Long-term D-Allose Administration Favorably Alters the Intestinal Environment in Aged Male Mice

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Abstract: D-Allose, a C3 epimer of D-glucose, has potential to improve human health as a functional food. However, its effect on the intestinal environment remains unknown. Aged humans progressively express changes in the gut, some of which deleteriously affect gastrointestinal health. In this study, we profiled the intestinal microbiome in aged mice and analyzed organic acids produced by bacteria in cecum contents after long-term ingestion of D-allose. D-Allose did not significantly change organic acid concentration. However, long-term ingestion did significantly increase the relative abundance of Actinobacteria and reduce the relative abundance of Proteobacteria. These results suggest that oral D-allose improves the proportion of favorable intestinal flora in aged mice. D-Allose significantly decreased the relative abundance of Lachnospiraceae bacteria, but increased the relative abundance of Bacteroides acidifaciens and Akkermansia muciniphila. Thus, D-allose might serve as a nutraceutical capable of improving the balance of gut microbiome during aging.

Key words: D-allose, intestinal microbiome, organic acid, Bacteroides acidifaciens, Akkermansia muciniphila

D-Allose is a rare sugar. Rare sugars are monosaccharides that occur in limited quantities in nature.1) D-Allose is a C3-epimer of D-glucose,2) and as with all rare sugars, is expected to have potential as a functional food.3) In rats4) and humans,5) D-allose is largely absorbed via a conventional sodium-dependent glucose cotransporter 15) after ingestion and then is rapidly excreted into the urine. Absorbed D-allose has some health benefits and disease modification properties.6) Its anti-hypertensive effect appears to occur through the modulation of glucose metabolism,7) and its anti-osteoporosis effect may occur by inhibiting osteoclast differentiation, as demonstrated in an in vitro study.3) D-Allose also appears to have antioxidative effects, as it has been demonstrated to suppress reactive oxygen species (ROS) production.8) The mechanisms underlying its antioxidative abilities may also contribute to its anti-hypertension and anti-osteoporosis effects.9,10) These beneficial effects led us to hypothesize that D-allose could potentially be a health-promoting dietary supplement. Intriguingly, majority of ingested D-allose is absorbed in the small intestine, and unabsorbed D-allose flows into the large intestine.3,4) The unabsorbed D-allose may act on the intestinal microbiome or intestinal epithelial cells and thereby play a physiological role. However, the effects of D-allose on the intestinal environment have yet to be determined.

Intestinal microbial flora comprises bacteria belonging to mainly four phyla: Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria.9) The balance and proportion of colonizing species in the intestines influence overall health.10) For instance, maintaining a good microbiota balance and possessing an abundance of Actinobacteria are believed to contribute to a healthy intestinal environment.11) The proportion of Bifidobacterium species, which include beneficial bacteria of the phylum Actinobacteria, decreases with aging.12) Bacteroidetes species influence intestinal immunity and weight maintenance.13) The beneficial bacteria in the Actinobacteria and Bacteroidetes phyla produce organic acids that improve the intestinal environment and help to maintain good health.13) On the other hand, an increase in the relative abundance of bacteria of the phylum Firmicutes appears to be associated with obesity.14)

In the present study, we examined the effect of long-term oral D-allose administration on age-related changes in the intestinal environment. Cecal-microbiome profile and...
concentrations of selected organic acids were evaluated in aged mice (22 months old) after oral administration of D-allose for 10 months.

**Experimental**

All animal experiments were approved by the Animal Experiment Committee of the Tokyo Metropolitan Institute of Gerontology (Animal Protocol Approval no. 17012 and 20018) and by the Matsutani Chemical Industry Co., Ltd. (Animal Protocol Approval no. 200918). The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council [United States] Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

One-month-old male C57BL/6J mice were obtained from CLEA Japan, Inc. (Tokyo, Japan). Mice were housed under specific pathogen-free conditions in groups of 5 per cage; cages were lined with paper chip bedding (Japan SLC, Inc., Shizuoka, Japan). The vivarium was maintained at 22 ± 1°C and 55 ± 5% humidity under a 12/12-h light-dark cycle. At 12 months of age, the mice were assigned to either the control diet (10 mice) or D-allose diet group (12 mice), in such a way that the group’s mean body weights were similar. The initial body weights of the control group and D-allose group were 39.7 ± 1.3 g and 39.4 ± 1.6 g, respectively (t(19.8) = 0.03, p = 0.976). The control group received regular mouse feed (CRF-1, Oriental Yeast Ltd., Tokyo, Japan), and the D-allose diet group received CRF-1 comprising 3% (w/w) of D-allose (Matsutani Chemical Industry Co., Ltd., Hyogo, Japan). Animals had ad libitum access to water for 10 months. The dose of D-allose fed to mice in the D-allose diet group was based on a previous toxicity study using rats, and calculated were the type and abundance of bacteria present in the cecum for both groups of mice, and the organic acids present and their abundance.

DNA from thawed individual cecal samples was extracted using a previously described method. This method uses a DNA isolation system (GENE PREP STAR PI-480; Kurabo, Osaka, Japan). The V3-V4 regions of bacterial 16S rRNA were amplified using PCR. Then, the amplified fragments were paired-end sequenced on a 2 × 284-bp cycle using a MiSeq system with MiSeq Reagent Kit version 3 (600 cycle) chemistry (Illumina, San Diego, CA, USA). The paired-end sequencing reads were merged using the fastq-join program set at default settings. Only joined reads with a quality value score ≥ 20 for more than 99% of the sequence were extracted using the FASTX-Toolkit. Chimeric sequences were deleted using the usearch6.1 tool. The taxonomy of the sequence reads was assigned using the Ribosomal Database Project (RDP) classifier tool (ver. 2.11) and the TechnoSurugaLab microbial identification database DB-BA13.0. The classifier tool is available from the RDP website (http://rdp.cme.msu.edu/classifier). Bacterial identification was performed using Metagenome@KIN ver. 2.2.1 analysis software (World Fusion; Tokyo, Japan). Bacterial identification at the phylum level was performed using the RDP Classifier tool (confidence, ≥ 0.8). Identification of bacterial genera and species was made possible by using data from the TechnoSuruga Lab microbial identification database DB-BA13.0 (homology, ≥ 97%). For each taxonomic level of bacteria identified, we calculated the average values of the relative abundance of the identified taxon and the fold change for each group.

Organic acid content analysis was performed as described previously. Briefly, 0.1 g of the collected cecal sample was mixed with zirconia beads and 2 mL of Milli-Q Water. The samples were heated at 85°C for 15 min, vortexed at 5 m/s for 45 s using FastPrep 24 5G (MP Biomedicals, LLC, Irvine, CA, USA), and centrifuged at 15,350 × g for 10 min at 24°C. The resulting supernatant was filtered through a 0.2-µm polytetrafluoroethylene membrane. Select organic acids (acetic acid, propionic acid, butyric acid, succinic acid, and lactic acid) from the filtered cecum samples were analyzed using high-performance liquid chromatography (Prominence HPLC System; Shimadzu Co., Kyoto, Japan) with two tandem columns (Shim-pack SCR-102, 8 mm × 300 mm; Shimadzu). The eluted organic acids were detected with a post-column pH Bufferd Electric Conductivity Detector (CDD-10A; Shimadzu).

All data are presented as means ± standard error of the mean (SEM). Statistical significance of group differences was evaluated using the two-sided Welch t-test. Statistical results showed the degree of freedom, test statistic, and p-value. All statistical analyses were done with version 27 SPSS software (IBM Corp., Armonk, NY, USA). P < 0.05 was considered statistically significant.

**No significant increase in body and cecum weight.** The final body weights of the control group and D-allose group after 10 months administration were 50.5 ± 1.7 g and 46.9 ± 2.1 g, respectively (t(18.8) = 1.12, p = 0.278). The cecum weights of the control group and D-allose group were 0.819 ± 0.055 g and 0.809 ± 0.046 g (t(18.5) = 0.14, p = 0.888).

**Favorable alteration in microbiome.** Our analyses revealed several significant findings in aged mice after long-term ingestion of D-allose. At the phylum level, compared to the control group, the abundance of Firmicutes and Bacteroidetes did not change significantly after D-allose administration. The Firmicutes (t(17.3) = 1.82, p = 0.086) and Bacteroidetes (t(18.2) = 1.91, p = 0.072) in the D-allose group of mice had a significantly higher relative abundance of Actinobacteria (t(14.5) = 2.42, p < 0.05) in comparison to the control group (Table 1), including the beneficial probiotic bacteria *Bifidobacterium*. In humans, the relative abundance of Actinobacteria decreases with increasing age. By contrast in aged mice, the D-allose group had a significantly lower relative abundance of Proteobacteria (t(17.5) = 2.94, p < 0.01) compared to the control group (Table 1), including *Escherichia*, *Salmonella*, and *Vibrio*. In both humans and C57BL/6J mice, Proteobacteria, which cause inflammation in the intestines, increases with aging. Our results indicate that D-allose could appear to improve intestinal microbiome balance at the phylum level.
in aged mice.

**Genus level.** The results of our analyses of the aged intestinal microbiome at the genus level are shown in Table 2. The D-allose group had a significantly lower relative abundance of *g. Lachnospiraceae bacterium KNHs209_incertae_sedis* (*t* (14.1) = 2.46, *p* < 0.05) and *Blautia* (*t* (11.3) = 3.39, *p* < 0.01). The presence of Lachnospiraceae bacteria in the human gut is associated with the development of metabolic syndrome, obesity, diabetes, liver disease, inflammatory bowel disease, and chronic kidney disease. While at the same time, Lachnospiraceae produces butyric acid that is involved in the improvement of atherosclerosis, maturation of the immune system, host histone epigenetic states, and fatty acid metabolism in colonic epithelial cells. *Blautia* bacteria have some beneficial effects in producing butyric acid and acetic acid in a host gut. On the other hand, *Blautia* bacteria after D-allose ingestion cannot be generalized to include all bacteria in the genus. Overall, our results suggest that D-allose might contribute to the abovementioned physiological functions partially by altering the intestinal microbiome at the genus level. The D-allose group also had a significantly lower relative abundance of one kind of *Acetatifactor* bacteria (*t* (14.7) = 2.35, *p* < 0.05), specifically *Acetatifactor muris* (see Table 3).

**Species level.** The results of our species-level analyses are shown in Table 3. The D-allose group had a significantly higher relative abundance of two species in the intestinal microflora. *Bacteroides acidifaciens* was more abundant compared to controls (*t* (13.2) = 3.05, *p* < 0.01). *B. acidifi-
ciens* helps maintain the intestinal immune system and produces propionic acid. *Akkrernansia muciniphila*, which resides in the human intestine as a typical mucin glycandegrading bacteria, was more abundant compared to controls (*t* (11.1) = 2.21, *p* < 0.05). *A. muciniphila* is expected to be a potential probiotic, and its abundance is inversely correlated with metabolic disorders, such as diabetes, obesity, and inflammatory conditions. The D-allose group had a significantly lower relative abundance of *Ruminococcus gnavus* (*t* (10.7) = 2.70, *p* < 0.05), which produces an inflammatory

### Table 1. Relative abundance of detected bacteria in aged mouse cecum after long-term ingestion of D-allose: Phylum level.

| Rank | Phylum               | Control         | D-Allose       | Fold-change†† |
|------|----------------------|-----------------|----------------|--------------|
| 1    | Firmicutes           | 74.4 ± 3.0      | 66.7 ± 2.4     | 0.897        |
| 2    | Bacteroidetes        | 21.2 ± 2.6      | 28.5 ± 2.2     | 1.34         |
| 3    | Actinobacteria*      | 1.38 ± 0.24     | 3.09 ± 0.60    | 2.24         |
| 4    | Proteobacteria*      | 2.45 ± 0.38     | 1.05 ± 0.28    | 0.429        |
| 5    | Verrucomicrobia      | 0.0132 ± 0.013  | 0.494 ± 0.26   | 37.4         |
| –    | Other Phyla          | 0.635 ± 0.11    | 0.205 ± 0.15   | –            |

Control group (10 mice) and D-allose group (12 mice). Mean values ± SEM; *p* < 0.05, **p** < 0.01 by Welch’s *t*-test.

† Rank from highest to lowest percentage in total of control and D-allose group. † † Quantity of change between the D-allose group and control group (i.e., D-Allose / Control).

### Table 2. Relative abundance of detected bacteria in aged mouse cecum after long-term ingestion of D-allose: Genus level.

| Rank | Genus                  | Relative abundance (%) |
|------|------------------------|------------------------|
| 1    | Lactobacillus         | 26.9 ± 4.6             |
| 2    | Bacteroides           | 20.1 ± 1.3             |
| 3    | *g. Lachnospiraceae bacterium KNHs209_incertae_sedis* | 18.0 ± 3.2 |
| 4    | Faecalibaculum        | 7.37 ± 2.0             |
| 5    | Bifidobacterium       | 3.87 ± 1.1             |
| 6    | Roseburia             | 2.53 ± 0.64            |
| 7    | Acetatifactor         | 3.37 ± 0.80            |
| 8    | Lachnolacostrium      | 1.75 ± 0.26            |
| 9    | Flintbacter           | 2.45 ± 0.24            |
| 10   | Enterorhabdus         | 1.66 ± 0.16            |
| 11   | Muribaculum           | 1.85 ± 0.27            |
| 12   | Clostridum            | 0.199 ± 0.13           |
| 13   | Blautia*              | 1.92 ± 0.40            |
| 14   | Akkrernansia          | 0.0461 ± 0.045         |
| 15   | Acetatifactor*        | 1.21 ± 0.31            |
| 16   | Maritococcus          | 0.262 ± 0.043          |
| 17   | Jeotgalicoccus        | 0.240 ± 0.053          |
| 18   | Harviflavia           | 0.253 ± 0.052          |
| 19   | Escherichia           | 0.397 ± 0.33           |
| 20   | Anaeroterrae          | 0.376 ± 0.17           |
| –    | Classified others     | 3.81 ± 1.1             |
| –    | Unclassified others   | 1.24 ± 0.22            |

Same conventions as in Table 1.
polysaccharide. In patients with Crohn’s disease, the relative abundance of *R. gnarus* increases during symptom flares. These results suggest that D-allose might contribute to maintaining intestinal health. The D-allose group had a significantly lower relative abundance of *Bacteroides caecimuris* (t (19.3) = 3.45, p < 0.01) and *Acetatifactor muris* (t (11.3) = 5.02, p < 0.05), which can be isolated from the cecal content of obese mice. The physiological function of these species is still unclear.

**Organic acid composition in aged cecum after long-term ingestion of D-allose.** D-Allose altered the relative abundance of several intestinal bacterial species and genera associated with organic acid production (Table 4). We measured increased *B. acidifaciens*, a species that produces propionic acids, but decreased *Lactobacillus gasseri*, a family that produces butyric acids. However, compared to the control group, the total organic acid content and the overall organic acid composition in the aged mouse cecum did not change significantly after D-allose administration: succinic acid (t (18.8) = 0.351, p = 0.730); lactic acid (t (16.1) = 1.38, p = 0.187); acetic acid (t (12.2) = 0.623, p = 0.545); propionic acid (t (15.2) = 1.76, p = 0.099); butyric acid (t (20.0) = 0.548, p = 0.590); total organic acids (t (19.0) = 0.453, p = 0.656). Thus, it appears that long-term ingestion of D-allose does not change organic acid concentration in the aged cecum as a result of altering the abundance of various intestinal bacteria.

**Possible mechanism for D-allose in altering intestinal bacteria.** Some sugar-related compounds and artificial sweeteners are reported to alter intestinal flora, and changes in inflammation and metabolism status and fermentability are reported to be involved. Although past studies showed that D-allose by itself is difficult to ferment, intestinal *Escherichia coli* K-12 has been found to possess the genes involved in metabolizing D-allose as an energy source, indicating that this species is capable of growing in the presence of D-allose. As for *B. acidifaciens* and *A. mcanziphila*, which were elevated in mice that ingested D-allose in our study, it is unknown whether they metabolize D-allose. Since D-allose may suppress intestinal α-glucosidase, and thus prevent the breakdown of other carbohydrates in the cecum, these carbohydrates can potentially undergo fermentation, which can affect intestinal flora.

As another possible mechanism, brain-gut interaction is considered, that might account for the way D-allose alters intestinal microbiota is through the central nervous system (CNS). Recently, accumulating evidence has suggested the interaction between the CNS and gut microbiota. Anatomically, a rich network of bidirectional neuronal connections exists between the central and enteric nervous system. Such an anatomical interplay within the gut-brain axis prompted us to hypothesize that the D-allose-induced alterations in intestinal microbiota are achieved through the CNS. Although little is known about the action of D-allose in the CNS, D-allose is reported to protect the brain from ischemic damage caused by ROS. These results suggest that D-allose may exert beneficial effects on cells in the CNS via the

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**Table 3.** Relative abundance of detected bacteria in aged mouse cecum after long-term ingestion of D-allose: Species level.

| Rank | Species | Control | D-Allose | Fold-change*† |
|------|---------|---------|----------|--------------|
| 1    | *Bacteroides acidifaciens* ** | 8.17 ± 0.98 | 17.9 ± 3.0 | 2.19 |
| 2    | *Lactobacillus gasseri* | 10.4 ± 3.9 | 13.1 ± 3.8 | 1.26 |
| 3    | *Bifidobacterium adolescentis* | 7.42 ± 2.0 | 7.09 ± 3.4 | 0.956 |
| 4    | *Bacteroides caecimuris* ** | 8.32 ± 0.72 | 4.14 ± 0.97 | 0.496 |
| 5    | *Butyricoccus autotrophicus* | 5.43 ± 1.8 | 4.81 ± 1.8 | 0.886 |
| 6    | *Bifidobacterium pseudolongum* | 3.42 ± 1.1 | 6.40 ± 1.5 | 1.87 |
| 7    | *Roseburia faeces* | 2.54 ± 0.64 | 3.24 ± 2.2 | 1.30 |
| 8    | *Bacteroides sartorii* | 2.99 ± 0.36 | 2.39 ± 0.54 | 0.799 |
| 9    | *Acetitijfaciens* | 3.38 ± 0.80 | 1.50 ± 0.41 | 0.443 |
| 10   | *Flanbitacter butyricus* | 2.45 ± 0.24 | 1.74 ± 0.50 | 0.710 |
| 11   | *Clostridium scindens* | 1.41 ± 0.26 | 1.95 ± 0.86 | 1.38 |
| 12   | *Muribaculum intestinate* | 1.85 ± 0.27 | 1.51 ± 0.23 | 0.816 |
| 13   | *Acidaminococcus intestini* | 0.0100 ± 0.0067 | 2.12 ± 2.1 | 212 |
| 14   | *Acetifactor muris* | 0.0458 ± 0.0045 | 1.95 ± 0.86 | 42.6 |
| 15   | *Ruminococcus gnavus* | 1.54 ± 0.41 | 0.391 ± 0.13 | 0.254 |
| 16   | *Acetitijfaciens* | 1.21 ± 0.31 | 0.212 ± 0.11 | 0.175 |
| 17   | *Enterorhabdus muris* | 0.677 ± 0.10 | 0.488 ± 0.14 | 0.721 |
| 18   | *Acetitijfaciens meyeri* | 0.00114 ± 0.0011 | 1.10 ± 1.1 | 965 |
| 19   | *Enterorhabdus mucosicola* | 0.618 ± 0.066 | 0.527 ± 0.16 | 0.853 |
| 20   | *Enterorhabdus caecimuris* | 0.361 ± 0.10 | 0.683 ± 0.20 | 1.89 |
| -    | Classified other species | 0.0346 ± 0.010 | 0.0406 ± 0.012 | – |
| -    | Unclassified other species | 31.4 ± 5.6 | 19.5 ± 1.8 | – |

Same conventions as in Table 1.

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**Table 4.** Organic acid concentration (µmol/g) in aged mouse cecum after long-term ingestion of D-allose.

|          | Succinic acid | Lactic acid | Acetic acid | Propionic acid | Butyric acid | Total |
|----------|---------------|-------------|-------------|----------------|--------------|-------|
| Control  | 2.02 ± 0.27   | 15.3 ± 3.0  | 63.3 ± 0.8  | 7.37 ± 0.70    | 17.7 ± 1.7   | 106 ± 3.7 |
| D-Allose | 1.89 ± 0.23   | 10.1 ± 2.0  | 65.6 ± 3.4  | 8.88 ± 0.43    | 16.2 ± 2.0   | 101 ± 5.2 |

Control group (10 mice) and D-allose group (12 mice). Mean values ± SEM.
Concluding remarks. In this study, we investigated the effect of long-term D-allose administration on the intestinal environment in aged mice. D-Allose favorably altered the balance of bacterial phyla of the intestinal microbiome. This improvement implies that D-allose might favorably modulate one feature of aging; that is, age-related decrements in beneficial gut bacterial, perhaps ultimately protecting against various gut-related diseases. Moreover, long-term D-allose ingestion altered several genera and species of intestinal bacteria related to metabolic disorders or intestinal health. Our study revealed the beneficial effect of D-allose at the genus and species level, however, we need to explore the biological significance of the changes in the intestinal environment to establish the physiological roles of D-allose. Also, we need to assess the biological parameters related to specific intestinal bacteria, such as \textit{B. acidifaciens} and \textit{A. muciniphila}. Further, clinical trials will be essential to establish the effect of D-allose in humans, considering known differences in the kind and proportions of intestinal bacteria between mice and humans.\textsuperscript{39}

CONFLICTS OF INTEREST

The authors declare the following financial interests/personal relationships that may be considered as potential competing interests: Tomoya Shintani, Akane Kanasaki, Misuzu Tanaka, and Tetsuo Iida are employees of the Matsutani Chemical Industry Co., Ltd. Genki Ozawa and Tadao Kunihiro are employees of Techno Suruga Laboratory Co., Ltd.

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