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Impact of D-allulose consumption on Enteric pathogens in human gut Microbiota: A randomized controlled trial study

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ABSTRACT

D-allulose, a rare sugar recognized as Generally Recognized as Safe, has emerged as a potential alternative to sucrose. Despite its growing popularity, research on its effects on the human gut microbiota, including pathogens, remains scarce. To address these concerns, we conducted a 12-week randomized, double-blind, parallel, placebo-controlled study assessing D-allulose's safety on gut microbiota in humans. Participants consumed 15 g/day of D-allulose or sucralose (placebo) for 12 weeks. Gut microbiota analysis in stool samples, performed through shotgun metagenomics sequencing before and after the intervention, evaluated microbial diversity, taxonomy of prevalent species, changes in pathogenic bacteria (*Clostridium difficile, Helicobacter hepaticus, Klebsiella pneumoniae, Bacteroides fragilis, Staphylococcus aureus, and Salmonella enterica*), and short-chain fatty acid production. Our findings revealed no significant differences in microbial diversity, pathogenic bacteria levels, or short-chain fatty acid production, suggesting that D-allulose consumption is safe and does not adversely affect the gut microbiome or pathogen presence.

1. Introduction

D-allulose, an epimer of D-fructose, is categorized as a rare sugar due to its limited occurrence in nature. It possesses 70 % of the sweetness of sucrose while being low in calories (Han et al., 2018) and has been associated with beneficial health effects, including an anti-hyperglycemic or anti-inflammatory effect (Hossain et al., 2015). The U.S. Food and Drug Administration (FDA) recognizes D-allulose as Generally Recognized as Safe (GRAS), under GRN 400, 498, 693, 828, and 1024.

As a sugar substitute, D-allulose has garnered considerable interest. Nonetheless, its interaction with the intestinal microbiota, particularly pathogenic organisms, remains incompletely elucidated. Previous research has suggested that foods containing trehalose may facilitate the spread of *Clostridium difficile* (Collins, Danhof, & Britton, 2019; Collins et al., 2018), a bacterium capable of causing severe gastrointestinal illnesses. Additionally, *in vitro* studies have indicated that certain bacteria, like *Klebsiella*, can metabolize D-allulose (Daniel, Hauner, Hornef, & Clavel, 2022; Martin et al., 2018). This is concerning since *Klebsiella pneumoniae* is known as an opportunistic human pathogen.

Given the potential for rare sugars to increase hospital infections with virulent bacteria such as *Clostridium difficile* or *Klebsiella pneumoniae*, our study explores the risk associated with D-allulose consumption in humans. We conducted a 12-week study comparing the effects of D-

Abbreviations: **ACE**, Abundance-based Coverage Estimator; **BMI**, Body Mass Index; **FDA**, U.S. Food and Drug Administration; **GC-FID**, Gas Chromatography-Flame Ionization Detector; **GRAS**, Generally Recognized as Safe; **PCoA**, Principal Coordinates Analysis; **PERMANOVA**, Permutational Multivariate Analysis of Variance; **SCFA**, Short-Chain Fatty Acid.

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allulose and a placebo. Han et al. (2020) previously conducted a 16-week animal study on high-fat-diet-induced obesity in mice, which was treated with diets supplemented with either 5 % allulose or 5 % erythritol (Han et al., 2020). Their findings suggested that dietary allulose altered the microbial community in a way that could ameliorate obesity and increase total fecal short-chain fatty acid (SCFA) production, as analyzed through 16S rRNA gene sequencing. Our human study, involving overweight and obese participants, aimed to verify these effects on body fat reduction and D-allulose's safety. Contrary to Han et al.'s findings, our analysis showed no increase in the microbial community or SCFA production after 12 weeks of D-allulose consumption compared to the placebo group. We observed that a daily intake of 15 g of dietary D-allulose does not alter the microbial community, including pathogenic bacteria, in a manner that affects total fecal SCFA production, based on whole-genome shotgun sequencing analysis. This study contributes to the understanding of the gut microbiome's composition, including pathogenic bacteria, in relation to dietary D-allulose and its interactions with gut bacteria.

2. Materials and Methods

2.1. Test substance

Test materials were provided in a 20 g stick pouch, in the form of a lemon-flavored powder, containing either 0.011 g sucralose (for placebo group) or 7.5 g D-allulose (for test groups) (Table 1). Participants consumed the placebo or D-allulose (two stick pouch per day). All materials were supplied by Samyang Corporation, Republic of Korea. Test materials were identified by different cod numbers, the identities of which were not revealed to the investigator or subjects until the completion of the study.

2.2. Subjects

All participants were aged 19–65 years and resided in the Republic of Korea. After an initial screening, 228 subjects with BMI $\geq$ 25 kg/m^2 and $\leq$ 35 kg/m^2 were selected. We selected randomly in 1:1 ratio to placebo control (n = 9) and D-allulose (n = 9) groups for the gut microbiome analysis (Table 2). The exclusion criteria were as follows: subjects with diagnosis such as hypertension, diabetes (potential diabetes), dysfunction of liver and kidney, central nervous system disorders, thyroid gland disease, allergy, cerebrovascular disease, cardiovascular disease, gastrointestinal disease, cancer, pulmonary disease, musculoskeletal disorders, immune disorder or inflammatory disease, and allergy or anaphylaxis to ingredients of test products. All subjects gave their written informed consent for inclusion before they participated in the study. The study was approved by the local ethical committee (IRB no. KHNMC 2021–03-029–020) The trial was registered with the Clinical Research Information Service (CRIS no. KCT0008184).

2.3. Design

This research was conducted from June 2021 to December 2022 as a randomized, double-blind, parallel, placebo-controlled study. The random allocation sequence for the larger pool of participants was created using computer-generated random numbers, aiming for broader demographic representation in the initial assessments related to safety and general health impacts. For the specific gut microbiome and SCFA analysis, a subset of 18 participants (9 in each group) was randomly selected from the larger study group for detailed analysis, maintaining the integrity of the randomized, controlled design. Allocation concealment was ensured through sequentially numbered containers prepared by an independent laboratory researcher. Participants were blinded to the sequence and randomization details until the study's conclusion. The supplements, whether containing D-allulose or placebo (sucralose), were indistinguishable in flavor and color, packaged in silver stick pouches. During the 12-week study, participants were advised to reduce their daily energy intake by 500 kcal from their usual consumption and encouraged to engage in physical activities burning approximately 300 kcal/day. They were required to consume two stick pouches of the supplement daily. The dosage of D-allulose was determined based on extrapolations from previous animal studies (Lee et al., 2021). Fecal samples for microbiota and SCFA analysis were collected before and after the intervention using provided kits (Fig. 1).

Table 1
Food composition used in this study (Unit g/20 g).

	Placebo (Sucralose)	Test (D-Allulose)
Sucralose	0.011	–
D-allulose	–	7.5
Citric acid	0.016	0.014
Trisodium citrate	0.006	0.006
L-Ascorbic acid	0.004	0.004
Lemon flavor	0.024	0.024
Purified water	19.939	12.452

Table 2
Characteristics of study participants (Mean ± SD).

Variables	Total (n = 18)	Placebo (n = 9)	D-allulose (n = 9)	P value
Sex (Female:Male)	12:6	6:3	6:3	1.00
Age (years)	41.7 ± 10.7	43.6 ± 9.0	39.8 ± 11.9	0.48
Weight (kg)	73.6 ± 8.7	71.0 ± 8.7	76.2 ± 7.9	0.22
BMI (kg/m^2)	27.4 ± 1.8	27.2 ± 1.5	27.7 ± 2.0	0.62
Morbid obesity (n, %)[#]	3 (16.7)	1 (11.1)	2 (22.2)	0.67

[#] Defined as BMI $\geq$ 30 kg/m^2.

2.4. Analysis of Short-Chain fatty acids (SCFAs)

Prior to sample analysis, a standard mixture of six types of SCFAs (acetic acid, propionic acid, *iso*-butyric acid, butyric acid, *iso*-valeric acid, valeric acid) was prepared at optimal analysis concentrations. This mixture was serially diluted with triple-distilled water from high to low concentrations and analyzed four times to normalize the gas chromatogram peak areas (peak area) to an internal standard. This normalization established a quantitative curve for the six SCFAs and determined the correlation coefficient values, allowing for the calculation of SCFA quantities present in the feces. The supernatant obtained after homogenizing the fecal mass with five times its volume of triple-distilled water and centrifugation, was mixed with an equal volume of buffer solution for gas chromatography analysis. This mixture was then added to a Gas Chromatography-Flame Ionization Detector (GC-FID) vial, sealed with a cap, and analyzed.

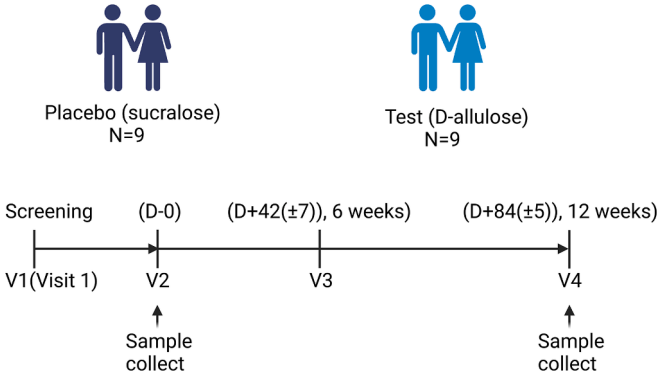

Fig. 1. Study scheme for the microbiome and SCFA.

2.5. Fecal shotgun metagenomics sequencing analysis

Fecal bacterial genomic DNA was extracted using the Mag-Bind® Universal Pathogen Kit (Omega Bio-tek). The process began by suspending fecal samples in 275 μL of SLX-Mlus Buffer, followed by mechanical disruption through bead beating in a MixerMill MM400 (Retsch). Subsequent steps involved isolation, purification, and elution of DNA, adhering strictly to the manufacturer's protocols. Library preparation for sequencing was conducted using the TruSeq DNA Nano Library Preparation Kit (Illumina), following the manufacturer's guidelines. The prepared libraries were sequenced on a NovaSeq 6000 platform utilizing a 2 × 150 bp paired-end (PE) configuration. Quality control of the sequencing data was conducted using FastQC software. Taxonomic assignment was carried out using Kraken version 2.0.9 beta (Wood, Lu, & Langmead, 2019) (database: k2_standard_20220607). The results of the taxonomic classification were analyzed in R software (version 4.3.0) for diversity analysis and pathogen identification. Statistical analyses were executed using nonparametric tests to ensure robust data interpretation.

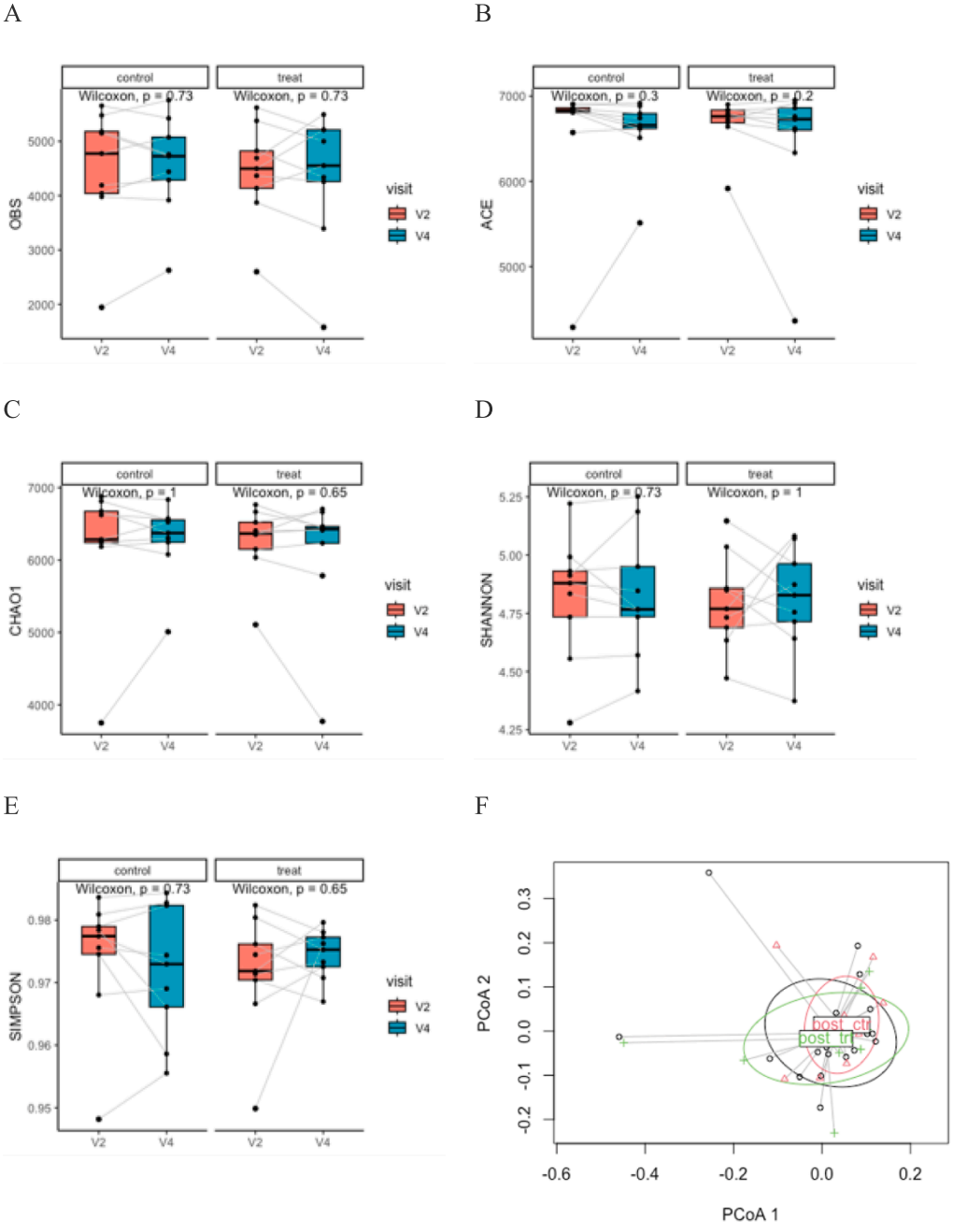

Fig. 2. Effect on alpha-diversity and beta-diversity of fecal microbiota. Observed species (OBS) (A), ACE index (B), CHAO1 index (C), SHANNON index (D), SIMPSON (E). Values indicate the means ± SEM. Data were analyzed by the paired Wilcoxon rank-sum test between the groups and the p value is shown in each panel. Beta diversity analysis of the D-allulose group and placebo group (control vs treat p-value: 0.3887, V2 vs V4 p-value: 0.9773) (Green is control group, Red is D-allulose group) (F). The Principal Coordinates Analysis (PCoA) is based on Bray-Curtis distance matrix. V2 represents before the intake of the test substance, and V4 represents after the intake. Control is the placebo group, and treat is the D-allulose group.

3. Results

3.1. Baseline Characteristics of study participants

The average age of total participants was 41.7 ± 10.7 years, 33.3 % were men, and the average BMI was 27.4 ± 1.8 kg/m^2. The age (43.6 ± 9.0 vs. 39.8 ± 11.9 years, p = 0.48) and the proportion of men (33.3 vs. 33.3 %, p = 1.00) did not differ between placebo and D-allulose groups. BMI (27.2 ± 1.5 vs. 27.7 ± 2.0 kg/m^2, p = 0.62) and the proportion of morbidly obese patients defined as BMI ≥ 30 kg/m^2 (11.1 vs. 22.2 %, p = 0.67) did not differ between placebo and D-allulose groups., either. (Table 2).

3.2. Microbial diversity

On average, each of the 36 fecal samples, collected from 18 participants at two visit points, generated 15.5 GB of raw sequencing data. This data was then preprocessed to remove low-quality reads and host-derived DNA sequences. To assess the impact of D-allulose on gut microbiota, we utilized alpha-diversity indices—observed species (actual species richness), ACE index (abundance-based coverage estimator), Chao1 index (estimated species richness), Shannon index, and Simpson index—alongside beta-diversity measures. No significant changes were observed in these alpha-diversity indices between the baseline (0 weeks) and 12 weeks after initiating the intervention in both the placebo and D-allulose groups (Fig. 2A–E). Principal Coordinates Analysis (PCoA) based on the Bray–Curtis distance matrix, both before and after consumption, and between the D-allulose and placebo groups, was validated through PERMANOVA analysis (p = 0.9773). Additionally, no clear increase was observed in the top 20 most abundant species between the D-allulose and placebo groups, as shown in Fig. 3.

3.3. Comparative analysis of differential abundance

To identify microorganisms exhibiting differential abundance before and after intake between the control and test groups, we conducted the Limma-Voom test (Ritchie et al., 2015). This method, one of several for analyzing differential abundance in the microbiome, transforms sequence counts to a log scale and then estimates the mean–variance relationship, allowing for highly precise analysis. Microorganisms were selected based on a nominal p-value of ≤ 0.05 in at least one group. This selection was made at the species level, and out of a total of 8201 species, 102 were identified as differentially abundant. Microorganisms marked with red dots indicate those within each group (top: Control, bottom: Test) that showed a statistically significant difference in abundance before and after intake, with a nominal p-value of ≤ 0.05. Among the microorganisms selected in the test group, *Lactobacillus sakei* showed a significantly higher abundance after intake. *Alistipes indistinctus* was found to be more abundant in the control groups than in the test groups.

When further refining the selection of the initially identified 102 microorganisms by lowering the threshold for the nominal p-value to ≤ 0.01, 6 species were found to show differential abundance before and after intake in the control group (*Shigella flexneri*, *Pseudomonas sp*. KNUC1026, *Staphylococcus saprophyticus*, *Staphylococcus saprophyticus*, *Alkalihalobacillus sp*. LMS39, *Mycolicibacterium vaccae*), and 5 species in the test group (*Thermoanaerobacterium sp*. RBIITD, *Sphingomonas koreensis*, *Ralstonia sp*. 56D2, *Aureimonas altamirensis*, *Geomonas subterranea*), totaling 11 species identified with significant differential abundance.

3.4. Changes in pathogen microbiome

Participants were instructed to consume supplements containing D-allulose or sucralose, and changes in the pathogen microbiome were monitored within the fecal microbiota. The nonparametric Wilcoxon rank-sum test was used to compare the relative abundance of *Helicobacter hepaticus*, *Klebsiella pneumoniae*, and *Bacteroides fragilis* before and after treatment in each group. While the relative abundance of *Clostridioides difficile* significantly increased in the placebo group, no significant increase was observed in the test group. No significant changes in *Helicobacter hepaticus*, *Klebsiella pneumoniae*, *Bacteroides fragilis*, *Staphylococcus aureus*, and *Salmonella enterica* were confirmed following the consumption of either placebo or D-allulose (Fig. 4).

3.5. Effects of D-allulose on SCFA production

We compared the amounts of short-chain fatty acids (SCFAs) before

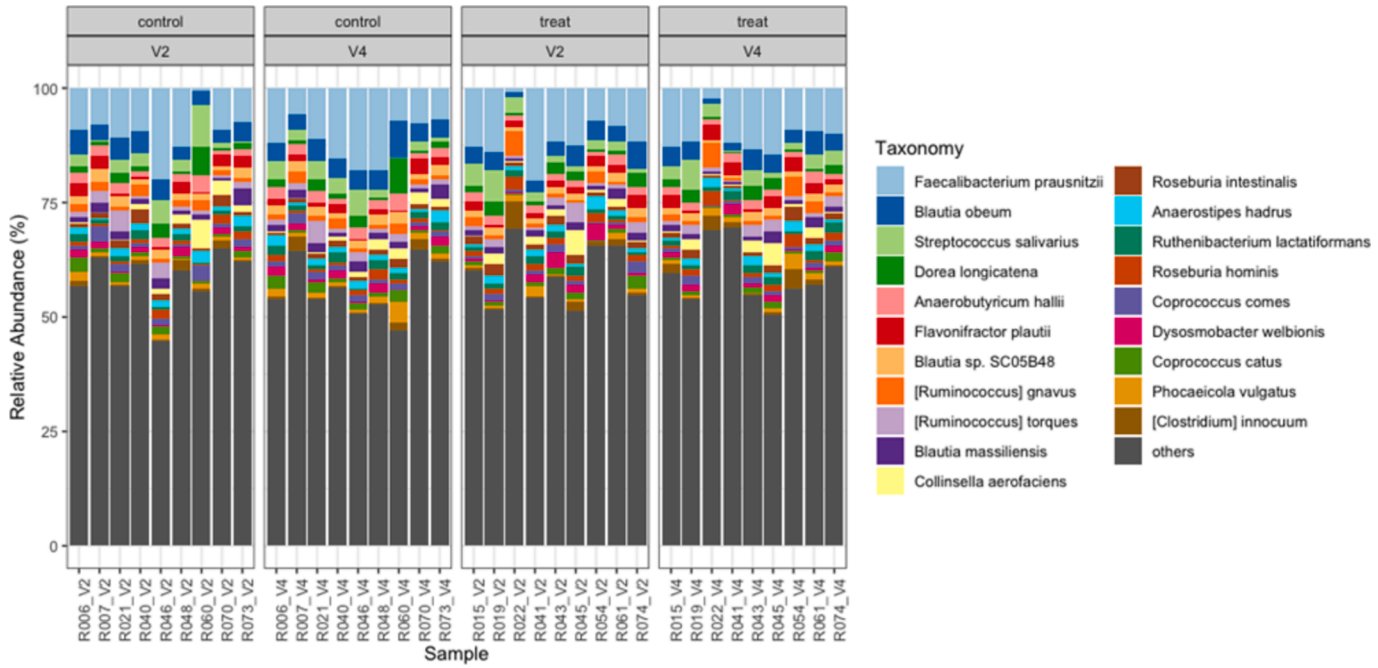

Fig. 3. Microbial taxonomy of the top 20 species with the highest abundance between the D-allulose and placebo groups. V2 represents before the intake of the test substance, and V4 represents after the intake. Control is the placebo group, and treat is the D-allulose group.

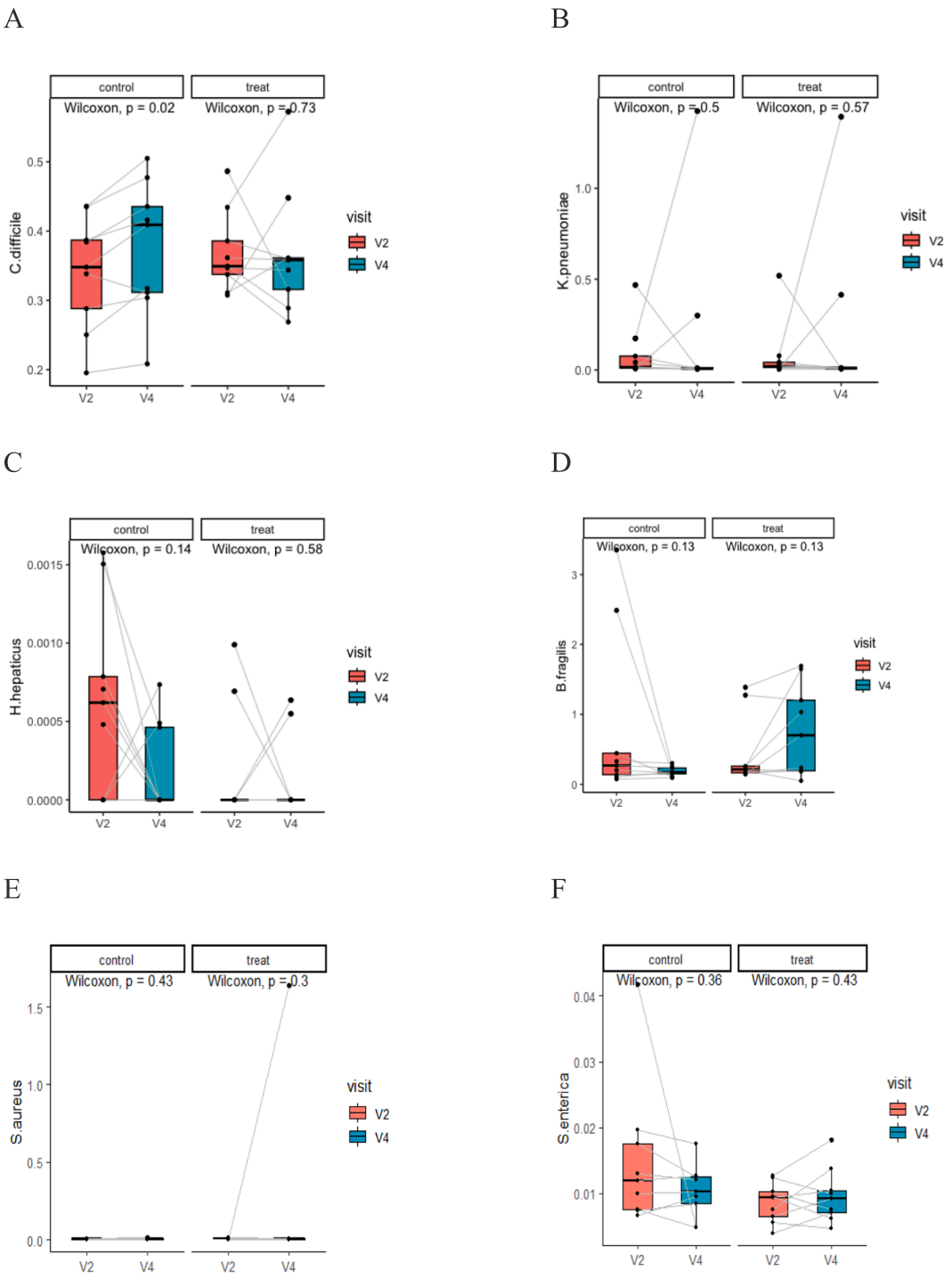

Fig. 4. Alteration of (A) *Clostridium difficile*, (B) *Klebsiella pneumoniae*, (C) *Helicobacter hepaticus* (D) *Bacteroides fragilis* (E) *Staphylococcus aureus*, and (F) *Salmonella enterica* between before and after consumption of placebo and allulose. V2 represents before the intake of the test substance, and V4 represents after the intake. Control is the placebo group, and treat is the D-allulose group.

and after consumption in both the control and test groups (Fig. 5A). The quantitative analysis of six SCFAs (acetic acid, propionic acid, *iso*-butyric acid, butyric acid, *iso*-valeric acid, and valeric acid) showed no significant differences in levels between the groups. Similarly, no significant differences were observed when comparing the pre-consumption (pre) levels with those after consumption in the control (post_ctr) and test (post_trt) groups (Fig. 5B). Although not statistically significant, the levels of acetic acid, propionic acid, and butyric acid, which are SCFAs produced by beneficial bacteria, showed a slight increase in the test group after consumption.

4. Discussion

Our investigation into the effects of D-allulose on the gut microbiome and short-chain fatty acid (SCFA) production yields important insights into its safety of D-allulose on potential implications for pathogenic infections in the gut. Consistent with prior research indicating the safety of D-allulose as a food additive (Iida et al., 2008), our study reinforces the notion that D-allulose does not adversely affect gut microbial diversity or SCFA production. These findings are pivotal, considering the growing interest in functional sugars as healthier alternatives to

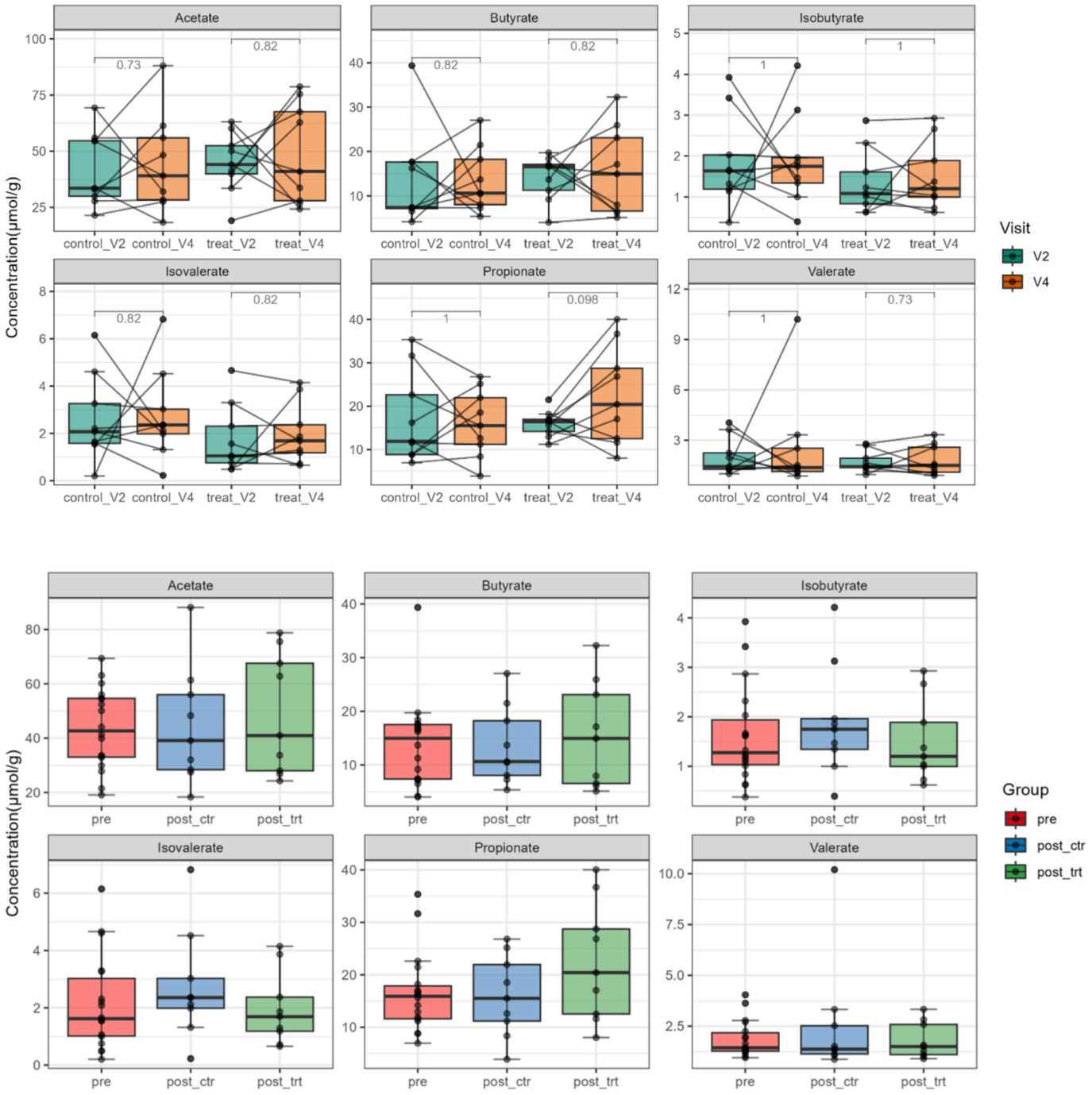

Fig. 5. Impact of D-allulose on Short-Chain Fatty Acid (SCFA) Production. This figure shows the levels of Acetate, Butyrate, Isobutyrate, Isovalerate, Propionate, and Valerate. Data are presented as mean ± Standard Error (SE); V2 indicates the period before intake of the substance under test, while V4 indicates after intake. The control group is represented by the placebo, and the treatment group by D-allulose. (A) Comparison of SCFAs by Group (B) Comparison of SCFAs Before and After Consumption in the Test Group.

traditional sweeteners.

The absence of significant changes in the gut microbiome profile after D-allulose consumption is noteworthy. The gut microbiota plays a crucial role in human health, influencing nutrient metabolism, immune function, and the risk of several diseases (Clemente, Ursell, Parfrey, & Knight, 2012). Disruptions in the microbial community, known as dysbiosis, have been linked to a variety of conditions, including obesity, diabetes, and inflammatory bowel disease (Tilg & Kaser, 2011). Our study's findings suggest that D-allulose does not contribute to such dysbiosis, indicating its non-disruptive nature on gut microbial homeostasis.

Moreover, our analysis revealed no significant alterations in the production of key SCFAs, including acetate, propionate, and butyrate. These metabolites are vital for maintaining gut barrier integrity, modulating immune responses, and providing energy to colonocytes (Koh, De Vadder, Kovatcheva-Datchary, & Backhed, 2016). Their steady levels post-D-allulose consumption align with the sugar's safety profile and suggest that it does not interfere with the microbial processes critical for SCFA production.

The differential abundance analysis, employing the Limma-Voom test, further enhances our understanding of D-allulose's impact on the gut microbiome. Despite identifying species that showed variations in

abundance, these did not translate into significant changes in overall microbial diversity or health-associated markers. *Lactobacillus sakei*, which showed a significantly higher abundance after intake in the test group, is known as a probiotic related to fat reduction (Lim, Moon, Shin, Jeong, & Kim, 2020). Notably, the identification of *Lactobacillus sakei* in the test group as differentially abundant, albeit not statistically significant, aligns with existing literature on its beneficial effects on obesity and metabolic health (Lim et al., 2020; Tilg & Kaser, 2011). *Alistipes indistinctus*, on the other hand, has been reported to be more abundant in control groups than in obese groups in studies related to obesity and gut microbiota (Bisanz, Upadhyay, Turnbaugh, Ly, & Turnbaugh, 2019).

No significant alterations were observed in pathogenic microorganisms such as *Clostridium difficile*, *Helicobacter hepaticus*, *Klebsiella pneumoniae*, *Bacteroides fragilis*, *Staphylococcus aureus*, and *Salmonella enterica* in the fecal microbiota. (Azimirad et al., 2020; Collins et al., 2019; Collins et al., 2018; Daniel et al., 2022; Martin et al., 2018; Mukherjee, Nolan, Dunn, & Banerjee, 2019; Shi et al., 2021).

Additionally, it is important to address concerns regarding the relevance of our findings to more vulnerable populations, such as patients with compromised immune systems or chronic illnesses. While the present study was designed as a pilot investigation involving healthy individuals, it serves as an important preliminary step in evaluating the safety of D-allulose. The studies by Blin et al. (2017) and Martin et al. (2018) have raised valid concerns regarding the potential interactions between sugars like D-arabinose and pathogenic bacteria. However, D-allulose and D-arabinose are structurally distinct, and the effects observed in the aforementioned studies cannot be directly extrapolated to D-allulose (Blin, Passet, Touchon, Rocha, & Brisse, 2017; Martin et al., 2018). Given these considerations, future research should aim to investigate the effects of D-allulose in more diverse and potentially vulnerable populations. Larger-scale clinical trials involving patients, such as those who are immunocompromised or suffering from chronic conditions, will be crucial to comprehensively assess the safety and potential health benefits of D-allulose.

Our study's findings contrast with those of Han et al., who reported increased fecal SCFA production following dietary intervention with D-allulose (Han et al., 2020). This discrepancy could be attributed to differences in study design, subject population, or dietary background, highlighting the complexity of gut microbiota responses to dietary interventions. It also suggests that the impact of D-allulose on SCFA production and the microbiome may be context-dependent, varying with individual gut microbiota compositions or dietary patterns.

Importantly, the safety profile of D-allulose, demonstrated through the lack of significant changes in pathogenic bacteria, adds to its appeal as a sugar substitute. The potential of sugar substitutes to alter gut microbiota composition and function has raised concerns, particularly regarding their impact on the proliferation of pathogens (Suez et al., 2014). Our findings alleviate such concerns for D-allulose, suggesting it does not promote the growth of harmful bacteria within the gut ecosystem.

In this study, while we have provided important insights into the safety of D-allulose on potential implications for pathogenic infections in the gut, it is crucial to acknowledge certain limitations, notably the small sample size of our groups (9 participants in the placebo group and 9 in the test group). This limitation may affect the generalizability of our results and the statistical power to detect significant differences. Therefore, our findings should be interpreted with caution and seen as a preliminary step that necessitates further research. Future studies with larger and more diverse populations are essential to validate and extend our observations, providing a more comprehensive understanding of D-allulose's impact on the gut microbiome and SCFA production. Such research will also help to explore the long-term effects of D-allulose consumption on metabolic health and disease risk in broader demographic contexts.

5. Conclusions

In summary, our study confirms the safety of D-allulose as a food ingredient, with no adverse effects on the gut microbiome or SCFA production. These results contribute valuable information to the ongoing discourse on dietary sweeteners and gut health, providing a scientific basis for the continued use and study of D-allulose in nutrition and health sciences. Future research should aim to explore the long-term effects of D-allulose consumption on the gut microbiome and metabolic health, with a focus on diverse populations and dietary contexts to fully understand its impact and potential benefits.

6. Ethics statement

All subjects gave their written informed consent for inclusion before they participated in the study. The study was approved by the local ethical committee (IRB no. KHNMC 2021-03-029-020) The trial was registered with the Clinical Research Information Service (CRIS no. KCT0008184).

CRediT authorship contribution statement

Heekuk Park: Writing – original draft, Data curation. **Jihye Baek:** Visualization, Validation, Software, Formal analysis. **Se Young Park:** Methodology, Investigation, Formal analysis. **Soonok Sa:** Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Ji Eun Jun:** Methodology, Investigation, Formal analysis. **Min Jeong Kim:** Resources, Methodology, Investigation, Formal analysis. **In-Kyung Jeong:** Writing – original draft, Supervision, Resources, Project administration, Investigation. **Wonyong Kim:** Writing – review & editing, Visualization, Validation, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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