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Kestose Increases the Relative Abundance of *Faecalibacterium* spp. and Nominally Increases Cow Milk Tolerant Dose in Children with Cow's Milk Allergy – Preliminary Results

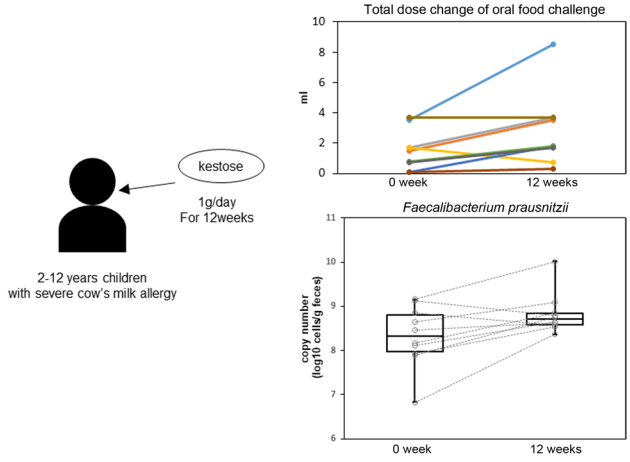
SHOHEI KUBOTA[1, 2], SHIRO SUGIURA[1], MAYUKO TAKAHASHI[3]*, YOSHIHIRO KADOTA[3], YOSHIHIRO TAKASATO[1], TERUAKI MATSUI[1], KATSUMASA KITAMURA[1], TAKUMI TOCHIO[3, 4] and KOMEI ITO[1, 5]

[1] Aichi Children's Health and Medical Center, Obu-shi, Japan
[2] Saiseikai Yokohamashi Tobu Hospital, Yokohama-shi, Japan
[3] B Food Science Co., Ltd., Chita-shi, Japan
[4] Fujita Health University, Toyoake-shi, Japan
[5] Nagoya University Graduate School of Medicine, Nagoya-shi, Japan

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Abstract

A single-arm study was conducted with 10 children aged 2–12 years with severe cow's milk allergy (CMA) requiring complete allergen elimination. Subjects were administered kestose, a prebiotic, at 1 or 2 g/day for 12 weeks. Results of a subsequent oral food challenge (OFC) showed a statistically significant increase in the total dose of cow's milk ingestion (1.6 ml vs. 2.7 ml, $p = 0.041$). However, the overall evaluation of the OFC results, TS/Pro (total score of Anaphylaxis Scoring Aichi (ASCA)/cumulative dose of protein), showed no statistically significant improvement, although the values were nominally improved in seven out of 10 subjects. The 16S rDNA analysis of fecal samples collected from the subjects revealed a statistically significant increase in the proportion of *Faecalibacterium* spp. (3.8 % vs. 6.8%, $p = 0.013$), a type of intestinal bacterium that has been reported to be associated with food allergy. However, no statistically significant correlation was found between *Faecalibacterium* spp. abundance and the results of the OFC.



K e y w o r d s: cow's milk allergy, gut microbiota, Anaphylaxis Scoring Aichi (ASCA), kestose, *Faecalibacterium*

Introduction

A food allergy is defined as an adverse health effect arising from a specific immune response, specifically, an effect that occurs reproducibly on exposure to a given food (Boyce et al. 2010). The gold standard in diagnosing food allergy is the oral food challenge (OFC), and management of the patient largely remains based on avoidance of the causative antigen (Sicherer and Sampson 2018). As a part of the diagnosis of food allergies, immunological tests such as the antigen-specific IgE antibody test are usually performed. The presence of specific IgE antibodies indicates an underlying immunological mechanisms when symptoms are provoked in an OFC; moreover, higher IgE titers have been reported to be related to a higher probability of challenge-positive results (Komata et al. 2007). On the other hand, specific IgG4 antibodies compete with specific IgE antibodies to bind allergens, thereby interfering in specific IgE antibody-mediated activation of basophils

* Corresponding author: M. Takahashi, B Food Science Co., Ltd., Chita-shi, Japan; **e-mail: m-takahashi@bfsci.co.jp**
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and mast cells and inhibiting allergic symptom (Santos et al. 2015). Cow's milk is the second-most-common cause of childhood food allergies in Japan. Although most children with cow's milk allergy (CMA) naturally acquire tolerance with age, severe cases, such as children who have high specific immunoglobulin E (sIgE) titer to milk, exhibit difficulty in acquiring tolerance (Wood et al. 2013).

Several reports show that intestinal microbiota is associated with the development of food allergy (FA). Fazlollahi et al. (2018) reported that the alpha-diversity index of the intestinal microbiota was high in patients with egg allergy and that the abundance ratios of *Lachnospiraceae*, *Streptococcaceae*, and *Leuconostocaceae* were high in such individuals. In addition, Berni Canani et al. (2016) reported that *Lachnospiraceae* and *Ruminococcaceae* are highly abundant in children with CMA. Those results suggest that different gut microbiome compositions occur in the intestines of patients with FA.

Several therapeutic intervention trials have investigated the possibility of alleviating FA symptoms by improving dysbiosis. Some probiotics, such as *Lactobacillus rhamnosus* GG, have been reported to improve symptoms (Berni Canani et al. 2017). Kestose is a fructooligosaccharide, a trisaccharide sugar consisting of a fructose residue of sucrose with a fructose β-1,1-glycoside bond. Kestose has been reported to support the proliferation of beneficial intestinal bacteria (Ose et al. 2018; Tanno et al. 2021). The ingestion of this sugar has been shown to improve atopic dermatitis in humans (Shibata et al. 2009; Kadota et al. 2022).

However, to our knowledge, no reports have indicated that three-month kestose ingestion affects the microbiota in children with food allergies or that this sugar alters the severity of milk allergy. Therefore, in the present study, we investigated the effects of kestose on patients with CMA who were on a complete elimination diet for the allergen.

Experimental

Materials and Methods

Study Design. Participants comprised 10 CMA patients, aged 2 to 12 years, who had wholly eliminated cow's milk from their diet. The subjects were recruited at the Aichi Children's Health and Medical Center between June and October 2020. Patients with chronic diseases other than the allergic disease, who were currently on any medication, or had a history of any severe condition were excluded. Patients were prohibited from taking concomitant probiotic and prebiotic preparations affecting gut microbiota and completely eliminated allergens. In addition, if antibiotics or other drugs were used, a record of drug use was carried out. The first OFC to whole milk was conducted within 2 weeks before initiating 1-kestose (B Food Science Co., Ltd., Japan) treatment. For the trial treatment, participants were instructed to take 1 g (< 10 years) or 2 g (≥ 10 years) of 1-kestose orally once per day. A second OFC was performed after 12 weeks (± 1 week) of the intervention. Fecal samples were collected at the start of the study and 12 weeks after the start of the intervention. This study was designed as a single-arm study without a control group because our data have shown that severe milk allergy could not be improved in such short terms (Sugiura et al. 2020); thus, OFCs could be unethical for such patients considering the risk of severe anaphylaxis.

Oral food challenge (OFC). An open OFC to whole milk was performed according to the Japanese guidelines (Ebisawa et al. 2020). The patients were instructed to ingest sequentially 0.2, 0.5, 1, and 2 ml of cow's milk at 40-minute intervals until obvious allergic symptoms were observed. The protocol was modified depending on the expected severity of each patient's reactions. The severity of symptoms was scored using the Anaphylaxis Scoring Aichi (ASCA) scale (Sugiura et al. 2016). In the ASCA, the total score (TS; maximum 240 points) is defined as the sum of the five organ scores (1 to 60 points) observed throughout OFC. To represent the overall severity of the OFC result, we used TS/Pro, which is divided by the allergen's cumulative protein dose (Pro). Pro was calculated as 3.3% of the weight of milk ingested (Sugiura et al. 2020).

Blood test. Blood samples were obtained at the start and the end of the study. We measured Thymus and Activation-Regulated Chemokine (TARC); the levels of total IgE (tIgE) and sIgE against cow's milk, casein, α-lactalbumin, and β-lactoglobulin; and the levels of specific immunoglobulin G4 (sIgG4) against casein. The sIgE and sIgG4 titers were measured using ImmunoCAP® kits (Thermo Fisher Scientific, Sweden).

Evaluation of skin rashes. Eczema was evaluated at the start of the study, and 6 and 12 weeks after the start of the study, using the Eczema Area and Severity Index (EASI) (Hanifin et al. 2001).

Genome extraction. Bacterial DNA was crudely extracted from 1 g of frozen stool sample. The extraction method followed the method of Takahashi et al. (2014). Extracted DNA was purified using a GENE PREP STAR PI-480 automated DNA isolation system (Kurabo Industries, Japan) with a reagent kit for animal tissues (NR-201; Kurabo Industries, Japan) according to the manufacturer's instructions. DNA concentrations were estimated by spectrophotometry using a NanoDrop™ 8000 instrument (Thermo Fisher Scientific Inc., USA).

PCR amplification and analysis of 16S rDNA sequences. Using DNA extracted from feces as a tem-

plate, approximately 430 bp of the 16S rDNA region common to bacteria and Archaea was amplified by polymerase chain reaction (PCR). PCR conditions were as described by Takahashi et al. (2014). PCR products were amplified using a MiSeq system (Illumina Inc., USA). Sequencing was performed using fastq, the sequencing data were assembled using fastq-join to link fastq paired ends, and the results were edited to exclude the primer sequences. Homology searches were performed using the Ribosomal Database Project (RDP) MultiClassifier ver.2.11.

Real-Time Quantitative PCR. Extracted DNA samples were used for quantitative analysis of the intestinal microbiota by real-time quantitative PCR (qPCR). Quick Taq™ HS DyeMix (Toyobo, Japan) was used for the qPCR reaction, following the manufacturer's instructions. Amplification targets were all Eubacteria (Muyzer et al. 1993), *Bifidobacterium* spp. (Gueimonde et al. 2004), *Bifidobacterium longum* (Matsuki et al. 2004), *Faecalibacterium prausnitzii* (Ramirez-Farias et al. 2009), and *Anaerostipes caccae* (Veiga et al. 2010), and primers were those previously reported, respectively.

Statistics. Statistical analysis was performed using IBM® SPSS® Statistics version 26 (IBM Japan, Ltd., Japan). Wilcoxon's signed-rank test was used to compare changes over time. Differences were considered statistically significant at $p < 0.05$.

Ethical consideration. This study was designed according to the Declaration of Helsinki for experiments with human beings. This study was approved by the Certified Clinical Research Review Committee of the Hattori Clinic (CRB3180027) and registered on the jRCT (jRCTs031200075). Written informed consent was obtained from the parents of each participant or the participant him/herself, depending on the participant's age.

Table I
Background information on the participants.

Number of participants	10
Age (median/minimum-maximum)	8/2–12
Male/female	7/3
Atopic dermatitis	8
Bronchial asthma	7
Allergic rhinitis	8
IgE (total) IU/ml (25%–75%)	1,157 (384–2,744)
IgE (milk) kU_A/l (25%–75%)	50 (27–66)
EASI score (25%–75%)	0.3 (0.05–2.1)
Serum TARC (pg/ml) (25%–75%)	551 (333–976)

IgE – Immunoglobulin E,
EASI – Eczema Area and Severity Index,
TARC – Thymus and Activation-Regulated Chemokine

Results

Characteristics of Participants. Ten patients with defined CMA were enrolled; all subjects completed the study. Patient demographics are shown in Table I. The median age was 8 (range, 2–12) years. Male patients accounted for 70% (7/10) of the subjects. Complications from other allergic diseases included atopic dermatitis (80%), bronchial asthma (70%), and allergic rhinitis (80%). The median days of ingestion of 1-kestose were 82 (range: 66–87) days. Two of the patients took antibiotics during the study period. The second OFC was performed at a median of 86 (range: 81–91) days after the first OFC.

OFC. OFC results are shown in Table II. The total dose of cow's milk ingested was higher in the second OFC than in the first OFC, and this difference was

Table II
Oral food challenge results for all patients.

	First OFC			Second OFC		
	TD (ml)	TS (points)	TS/Pro	TD (ml)	TS (points)	TS/Pro (points/g)
Patient 1	3.5	10	94	8.5	5	19
Patient 2	1.5	25	550	3.5	25	236
Patient 3	1.7	15	291	3.7	1	9
Patient 4	1.7	31	601	0.7	20	943
Patient 5	0.1	10	3300	1.8	0	0
Patient 6	0.8	16	660	1.8	20	367
Patient 7	3.7	15	134	3.7	15	134
Patient 8	0.1	10	3300	0.3	25	2750
Patient 9	0.7	11	519	1.7	20	388
Patient 10	3.7	15	134	3.7	40	357

OFC – Oral Food Challenge, TD – Total Dose, TS – Total Score of ASCA,
Pro – cumulative protein dose

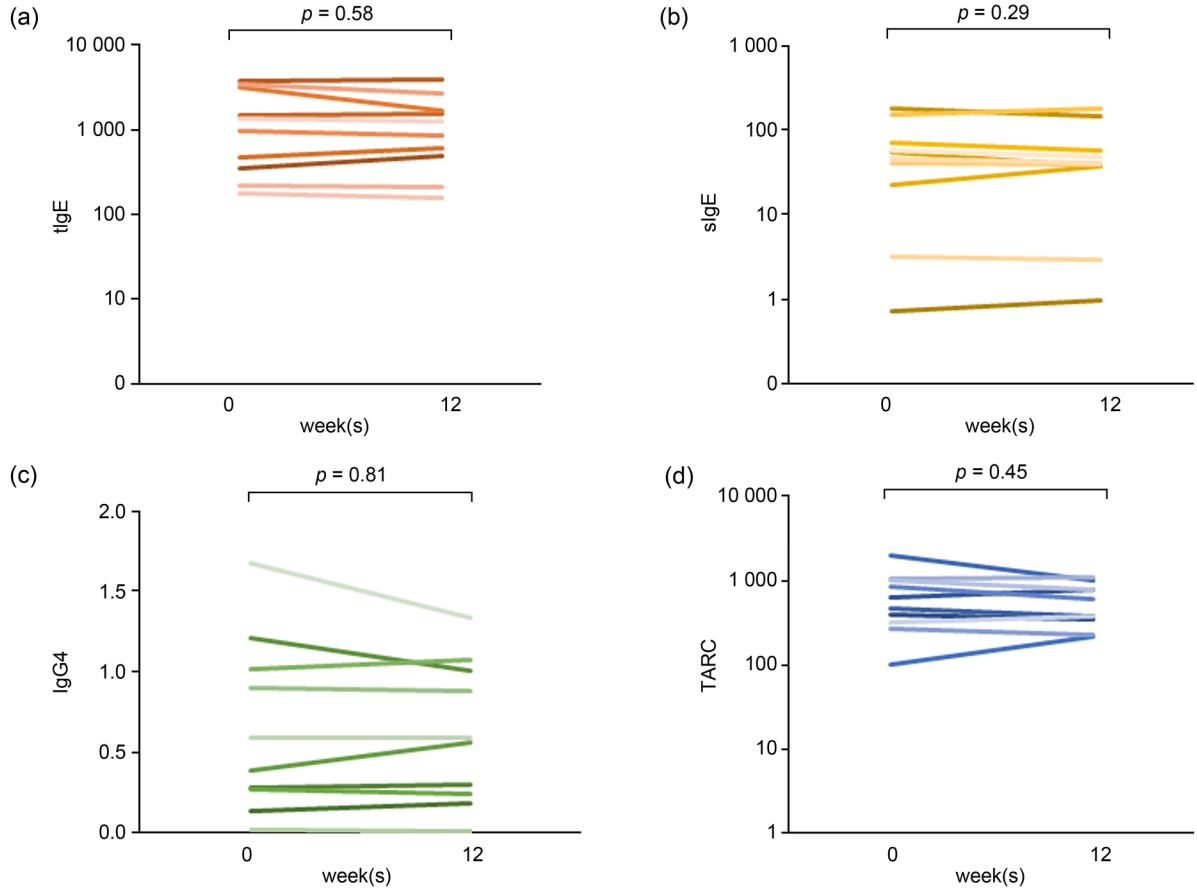


Fig. 1. Changes in the patients' immunological parameters.

The laboratory data from 10 participants before and after the study are shown. a) Total Immunoglobulin E (tIgE), b) milk-specific Immunoglobulin E (sIgE), c) casein-specific Immunoglobulin G4 (IgG4), d) Thymus and Activation-Regulated Chemokine (TARC). No significant changes were seen after 12 weeks ($p > 0.05$, Wilcoxon's signed-rank test).

statistically significant (1.6 vs. 2.7 ml, respectively; $p = 0.041$). The TS/Pro index for the second OFC was nominally decreased compared to that for the first OFC in seven of the 10 patients, although this change fell short of statistical significance. Intriguingly, one of the 10 subjects no longer exhibited allergic symptoms at the second OFC, so TS/Pro was 0.

Immunological parameters. There were no statistically significant differences in any of the tested immunological parameters when compared before and after the study (tIgE: $p = 0.58$, sIgE against cow's milk: $p = 0.29$, sIgG4 against casein: $p = 0.81$, TARC: $p = 0.45$; Fig. 1). In addition, no statistically significant correlation was observed between any of the immunological parameters and the results of the OFCs, including the TS/Pro values (data not shown).

Eczema. No significant change in EASI scores was observed when comparing values at 6 or 12 weeks of intervention to those at the study start (data not shown). Also, no remarkable change in the amount and rank of the ointment used to treat eczema was observed.

Gut Microbial Composition. The relative abundance ratios of the stool microbiota of the 10 participants at the start of the study and after 12 weeks are shown in Fig. 2. The median (quartiles) Shannon's alpha diversity index was 2.1 (2.0–2.5) at the beginning of the study and 2.3 (2.1–2.4) after 12 weeks. No significant change over time was observed in these values. Seventeen species of bacteria had a median occupancy of at least 0.1% at the start of the study or after 12 weeks. *Faecalibacterium* spp. showed a significant increase in occupancy (median (quartiles)) from 3.8% (1.4–7.0) to 6.8% (4.4–9.7) between baseline and 12 weeks (Table III, $p = 0.013$). No differences in changes in gut microbiota were identified with or without antibiotic intake (data not shown).

Real-Time qPCR. Absolute quantification using qPCR was performed for the *Faecalibacterium*, for which a significant increase in occupancy was identified in the microbiota. While a nominal increase in genome copy number at 12 weeks compared to baseline in the respective subjects was observed in 8 of 10 specimens, this change was not statistically significant (Fig. 3). No statistically significant correlation was identified between *Faecalibacterium* copy number and the OFC results.

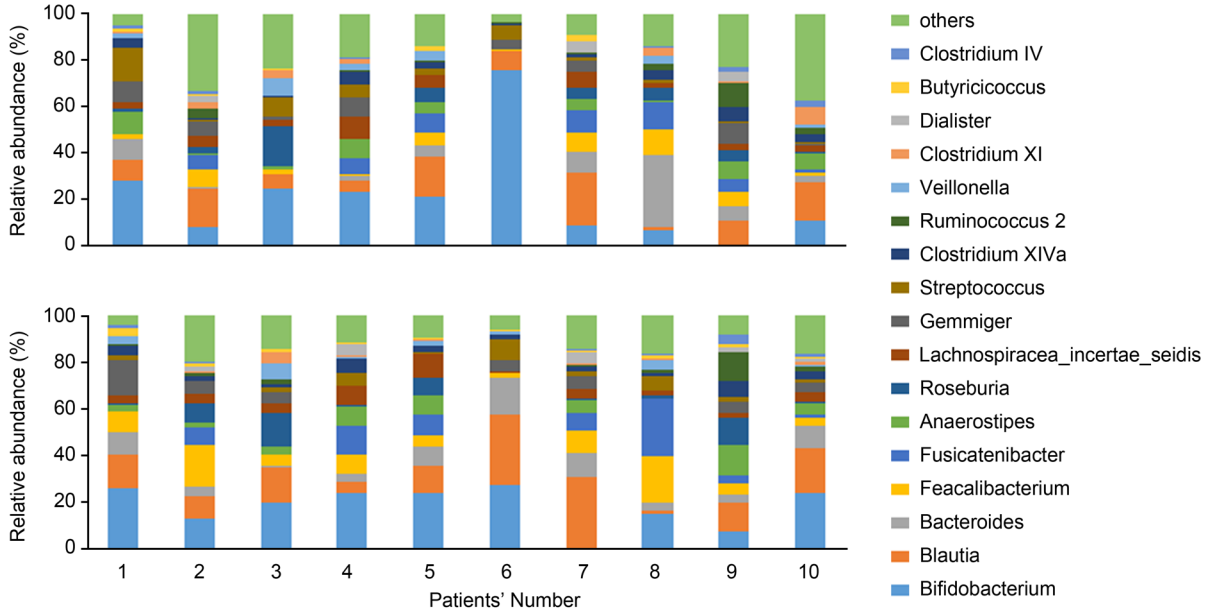


Fig. 2. Relative abundance of gut microbiota at 0 and 12 weeks.

This bar graph shows the composition of the intestinal microbiota in each of the 10 subjects. The numbers on the horizontal axis of the graphs represent the patient number, and the graphs in the same column represent the results for the same subject. The results for the top 17 genera with the highest occupancy are shown. The top row shows the results at the beginning of the study, and the bottom row shows the results 12 weeks into the study.

Discussion

We showed that continuous ingestion of kestose for three months resulted in a statistically significant change in the subjects' microbiota compared to baseline. This regimen also resulted in a nominal increase in the tolerable amount of cow's milk in seven of ten patients with severe CMA. We consider these data a promising result, although we acknowledge that this trial is preliminary work.

In a cross-sectional study of Japanese subjects, the predominant species of the intestinal microbiota of

Table III
Relative abundance of gut microbiota.

	0w	12w	*p*-value*
	Median (25%–75%)	Median (25%–75%)	
x*Bifidobacterium*	16.2 (8.3–24.2)	21.7 (13.3–24.1)	0.72
Blautia	9.5 (6.8–16.9)	13.6 (10.0–22.7)	0.24
Bacteroides	4.0 (0.8–8.3)	6.6 (3.7–9.8)	0.20
Faecalibacterium	3.8 (1.4–7.0)	6.8 (4.4–9.7)	0.01
Fusicatenibacter	6.0 (0.3–8.1)	5.3 (0.4–8.8)	0.29
Anaerostipes	5.0 (1.0–7.7)	4.4 (2.3–7.3)	0.39
Roseburia	3.8 (0.5–5.3)	0.8 (0.4–8.0)	0.88
Lachnospiracea_incertae_sedis	3.0 (2.7–5.3)	3.8 (2.5–4.5)	0.65
Gemmiger	4.6 (1.0–7.5)	4.7 (1.1–5.6)	0.67
Streptococcus	2.3 (0.9–6.5)	2.4 (1.6–4.7)	0.88
Clostridium XlVa	2.9 (1.1–4.5)	2.2 (1.8–3.9)	0.24
Ruminococcus2	0.6 (0.4–3.1)	0.8 (0.3–1.7)	0.51
Veillonella	1.8 (0.2–3.4)	0.8 (0.2–3.2)	0.96
Clostridium XI	1.4 (0.1–3.0)	0.6 (0.4–0.9)	0.33
Dialister	0.2 (0.0–2.2)	0.9 (0.0–1.9)	0.59
Butyricicoccus	0.4 (0.1–1.3)	0.9 (0.6–1.6)	0.20
Clostridium IV	0.6 (0.1–1.5)	0.3 (0.2–1.0)	0.45

* – *p*-values calculated by Wilcoxon's signed rank test for comparison over time at 0 and 12 weeks

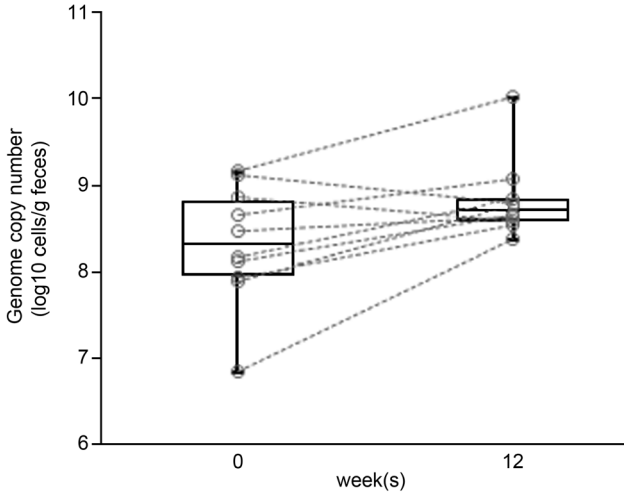


Fig. 3. Absolute quantification of intestinal microbiota (qPCR).
The logarithm of the genome copy number of Faecalibacterium prausnitzii per gram of feces is shown at baseline (0w) and after 12 weeks of intervention (12w). Dotted lines connect changes in the same participants. Eight of 10 patients exhibited nominal increases from 0 to 12 weeks, but this difference was not statistically significant ($p > 0.05$, Wilcoxon's signed-rank test).

4- to 9-year-olds (mean, 6.1 years) were *Lachnospiraceae*, *Blautia*, and *Bifidobacterium*, which were present in median proportions of 21.52, 16.83, and 12.65%, respectively (Odamaki et al. 2016). In another study, Fieten et al. (2018) reported that the intestinal microbiota of children with FA exhibited decreased abundances of *Bifidobacterium breve*, *Bifidobacterium adolescentis*, and *F. prausnitzii* compared to those of control children. Compared to those previous studies, participants at the beginning of the present study had lower proportions of *Blautia* spp. and more significant proportions of *Bifidobacterium* spp. and *Faecalibacterium* spp. However, no statistically significant differences were detected that might be considered characteristic of CMA. The gut microbiota varies daily, even in the same person, while maintaining a unique composition (Wu et al. 2011), and the results of the present study are considered intervention-induced variation.

The genus *Faecalibacterium* includes the largest number of butyrate-producing bacterial species in the human gut, and some of the butyrate produced in the gut has been reported to be beneficial, inducing roles in regulatory T cell differentiation and regulating immune responses (Furusawa et al. 2013). In the present study, we hypothesize that the increase in butyrate-producing bacteria may have increased intestinal butyrate concentrations, resulting in better OFC results in some subjects.

Patients with severe milk allergy were not expected to show improvements in disease severity, not to mention the acquisition of natural tolerance in the short term. In fact, the complete-avoidance group in a slow, low-dose oral immunotherapy (OIT) trial did not change the subjects' TS/Pro indices, even after one year (Sugiura et al. 2020). Therefore, the significant increases in the tolerable amount of cow's milk and the nominally improved TS/Pro values in seven of ten patients that did not employ OIT were considered the promising effect of kestose on the acquisition of immune tolerance, although these results did not reach statistical significance. While increased proportions of butyrate-producing bacteria have been reported to correlate with attenuation of FA, butyrate has a strong odor, and butyrate-producing bacteria have very low stability characteristics (Zmora et al. 2018), precluding the oral administration of butyrate itself or such bacteria as probiotics. Instead, prebiotics have been proposed as an effective method for increasing intestinal butyrate levels via the effects of the prebiotics on the intestinal microbiota.

No changes in immunological parameters such as tIgE and sIgE titers were observed in the present study. These immunological parameters are not expected to decrease within one year of prebiotic intervention, unless patients are undergoing OIT (Sugiura et al. 2020). The TARC index represents the severity of atopic dermatitis, which has been shown to decrease rapidly after successful treatment with topical steroids (Renert-Yuval et al. 2021). In the present study, however, no statistically significant improvement in EASI score was detected, consistent with a lack of decrease in the TARC value and with the fact that we did not change the treatment policy for atopic eczema in our subjects. Despite a lack of change in the immunological parameters, the statistically significant increase in the tolerable intake of cow's milk may reflect a non-immune mechanism. For instance, the observed prebiotic effect may have resulted from enhanced tight junctions of intestinal epithelial cells and/or enhanced mucosal barriers in the gut. Given the beneficial nature of these effects, using such prebiotics is expected to complement the effectiveness and safety of OITs.

Tang et al. (2015) were the first to report the efficacy of a combination of probiotics and OIT, showing that co-administration of a probiotic with peanut OIT was effective compared to placebo in inducing possibly sustained tolerance and immune changes. More recently, Loke et al. (2022) have demonstrated that combining probiotics and OIT may have safety advantages compared

to OIT alone. Based on the results of the present study, FA may be improved by combining OIT for cow's milk with a prebiotic, kestose; this hypothesis should be tested in future trials with an appropriate control group.

ORCID
Mayuko Takahashi https://orcid.org/0000-0002-2607-2139

Author contributions

Conceptualization by KI; data curation by SS, YT, TM, KK; formal analysis by SK, SS, MT, YK; funding acquisition by TT; investigation by SK, SS; methodology by SS, YK, KI; project administration by KI; supervision by KI; visualization by SK; writing – original draft by SK, SS, MT; writing – review and editing by SS, KI.

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Conflict of interest

MT, TK, and TT are employees of B Food Science Co., Ltd, which was the producer of the 1-kestose used in this study and provided the funding source of this study. The remaining authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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