BUSCO 3.0.2. The complete sequence of *L. acidophilus* EG004 that is
624 deposited in the NCBI database with accession number PRJNA657145 was used.
625
626 **Comparative analysis of bacterial genome sequences**
627 Genetic map was generated by CGView server (61). To check safety and functionality as
628 probiotics, genetic factors were identified by whole-genome sequences. Virulence factor and
629 prophage gene were detected by VirulenceFinder 2.0 and PHASTER, respectively.
630 IslandViewer4 identified genomic island and crisprfinder searched CRISPR region. Bacteriocin
631 detection was conducted by BAGLE4. To compare functional gene contents, protein prediction
632 was performed by the RAST server. Predicted protein sequences were classified by the SEED
633 system. Categorized protein sequences showed as the proportion in the total predicted sequences.
634
635 **Data availability**
636 The complete sequences of *Lcb. paracasei* EG005 and *Lcb. rhamnosus* EG006 are available

in the NCBI database with accession numbers, SAMN23227569 and SAMN23227570, respectively. The metagenomic sequences are available in the NCBI database under the accession number PRJNA781018.

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Tables

Table 1. Metagenomic sequencing statistics of *L. acidophilus group* and control

	The number of samples	Total number of reads	Estimated base (Mb)	N50	Total number of counts	Total number of OTUs
LA[a]	5	312,384±31,887	1,434±143	4,872±90	252401.6±25,171	528.4±40
W[b]	5	335,356±45,814	1,485.6±215	4,748±40	259945.6±35,117	539.8±25
Total	10	323,870±37,604	1,459.8±173	4810±72	256173.6±28,860	534.1±32

[a]: *L. acidophilus* group, [b]: control group. There was no significant difference between groups. All values were presented as average ± standard error of the mean. Fecal samples compiled after 8 weeks of probiotic ingestion were used for metagenome sequencing.

Figure legends

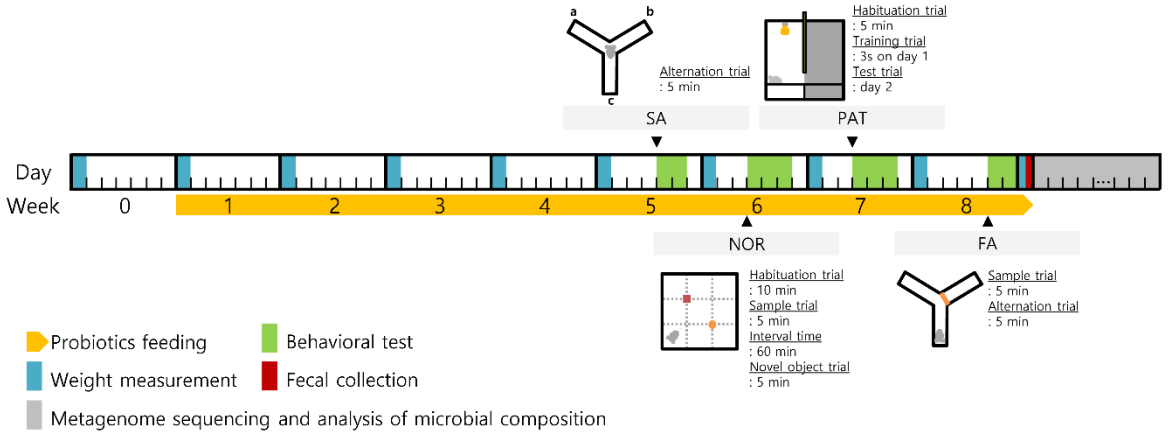
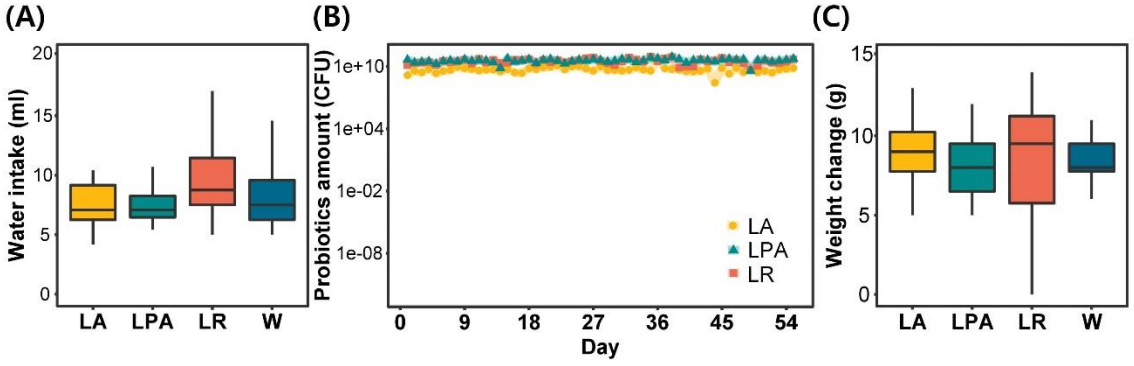


Figure 1. Schematic diagram of the study to discover a new probiotic strain with improved cognitive ability

The diagram displays the experimental schedule by day and week for identifying probiotic strain with improved cognitive ability. Cognitive ability was measured once a week by four behavioral tests. The diagram of each experiment shows the first position of the animal.

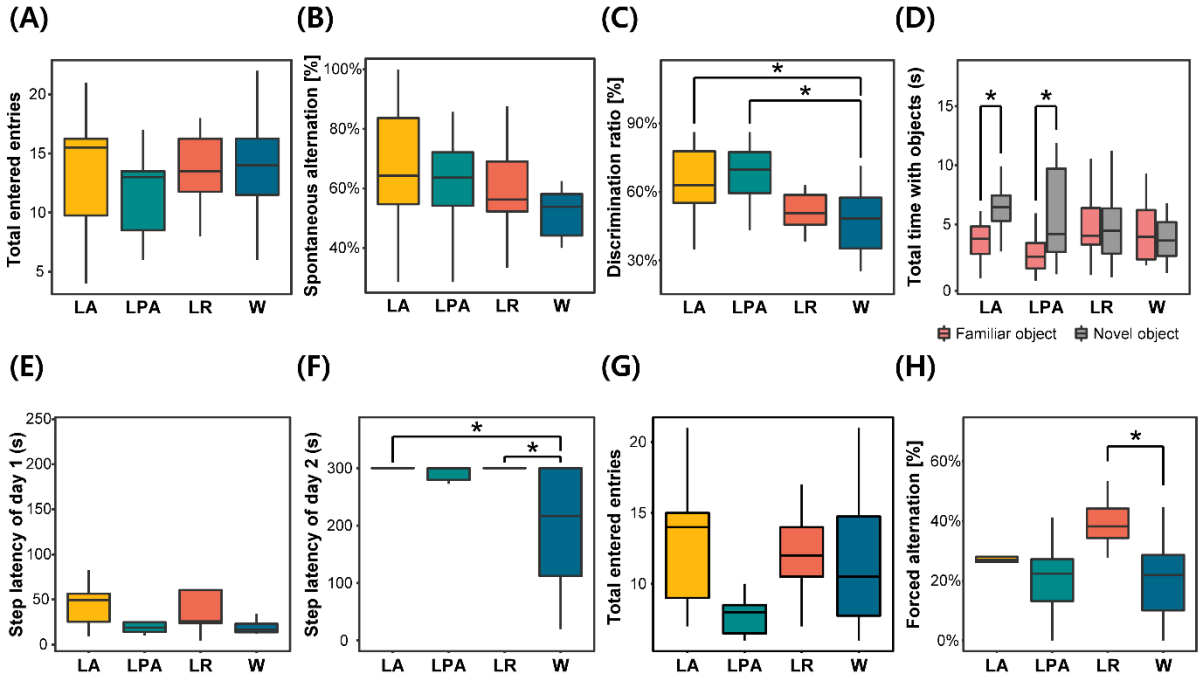


817

818 **Figure 2. Measurement of additional effect after probiotic consumption**

819 Experimental groups are expressed in abbreviations. LA: *L. acidophilus* group, LPA: *Lcb.*
820 *Paracasei* group, LR: *Lcb. Rhamnosus group*, and W: tap water-fed group (control). **(A)** The
821 average daily water intake. All groups showed a similar average. **(B)** The change of daily intaken
822 probiotic amount by timeline. *L. acidophilus* was ingested in smaller amounts compared to the
823 other two strains. **(C)** The average body weight change for 8 weeks. All groups showed similar
824 averages.

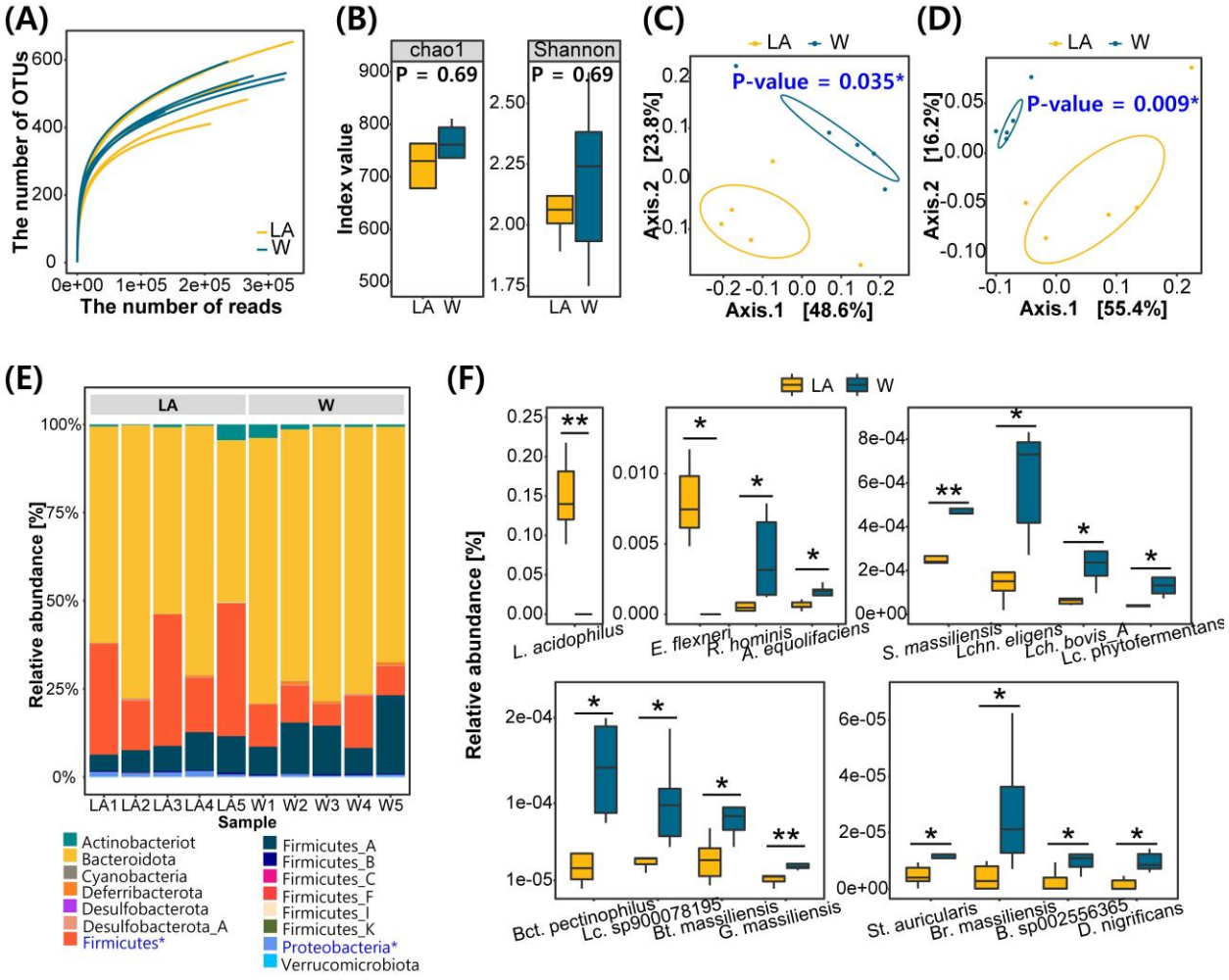
825



826

827 **Figure 3. Results of cognitive behavioral tests**

828 Experimental groups are expressed in abbreviations. LA: *L. acidophilus* group, LPA: *Lcb.*
829 *Paracasei* group, LR: *Lcb. rhamnosus* group, and W: the group fed on tap water (control). (**A)**
830 Total arm entries during spontaneous alternation test. **(B)** Spontaneous alternation. This is the
831 representative value of spontaneous alternation test. **(C)** Discrimination ratio. It is the
832 representative value of the novel object recognition test. **(D)** Comparison of the total time to
833 observe two objects. **(E)** Step-through latency of day 1. **(F)** Step-through latency of day 2. This is
834 the representative result of the passive avoidance task. **(G)** Total arm entries during forced
835 alternation test. **(H)** Forced alternation. This result is a representative value of forced alternation.
836 All comparison of average between experimental groups was measured by Wilcoxon rank-sum
837 test. Significant difference is presented with symbol (Adjusted P-value* < 0.05).

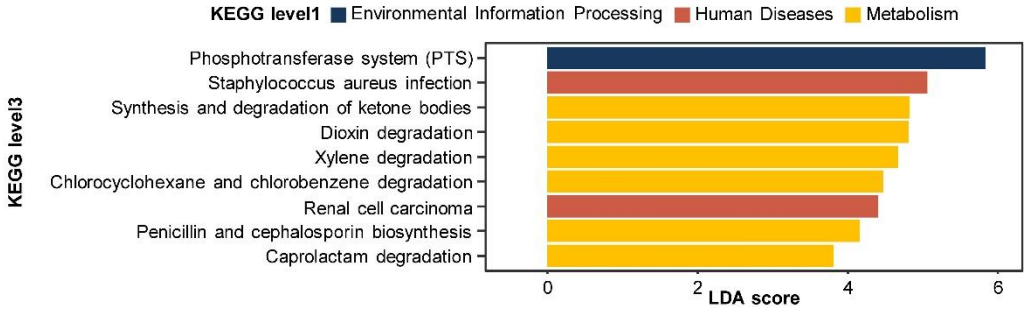


838

839 **Figure 4. Results of metagenomics sequencing**

840 Experimental groups are expressed in abbreviations. LA: *L. acidophilus group* and W: the group
841 fed on tap water (control). **(A)** Rarefaction curve of metagenome sequencing. **(B)** Alpha-diversity
842 of the *L. acidophilus* group and control. **(C)** Beta-diversity using Bray-Cutis distance between
843 the *L. acidophilus* group and control. **(D)** Beta-diversity using Unifrac distance between both
844 groups. **(E)** Comparison of microbial composition at the phylum level. The blue-colored phylum
845 with the (*) symbol showed a significant difference compared to the two experimental groups. **(F)**
846 Comparison of microbial composition at the species level. L. acidophilus: *Lactobacillus*

acidophilus, E. flexneri: *Escherichia flexneri*, R. hominis: *Roseburia hominis*, A. equolifaciens: *Adlercreutzia equolifaciens*, S. massiliensis: *Soleaferrea massiliensis*, Lchn. Eligens: *Lachnospira eligens*, Lch. Bovis_A: *Lachnobacterium bovis_A*, Lc. Phytofermentans: *Lachnoclostridium phytofermentans*, Bct. Pectinophilus: *Bacteroides_F pectinophilus*, Lc. Sp900078195: *Lachnoclostridium sp900078195*, Bt. Massiliensis: *Bittarella massiliensis*, G. massiliensis: *Gemella massiliensis*, St. auricularis: *Staphylococcus auricularis*, Br. Massiliensis: *Bariatricus massiliensis*, B. sp002556365: *Bacillus_AW sp002556365*, D. nigrificans: *Desulfotomaculum nigrificans.* All comparisons of average between experimental groups were measured by independence t-test. Significant difference is presented with symbol (Adjusted P-value* < 0.05, P-value** < 0.01).



858

859 **Figure 5. Results of functional profiling**

860 Predictive functional profiling of microbiome. All predicted functions have a positive LDA score

861 for the *L. acidophilus* group.

862

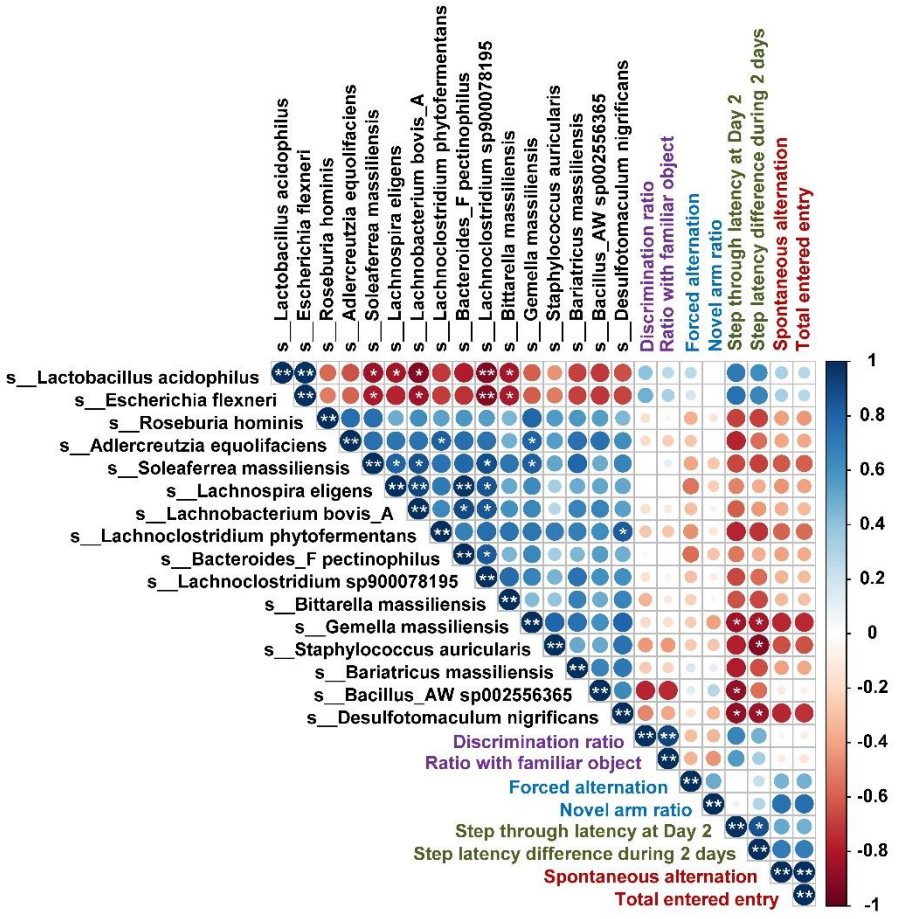
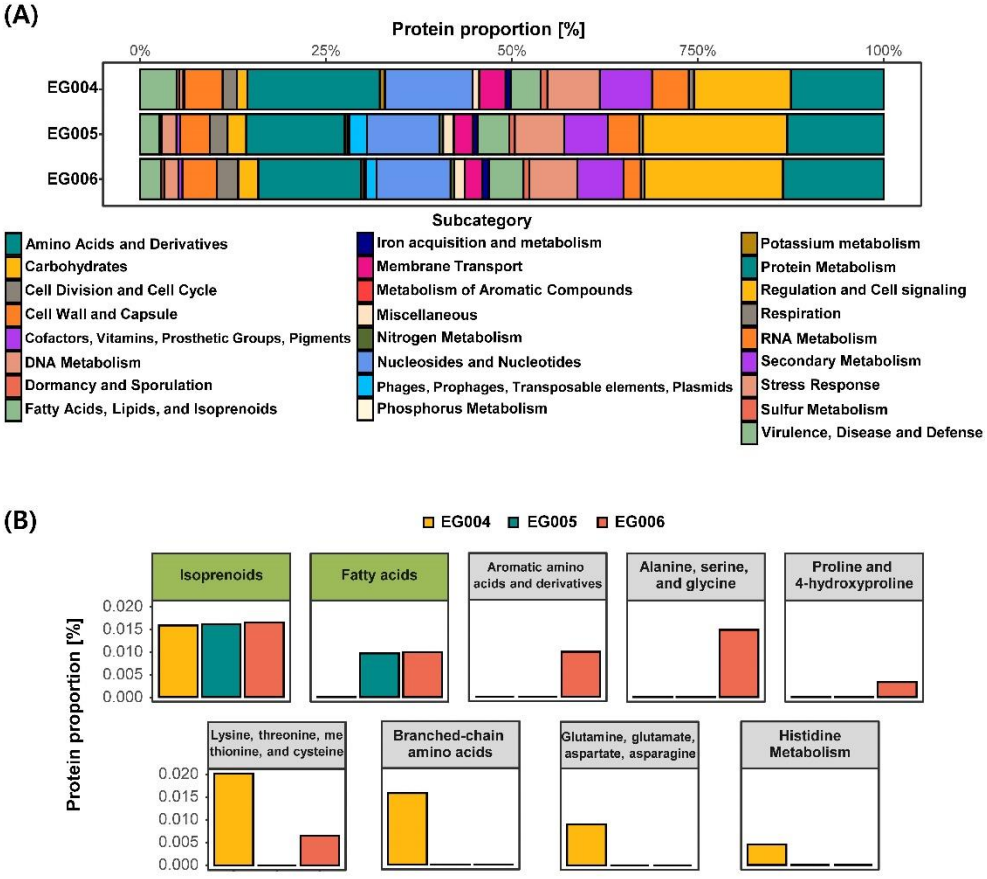


Figure 6. Spearman's rank correlation analysis

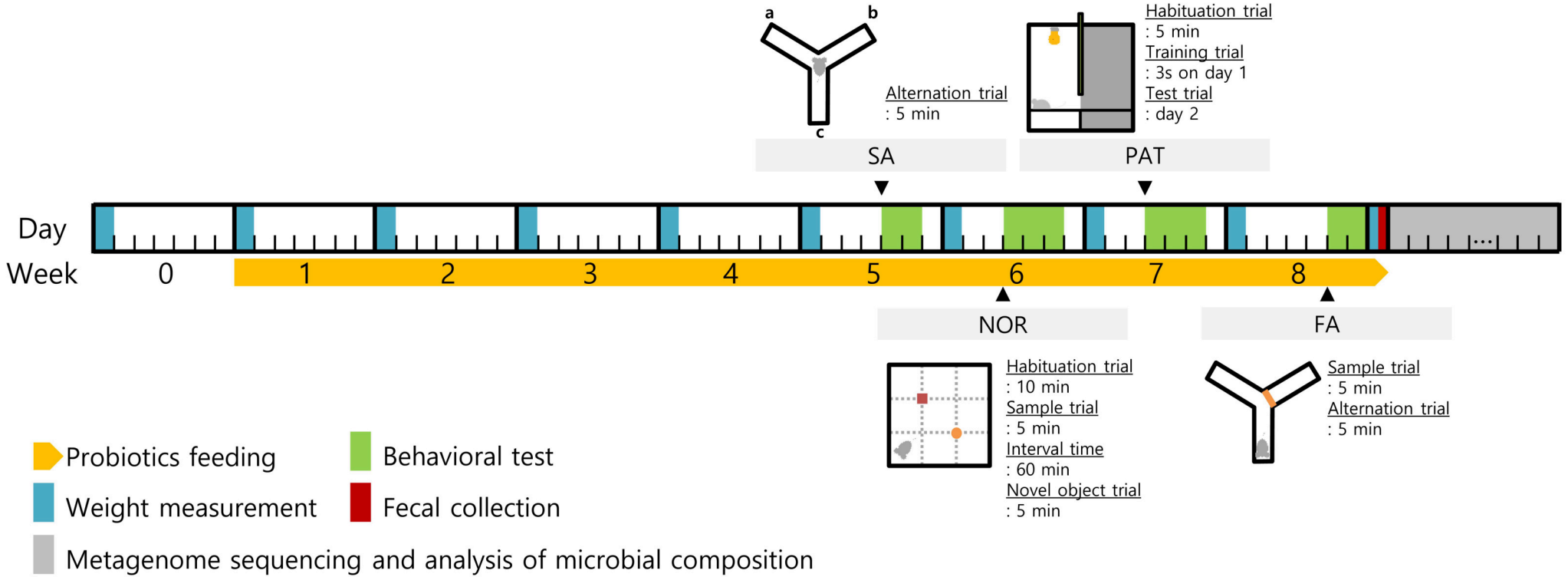
Correlation analysis was conducted to detect association among bacterial OTUs, measured cognitive abilities, and fermentation products. The color intensity and circle size show the strength of the correlation. Red color represents a negative correlation, and blue color is a positive correlation. Only circles with adjusted P-value under 0.01 are illustrated in the matrix. Results of cognitive ability evaluation were classified by 4 colors: NOR (purple), FA (blue), PAT (deep green), and SA (brown). Significant P values indicated by the symbol * (<0.05) and ** (<0.01).

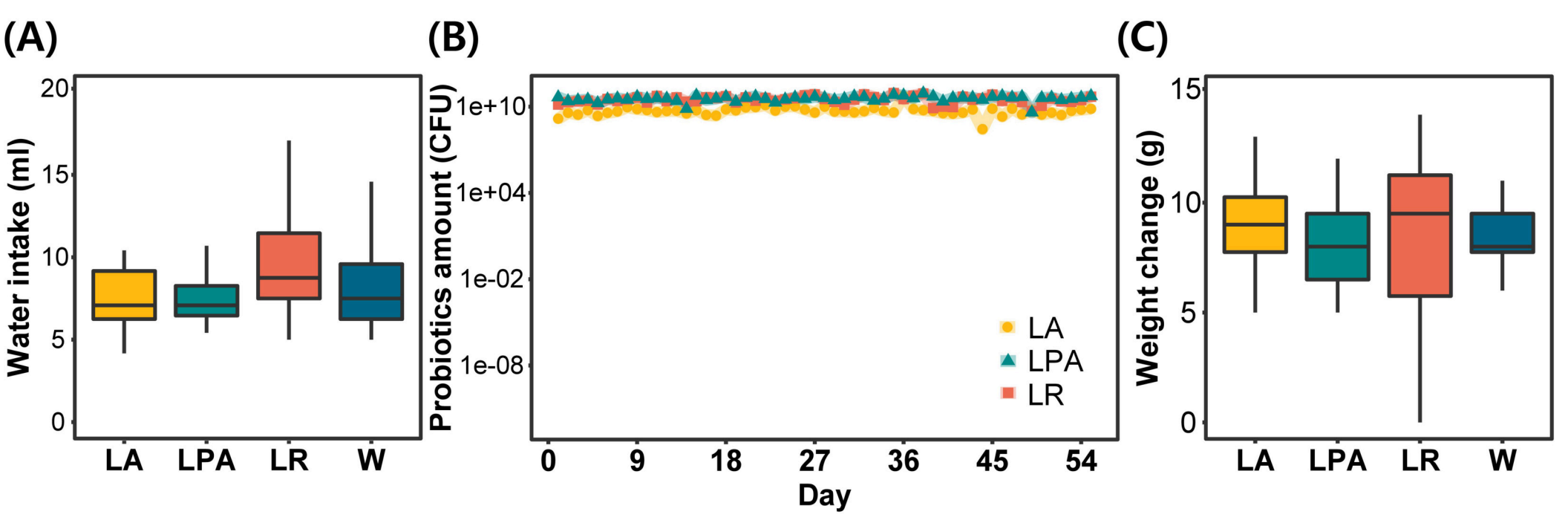


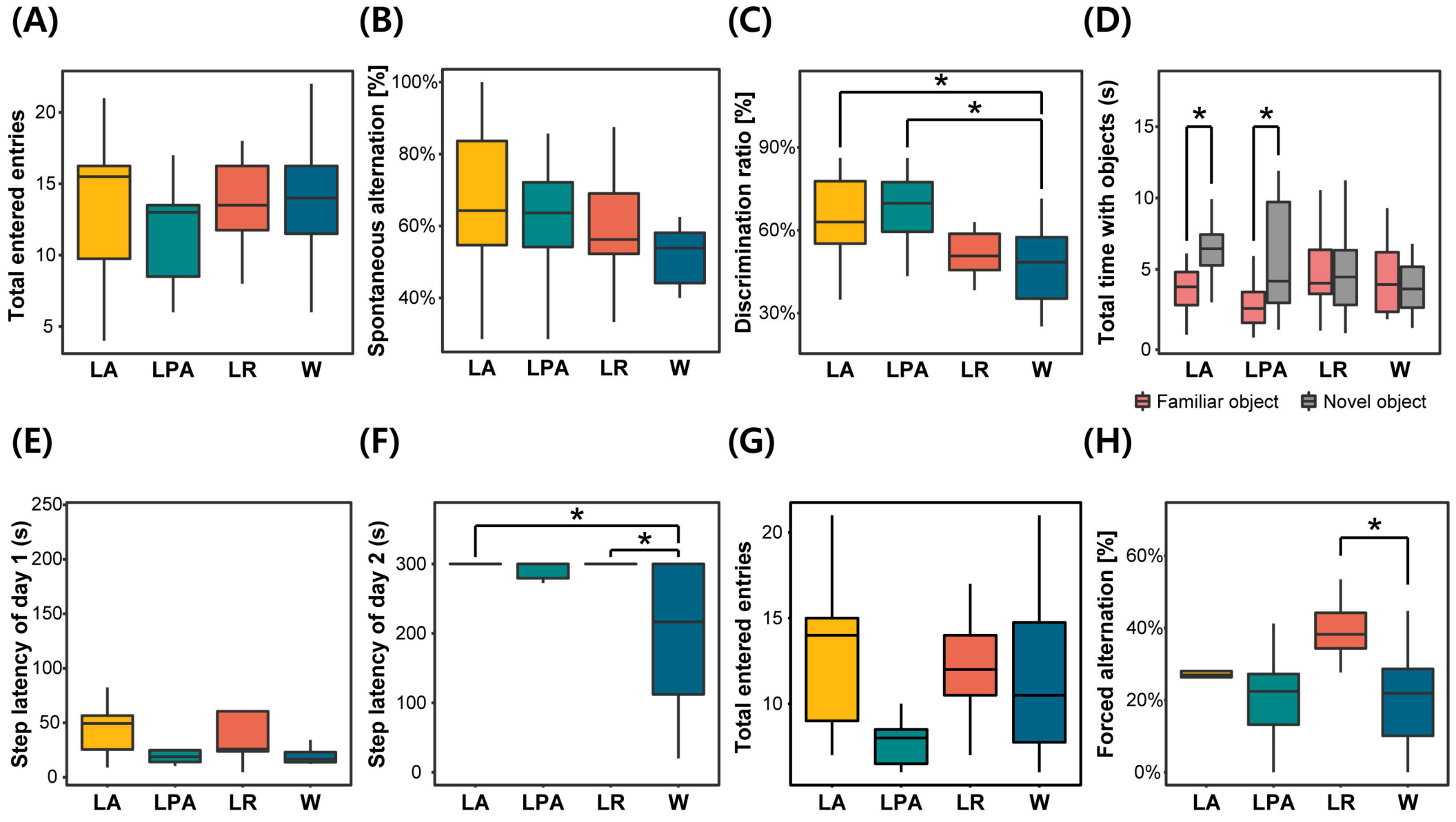
873

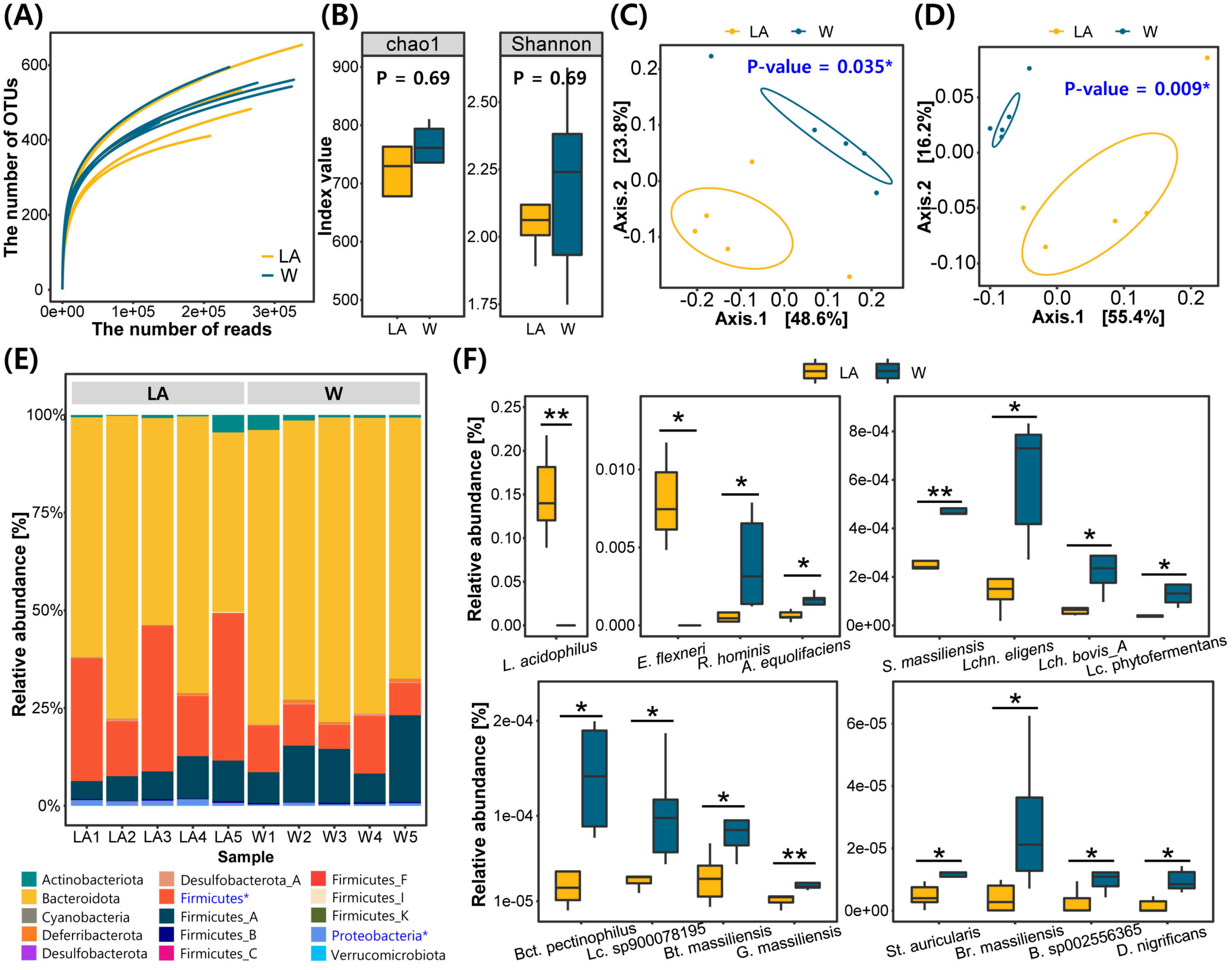
874 **Figure 7. Genomic comparison of 3 probiotic strains**

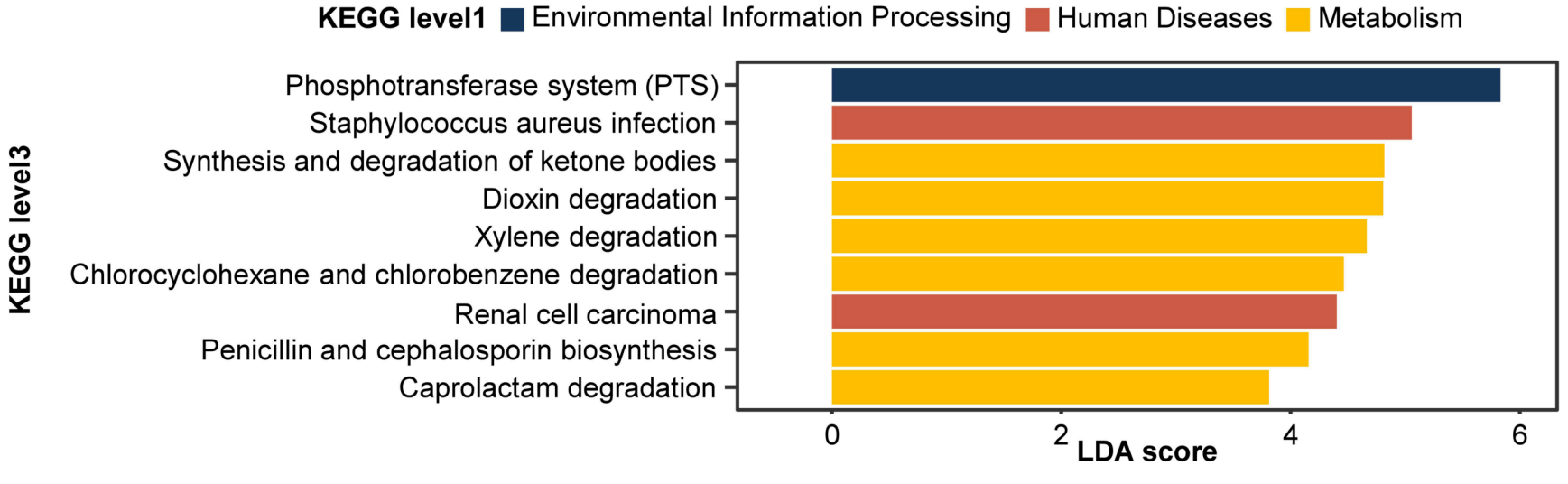
875 (A) Functional classification of protein coding sequences. All predicted protein sequences were
876 classified by categories by SEED system. (B) Subcategories in [Fatty Acids, Lipids, and
877 Isoprenolds] and [Amino Acids and Derivatives]. [Fatty Acids, Lipids, and Isoprenolds]
878 subcategory showed yellow-green colored head and [Amino Acids and Derivatives] category
879 presented light gray colored head.

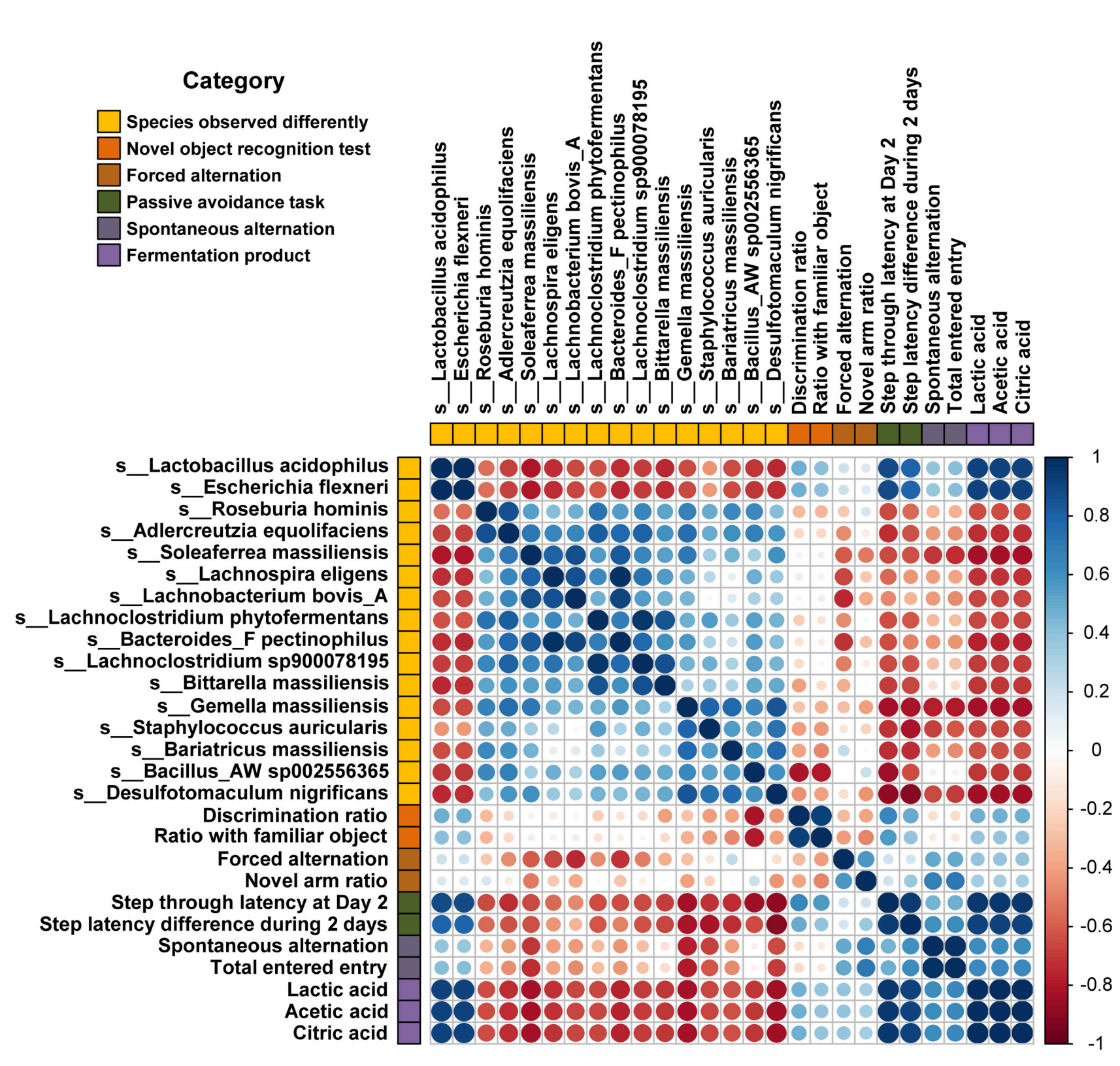

a
b
c
Alternation trial
: 5 min
Habituation trial
: 5 min
Training trial
: 3s on day 1
Test trial
: day 2
SA
PAT
Day
Week
0
1
2
3
4
5
6
7
8
NOR
FA
Habituation trial
: 10 min
Sample trial
: 5 min
Interval time
: 60 min
Novel object trial
: 5 min
Sample trial
: 5 min
Alternation trial
: 5 min
Probiotics feeding
Behavioral test
Weight measurement
Fecal collection
Metagenome sequencing and analysis of microbial composition

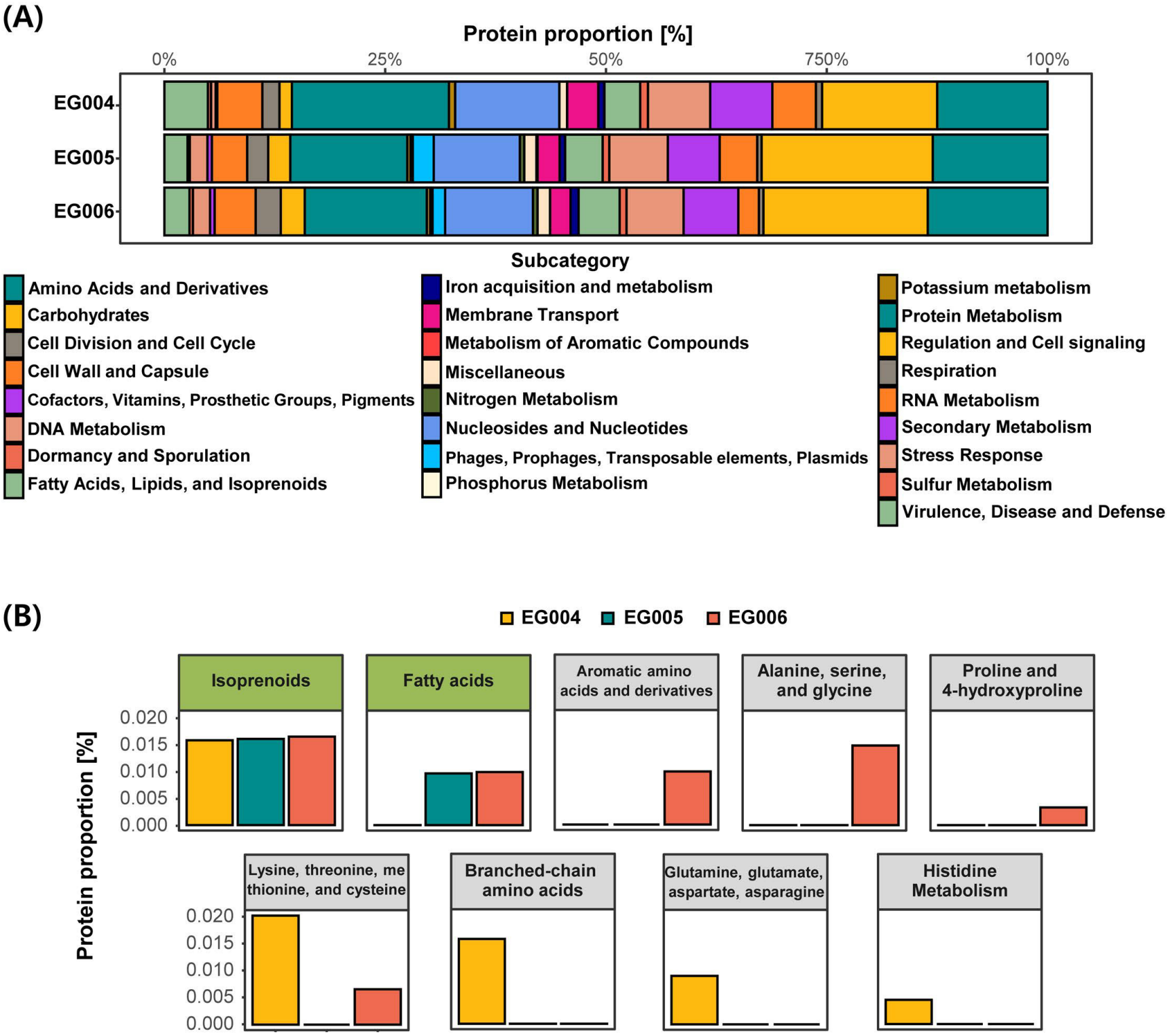

(A)
Water intake (ml)
20
15
10
5
0
LA
LPA
LR
W
(B)
Probiotics amount (CFU)
1e+10
1e+04
1e-02
1e-08
LA
LPA
LR
0
9
18
27
36
45
54
Day
(C)
Weight change (g)
15
10
5
0
LA
LPA
LR
W


(A)
Total entered entries
20
15
10
5
LA
LPA
LR
W
(B)
Spontaneous alternation [%]
100%
80%
60%
40%
LA
LPA
LR
W
(C)
Discrimination ratio [%]
90%
60%
30%
*
*
LA
LPA
LR
W
(D)
Total time with objects (s)
15
10
5
0
*
*
LA
LPA
LR
W
Familiar object
Novel object
(E)
Step latency of day 1 (s)
250
200
150
100
50
0
LA
LPA
LR
W
(F)
Step latency of day 2 (s)
300
200
100
0
*
*
LA
LPA
LR
W
(G)
Total entered entries
20
15
10
LA
LPA
LR
W
(H)
Forced alternation [%]
60%
40%
20%
0%
*
LA
LPA
LR
W


(A)
The number of OTUs
The number of reads
LA
W
(B)
chao1
Shannon
P = 0.69
P = 0.69
Index value
LA W
(C)
LA W
P-value = 0.035*
Axis.2 [23.8%]
Axis.1 [48.6%]
(D)
LA W
P-value = 0.009*
Axis.2 [16.2%]
Axis.1 [55.4%]
(E)
LA
W
Relative abundance [%]
Sample
LA1 LA2 LA3 LA4 LA5 W1 W2 W3 W4 W5
Actinobacteriota
Bacteroidota
Cyanobacteria
Deferribacterota
Desulfobacterota
Desulfobacterota_A
Firmicutes*
Firmicutes_A
Firmicutes_B
Firmicutes_C
Firmicutes_F
Firmicutes_I
Firmicutes_K
Proteobacteria*
Verrucomicrobiota
(F)
LA W
Relative abundance [%]
L. acidophilus
E. flexneri
R. hominis
A. equolifaciens
S. massiliensis
Lchn. eligens
Lch. bovis_A
Lc. phytofermentans
Bct. pectinophilus
Lc. sp900078195
Bt. massiliensis
G. massiliensis
St. auricularis
Br. massiliensis
B. sp002556365
D. nigrificans


KEGG level1
Environmental Information Processing
Human Diseases
Metabolism
KEGG level3
Phosphotransferase system (PTS)
Staphylococcus aureus infection
Synthesis and degradation of ketone bodies
Dioxin degradation
Xylene degradation
Chlorocyclohexane and chlorobenzene degradation
Renal cell carcinoma
Penicillin and cephalosporin biosynthesis
Caprolactam degradation
0
2
4
6
LDA score


Category
Species observed differently
Novel object recognition test
Forced alternation
Passive avoidance task
Spontaneous alternation
Fermentation product
s__Lactobacillus acidophilus
s__Escherichia flexneri
s__Roseburia hominis
s__Adlercreutzia equolifaciens
s__Soleaferrea massiliensis
s__Lachnospira eligens
s__Lachnobacterium bovis_A
s__Lachnoclostridium phytofermentans
s__Bacteroides_F pectinophilus
s__Lachnoclostridium sp900078195
s__Bittarella massiliensis
s__Gemella massiliensis
s__Staphylococcus auricularis
s__Bariatricus massiliensis
s__Bacillus_AW sp002556365
s__Desulfotomaculum nigrificans
Discrimination ratio
Ratio with familiar object
Forced alternation
Novel arm ratio
Step through latency at Day 2
Step latency difference during 2 days
Spontaneous alternation
Total entered entry
Lactic acid
Acetic acid
Citric acid
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1


(A)
Protein proportion [%]
0%
25%
50%
750%
100%
EG004
EG005
EG006
Subcategory
Amino Acids and Derivatives
Carbohydrates
Cell Division and Cell Cycle
Cell Wall and Capsule
Cofactors, Vitamins, Prosthetic Groups, Pigments
DNA Metabolism
Dormancy and Sporulation
Fatty Acids, Lipids, and Isoprenoids
Iron acquisition and metabolism
Membrane Transport
Metabolism of Aromatic Compounds
Miscellaneous
Nitrogen Metabolism
Nucleosides and Nucleotides
Phages, Prophages, Transposable elements, Plasmids
Phosphorus Metabolism
Potassium metabolism
Protein Metabolism
Regulation and Cell signaling
Respiration
RNA Metabolism
Secondary Metabolism
Stress Response
Sulfur Metabolism
Virulence, Disease and Defense
(B)
EG004
EG005
EG006
Protein proportion [%]
Isoprenoids
Fatty acids
Aromatic amino acids and derivatives
Alanine, serine, and glycine
Proline and 4-hydroxyproline
Lysine, threonine, methionine, and cysteine
Branched-chain amino acids
Glutamine, glutamate, aspartate, asparagine
Histidine Metabolism
0.020
0.015
0.010
0.005
0.000

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Republic of Korea

Brief Rebuttal to the remarks of the reviewer3

Comment #1 of the reviewer3: Line 54 - Cognition is one of the functions of the brain. The authors should write in the Manuscript the idea that they study bacterial strain that has positive effects on brain function, which can be recognized through changes in cognitive processes.

Amendment for comment #1
We appreciate the reviewer for pointing out the most important part of understanding the experimental design. The context has been added in the Abstract and Introduction parts, and it will help readers naturally understand the research aim (***Line 54-56, page 4***).

Comment #2 of the reviewer3: Line 68 - In the annotation, you do not say a word about strains EG005 and EG006. Why? Also, add into the discussion part more information about comparison and differences in the action of these three strains. Explain the reasons for these differences.

Amendment for comment #2
Thank you for your valuable advice. In order to focus on *L. acidophilus* EG004, the results of the other two strains were omitted in the abstract of the previous manuscript. To increase the overall understanding of the study, we have added results for *Lcb. paracasei* EG005 and *Lcb. rhamnosus* EG006 to Abstract (***Line 58-60, page 4***). In addition, referring to the reviewer's advice, discussion was revised to provide comparative information on the effects after ingestion of the three strains (***Line 294-319, page 16-17,*** *and* ***Supplementary Table3***). This additional explanation will provide the reader with a richer understanding of the cognitive abilities of each Lactic acid bacteria.

Comment #3 of the reviewer3: Line 130 - will be better if you use the word - healing effects

Amendment for comment #3
Based on the reviewer comments, the word was modified to a more appropriate word (***Line 129-131, page 7***).

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Comment #4 of the reviewer3: Line 150 - what kind of molecular method? add the explanation into the text.

Amendment for comment #4
Thanks for your kind comments. The previous expression as “molecular method” has been replaced by “16S rRNA sequencing” (***Line 149-150, page 9***). A detailed description of this method can be found in the Materials and methods section. We expect that this clear statement will help the reader's understanding.

Comment #5 of the reviewer3: Line 390 - you wrote - that the antibacterial activity of L. acidophilus EG004 was the potential reason for cognitive ability enhancement. - how it is possible? Why do you assume this?

Amendment for comment #5
We thank the reviewer for raising this issue. We assumed that the low levels of the microorganisms (such as *Adlercreutzia equolifaciens* and *Roseburia hominis*) were affected by ingested *L. acidophilus* EG004. The only difference was the intake of *L. acidophilus* between the control group and the *L. acidophilus* group. Based on the function of *L. acidophilus* indicated in previous studies, we estimated that *L. acidophilus* interfered with the habitat and growth of other microorganisms through preoccupation of habitat and antibacterial activity. However, we did not provide experimental evidence for the process in this study. We acknowledge that the current argument has some leaps and bounds. This may be misleading to readers. Accordingly, we omitted the detailed explanation of the presumed mechanism, leaving only the assumption that *L. acidophilus* may have been affected with toning down of suggestion (***Line 391-395, page 20***). The revised manuscript will be able to more accurately convey the effects of *L. acidophilus* to the reader.

Comment #6 of the reviewer3: Line 407 - Line 54 - Cognition is one of the functions of the brain. The authors should write in the Manuscript the idea that they study bacterial strain that has positive effects on brain function, which can be recognized through changes in cognitive processes.

Amendment for comment #6
Thanks for pointing out the most important part of understanding the experimental design (***Line 54-56,***

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page* 4** and ***Line 411-415, page 21). The content has been added to the Abstract and introduction so that the purpose of the study can be understood naturally.

Comment #7 of the reviewer3: Line 421 - why male?

Amendment for comment #7

We thank the reviewer for raising this issue. In an animal experiment, it is an ideal experiment by setting females and males as separate groups. However, our experiment was performed using only male subjects with consideration of some concerns.

- Simplification of the experimental variation affecting the interpretation of results[1]
- Prevention of statistical power loss due to small subsamples for each sex[2]
- Estimation that there is no difference between the intestinal environment and the brain-gut axis system between female and male
- Male is mainly used in animal experiments for the brain-gut axis
- Restrictions on money, time, and the skill level of the experimenter.

Ideally, it is appropriate to use both males and females, but in consideration of these concerns, male mice were used. In order to provide this specific information to readers, this information was added to the manuscript (***Line 426-430, page 22***). We believe that it will help the reader's understanding.

Comment #8 of the reviewer3: Line 605 - Why is EG004 do not present here?

Amendment for comment #8

Thank you for your valuable advice. Since *L. acidophilus* EG004 was previously sequenced using the PacBio platform, only *Lcb. paracasei* EG005 and *Lcb. rhamnosus* EG006 were newly sequenced for this study. The sequence information of *L. acidophilus* EG004 is mentioned in ***Line 634-636*** and ***page 31***, and related papers were cited to provide the sequencing information to readers. Also, since the paragraph indicated by the reviewer is about sequence information of the three strains, the sentence was changed to

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'Whole-genome sequencing of EG005 and EG006 and Whole-genome sequence of EG004' (***Line 614, page 30***).

Comment #9 of the reviewer3: Line 623 - Add here the information from Data availability - The complete sequences of Lcb. paracasei EG005 and Lcb. rhamnosus EG006 is available in the NCBI database with accession numbers, SAMN23227569 and SAMN23227570, respectively. The metagenomic sequences are available in the NCBI database under the accession number PRJNA781018.

Amendment for comment #9
Thank you for the reviewer's advice. Data availability information was added to the appropriate part (Whole-genome sequences of three probiotics; ***Line 632-636, page 31***, and metagenomics data; ***Line 586-587, page 30***).

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2 Richardson, S. S., Reiches, M., Shattuck-Heidorn, H., LaBonte, M. L. & Consoli, T. J. P. o. t. N. A. o. S. Opinion: focus on preclinical sex differences will not address women's and men's health disparities. **112**, 13419-13420 (2015).

December 7, 2021

Prof. Heebal Kim
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Seoul
Korea (South), Republic of

Re: Spectrum01815-21R2 (Positive effect on cognitive ability of Lactobacillus acidophilus EG004 in healthy mouse and fecal microbiome analysis using full-length 16S-23S rRNA metagenome sequencing)

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Thanks for addressing the Reviewer's comments and congratulations on the acceptance of your manuscript for publication at Spectrum!

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Supplemental material: Accept