

DR. SHOHEI AKAGAWA (Orcid ID : 0000-0002-1659-4187)

PROF. KAZUNARI KANEKO (Orcid ID : 0000-0002-8215-0832)

Article type : Letter to the Editor

Letter to the Editor

Decreased butyric acid-producing bacteria in gut microbiota of children with egg allergy

Manuscript Acceptance Date: 25-Feb-2021

Mitsuru Yamagishi^{a*}, MD yamagism@hirakata.kmu.ac.jp

Shohei Akagawa^{a*}, MD, PhD akagawas@hirakata.kmu.ac.jp

Yuko Akagawa^a, MD akagaway@hirakata.kmu.ac.jp

Yoko Nakai^a, MD yokodayo.co@gmail.com

Sohsaku Yamanouchi^a, MD, PhD yamanous@hirakata.kmu.ac.jp

Takahisa Kimata^a, MD, PhD kimatat@hirakata.kmu.ac.jp

Masaki Hashiyada^b, PhD hashiyam@hirakata.kmu.ac.jp

Atsushi Akane^b, MD, PhD akane@hirakata.kmu.ac.jp

Shoji Tsuji^a, MD, PhD tsujis@hirakata.kmu.ac.jp

Kazunari Kaneko^a, MD, PhD kanekok@hirakata.kmu.ac.jp

*Both authors contributed equally to this manuscript

^aDepartment of Pediatrics, Kansai Medical University, 2-5-1 Shin-machi, Hirakata-shi, Osaka

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/ALL.14795](#)

This article is protected by copyright. All rights reserved

5731010, Japan

^bDepartment of Legal Medicine, Kansai Medical University, 2-5-1 Shin-machi, Hirakata-shi,
Osaka 5731010, Japan

Corresponding author:

Kazunari Kaneko, MD, PhD

2-5-1 Shin-machi, Hirakata-shi, Osaka, 5731010, Japan

Tel: +81 72 804 0101; Fax: +81 72 804 2569

E-mail: kanekok@hirakata.kmu.ac.jp

To the Editor,

Recent research advances on human gut microbiota have revealed that imbalanced gut microbiota (also known as dysbiosis) can contribute to the development of various chronic diseases.

Butyric acid, a short-chain fatty acid produced by butyric acid-producing bacteria (BAPB) residing in the intestine induces the maturation of regulatory T cells (Tregs) from naïve T cells. Tregs are critical for suppressing excessive immune responses and are believed to suppress the onset of allergic diseases by secreting inhibitory cytokines.¹

This study aimed to determine whether children with chicken egg allergy have dysbiosis characterized by a reduced number of BAPB. Stool samples were collected from 18 children with egg allergy (median age: 3.1 years, interquartile range [IQR]: 1.5–5.5) and 22 healthy controls (median age: 4.0 years, IQR: 2.9–6.1). Blood samples were also collected from allergy patients, who were diagnosed with egg allergy based on either a positive result in oral food challenge or a convincing reaction and sensitization with an egg-specific immunoglobulin E level (> 0.35 kU_A/L). The proportion of BAPB, alpha and beta diversity, and relative abundance of gut microbiota were assessed using 16S rRNA sequencing. The percentage of Tregs in CD4⁺ cells was assessed via flow cytometry.

No significant differences in age and sex between the groups were observed (Supplementary Table 1). The allergy group was found to have less diversity with significantly lower values for observed species, Shannon index, and Simpson index (Figure 1A, Supplementary Table 2). The visible and apparent clustering distances observed in a principal coordinate analysis plot of Bray-Curtis dissimilarity revealed distinct gut microbiota structures in the two groups (Figure 1B). In the allergy group, the percentage of Enterobacteriales was significantly higher [17.0% (9.5–22.3) vs. 1.8% (0.9–10.9), $p = 0.029$], whereas the percentage of Lactobacillales was

significantly lower [7.1% (3.6–10.1) vs. 11.5% (7.5–18.5), $p = 0.012$] (Figure 2A,B, Supplementary Table 3). The proportion of BAPB in the gut microbiota was significantly lower in the allergy group than in the control group [2.3% (1.0–5.2) vs. 6.9% (2.5–9.6), $p = 0.013$] (Figure 2C). The median percentage of Tregs in the allergy group was 2.7%, which was lower in six of nine patients compared with the reference range (Figure 2D).²

In summary, this study revealed that children with egg allergies have less BAPB and tendentially fewer circulating Treg than the reported reference range. Several studies have reported dysbiosis in patients with allergy. Hua et al. analyzed 1,879 patient samples and found that those with allergy had reduced abundance of Clostridiales.³ Concerning children, Savage et al. analyzed 216 samples from infants aged 3–6 months with and without food sensitization at 3 years of age and found that *Clostridium* were underrepresented in those with food sensitization.⁴ *Clostridium* (belonging to the order Clostridiales) clusters IV, XIVa, and XVIII are known to produce butyric acid;⁵ therefore, decreased butyric acid in the gut may have contributed to the allergy or food sensitization. Although we cannot conclude if decreased BAPB abundance is the cause or result of food allergy, studies in birth cohorts have shown that changes in gut microbiota and organic acid concentrations occur before the onset of allergies;^{6, 7} rendering dysbiosis as the possible cause for allergies.

To our knowledge, this is the first report focusing on BAPB. Although fecal butyric acid concentrations were not measured, a study by Sandin et al. reported that allergy pediatric patients had lower fecal butyric acid levels compared with non-allergic children, which supports the possibility that fecal butyric acid is lower in children with egg allergies due to reduced BAPB abundance.⁸

In conclusion, children with egg allergies can experience dysbiosis characterized by the decreased abundance of BAPB. Administration of organic acids, prebiotics, probiotics, and

synbiotics may lead to the prevention and treatment of food allergies in the future.

References

1. Noval Rivas M, Chatila TA. Regulatory T cells in allergic diseases. *J Allergy Clin Immunol.* 2016;138:639-652.
2. Tsuji S, Kimata T, Yamanouchi S, et al. Regulatory T cells and CTLA-4 in idiopathic nephrotic syndrome. *Pediatr Int.* 2017;59:643-646.
3. Hua X, Goedert JJ, Pu A, Yu G, Shi J. Allergy associations with the adult fecal microbiota: Analysis of the American Gut Project. *EBioMedicine.* 2016;3:172-179.
4. Savage JH, Lee-Sarwar KA, Sordillo J, et al. A prospective microbiome-wide association study of food sensitization and food allergy in early childhood. *Allergy.* 2018;73:145-152.
5. Atarashi K, Tanoue T, Oshima K, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature.* 2013;500:232-6.
6. Björkstén B, Sepp E, Julge K, Voor T, Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. *J Allergy Clin Immunol.* 2001;108:516-20.
7. Roduit C, Frei R, Ferstl R, et al. High levels of butyrate and propionate in early life are associated with protection against atopy. *Allergy.* 2019;74:799-809.
8. Sandin A, Bråbäck L, Norin E, Björkstén B. Faecal short chain fatty acid pattern and allergy in early childhood. *Acta Paediatr.* 2009;98:823-7.

Acknowledgements: The authors would like to thank Yuki Kawanaka for technical assistance with the experiments and Editage (www.editage.com) for English language editing. This research was presented as a mini symposium at the JSA/WAO Joint Congress 2020. Dr. Yamagishi has nothing to disclose; Dr. Shohei Akagawa reports grants from Toshiko Kitanishi Foundation during

the conduct of the study; Dr. Yuko Akagawa has nothing to disclose; Dr. Nakai has nothing to disclose; Dr. Yamanouchi has nothing to disclose; Dr. Kimata has nothing to disclose; Dr. Hashiyada has nothing to disclose; Dr. Akane has nothing to disclose; Dr. Tsuji has nothing to disclose; and Dr. Kaneko has nothing to disclose.

Authors' contributions: MY, SA, and KK designed the study. MY, SA, and YA wrote the initial draft of the manuscript and produced the figures. MY, SA, and YN recruited subjects and acquired data. MY, SA, YA, MH, AA, and ST contributed to the analysis and interpretation of data. MY, SA, YA, SY, TK, MH, AA, ST, and KK revised the work critically for important intellectual content. All authors approved the final version of the manuscript, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Funding sources: This study was supported by a grant from the Toshiko Kitanishi Foundation.

Conflict of interest: The authors have no conflicts of interest to declare.

Ethical approval: The study protocol was approved by the Ethics Committees of Kansai Medical University (No. 2019250). Written informed consent was obtained from the parents of all participants before enrollment.

Figure legends

Figure 1. Differences in alpha and beta diversity indices in children with egg allergy. (A)

Observed species, Shannon index, and Simpson index were significantly lower in the FA group.

The edges of the boxes represent the 25th and 75th percentiles. Vertical lines extend to the maximum and minimum values. (B) Principal coordinate (PC) analysis plot of Bray-Curtis dissimilarity was created for both groups. Pink and blue dots represent samples from the FA and HC groups, respectively. The visible and apparent clustering distances reveal the distinct structures of the gut microbiota in the two groups. FA: food allergy, HC: healthy control.

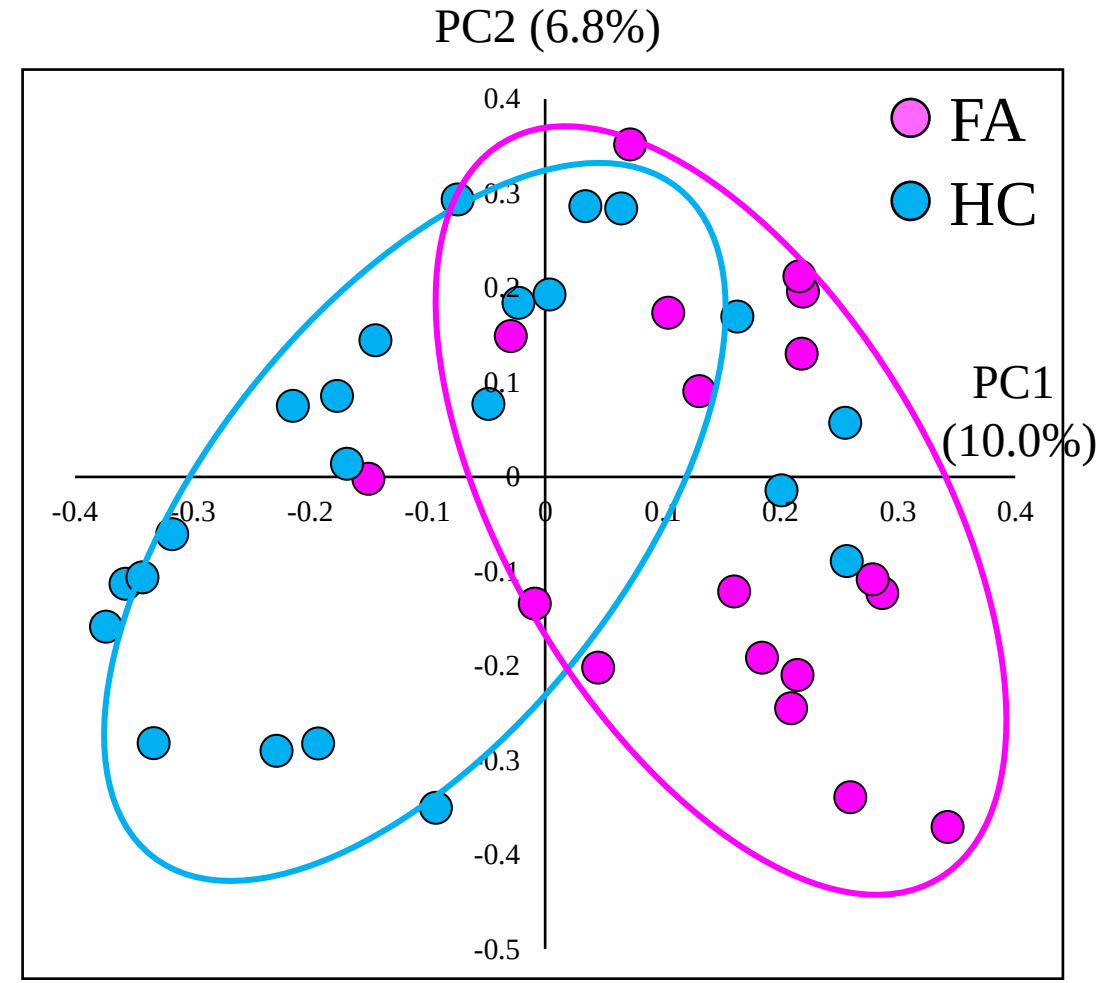
Figure 2. Composition of gut microbiota and abundance of BAPB and regulatory T cells in children with food allergy.

Gut microbiota representation of (A) a group or (B) an individual

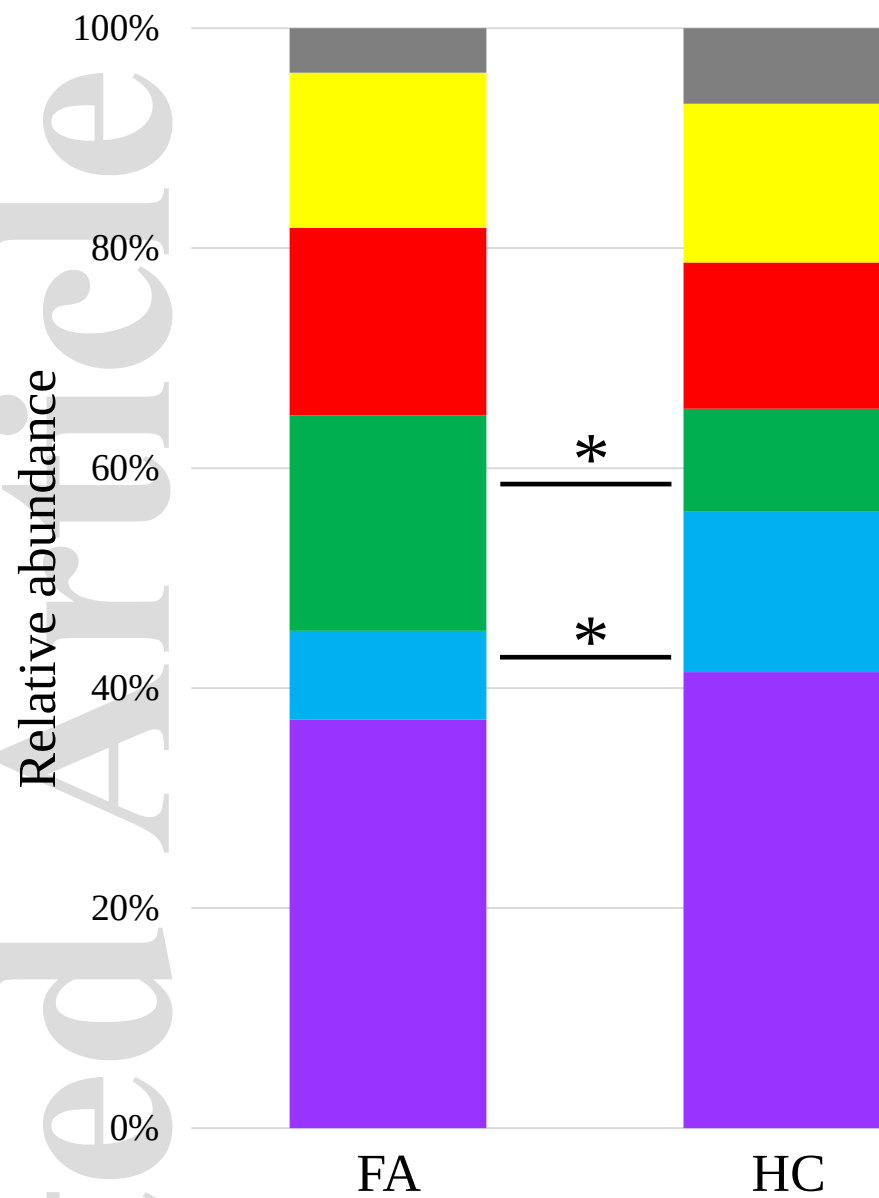
sample. (C) The relative abundance of BAPB was significantly lower in the FA group ($p = 0.013$).

(D) The median percentage of regulatory T cells in CD4⁺ cells in the FA group was lower than the reference range. The central horizontal line represents the median value. The edges represent the minimum and maximum values. * $p < 0.05$. FA: food allergy, HC: healthy control, BAPB: butyric acid-producing bacteria.

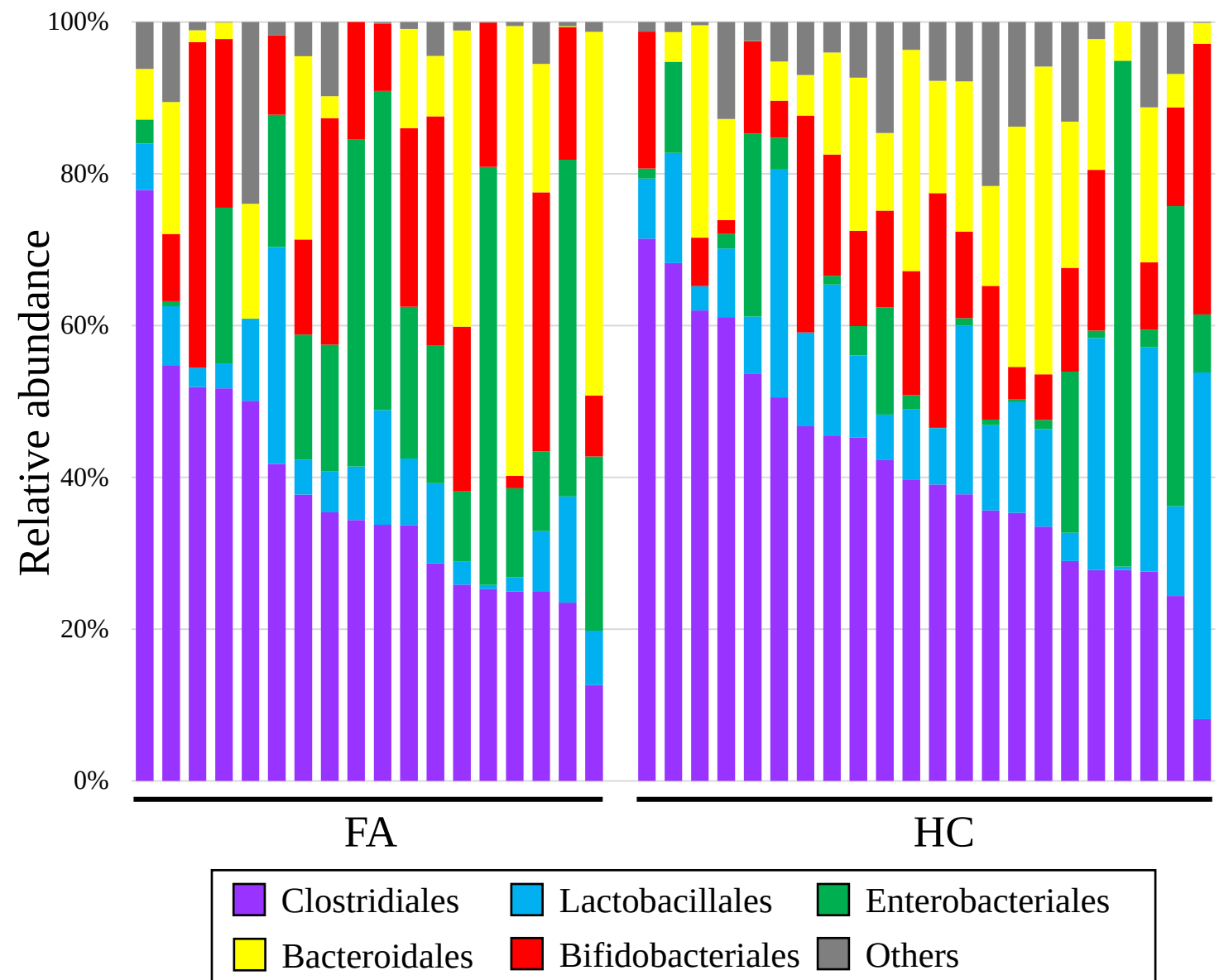
B



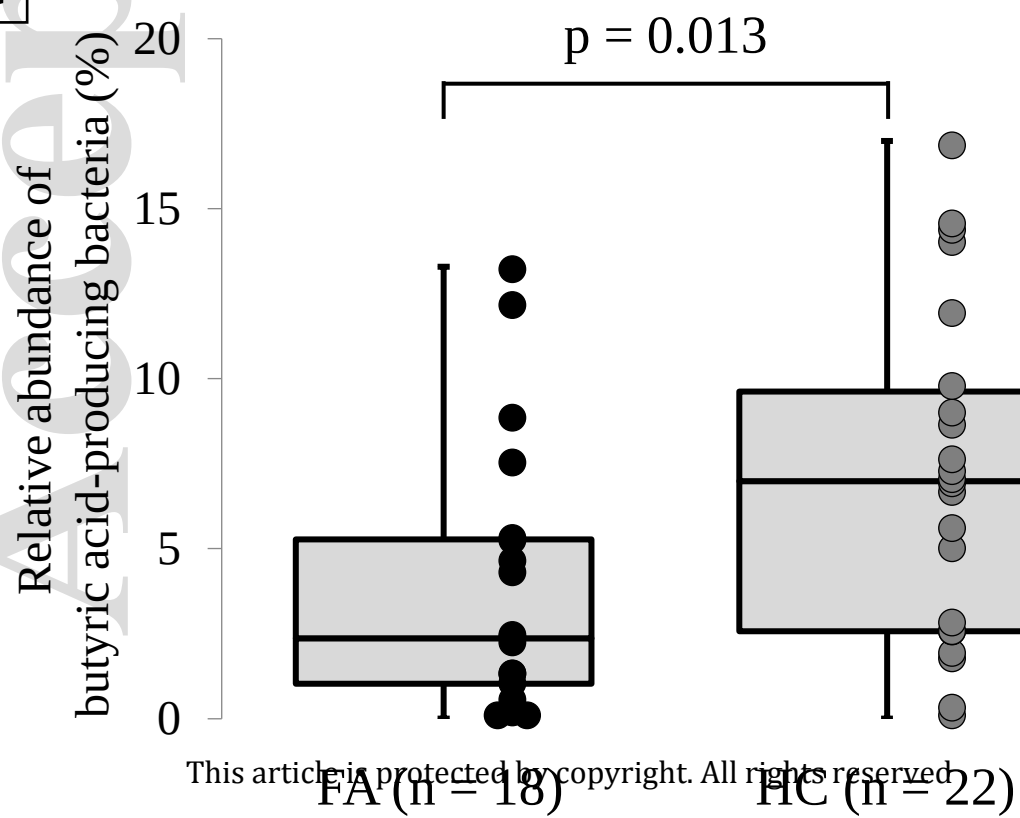
A



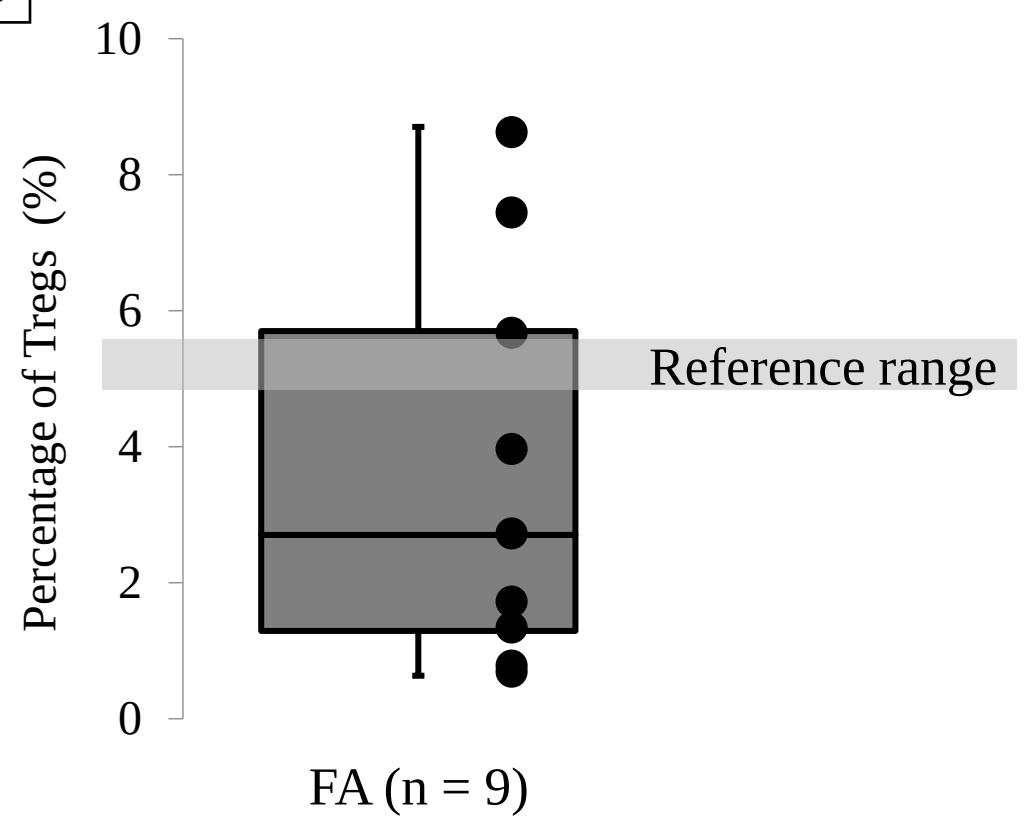
B



C



D



*Supplementary data***Decreased butyric acid-producing bacteria in gut microbiome of children with egg allergy**

Mitsuru Yamagishi ¹ , MD	yamagism@hirakata.kmu.ac.jp
Shohei Akagawa ¹ , MD, PhD	akagawas@hirakata.kmu.ac.jp
Yuko Akagawa ¹ , MD	akagaway@hirakata.kmu.ac.jp
Yoko Nakai ¹ , MD	yokodayo.co@gmail.com
Sohsaku Yamanouchi ¹ , MD, PhD	yamanous@hirakata.kmu.ac.jp
Takahisa Kimata ¹ , MD, PhD	kimatat@hirakata.kmu.ac.jp
Masaki Hashiyada ² , PhD	hashiyam@hirakata.kmu.ac.jp
Atsushi Akane ² , MD, PhD	akane@hirakata.kmu.ac.jp
Shoji Tsuji ¹ , MD, PhD	tsujis@hirakata.kmu.ac.jp
Kazunari Kaneko ¹ , MD, PhD	kanekok@hirakata.kmu.ac.jp

¹ Department of Pediatrics, Kansai Medical University,

2-5-1 Shinmachi, Hirakata, Osaka, Japan

² Department of Legal Medicine, Kansai Medical University,

2-5-1 Shinmachi, Hirakata, Osaka, Japan

Corresponding author: Kazunari Kaneko, MD, PhD

2-5-1 Shinmachi, Hirakata, Osaka, Japan

Tel: +81 72 804 0101; Fax: +81 72 804 2569

E-mail: kanekok@hirakata.kmu.ac.jp

Methods

Participants and study design

The study protocol was approved by the Ethics Committees of Kansai Medical University (No. 2019250). Written informed consent was obtained from the parents of all participants before enrollment.

The participants were 18 outpatients who were diagnosed with chicken egg allergy at the authors' clinic between August 2019 and January 2020 (FA group) and 22 healthy children of the same age group (HC group). The diagnosis of egg allergy was based on either a positive result in a physician-supervised oral food challenge or a convincing reaction and sensitization, with an egg white-specific IgE level > 0.35 kU_A/L.

Stool samples were collected once from each subject. Bacterial DNA was extracted for next-generation 16S rRNA sequencing. Gut microbiota alpha and beta diversity, relative abundance of bacteria by order, and ratio of butyric acid-producing bacteria (BAPB) were compared between the two groups. The percentage of regulatory T cells (Tregs) in CD4⁺ cells was calculated via flow cytometry and compared to a previously reported reference range.

Fecal and blood sampling

For children who were not yet potty trained, approximately 0.5 g stool samples were collected directly from a disposable diaper using a small sterile spoon attached to the sample collection cup. For children who were potty trained, a sample of naturally voided stool was collected. Stool samples were stored at -80°C until analysis. For measurement of Tregs through flow cytometry, blood samples were collected through venipuncture from the FA group participants on the same day as that of the stool sample collection. A 50- μL aliquot was taken from each blood sample and was immediately transferred to a sterile 6 mL centrifuge tube.

16S rRNA gene sequencing

Stool samples were thawed, and DNA was extracted using a NucleoSpin DNA Stool Kit (MACHEREY-NAGEL, Düren, Germany). The seven hypervariable regions of the DNA, excluding V1 and V5 regions of the 16S rRNA region, were amplified using a 16S metagenomics kit (Thermo Fisher Scientific, Waltham, MA, USA). After refinement, a library was produced using the Ion Plus Fragment Library Kit and Ion Xpress Barcode Adapters Kit (Thermo Fisher Scientific). The barcoded library was quantified and pooled to generate the final concentration of 30 pM per target using the Agilent Bioanalyzer 2000 (Agilent, Santa Clara, CA, USA). The Ion Chef Instrument and Kit (Thermo Fisher Scientific) were used to achieve target concentrations for

template preparation and emulsion PCR, and sequence analysis was performed using the Ion PGM sequencer and Ion 318 chip (Thermo Fisher Scientific). The sequence data were analyzed using Ion Reporter Software with the Metagenomics 16S w1.1 v5.16 workflow (Thermo Fisher Scientific).

Flow cytometric analysis of CD4⁺ cells and Tregs

The ratio of Treg to CD4⁺ cells was measured using flow cytometry to evaluate peripheral Treg differentiation in FA group. Fluorescein isothiocyanate conjugate anti-CD4 antibody and phycoerythrin-Cy5 conjugate anti-CD25 monoclonal antibody (Beckman Coulter, Fullerton, CA, USA) were added to the tubes containing the blood samples. After membrane staining and washing, the samples were permeabilized using IntraPrep Reagent 2 (Beckman Coulter) and the remaining red blood cells were simultaneously dissolved. Next, the cells were incubated with phycoerythrin conjugate anti-FOXP3 monoclonal antibody (Imgenex, San Diego, CA, USA). After intracellular staining, the cells were washed twice with phosphate-buffered saline and promptly analyzed using the Cytomics FC 500 flow cytometer equipped with CXP Cytometer Software (Coulter, Hialeah, FL, USA). First, we used scatter-gating to identify the lymphocytes based on the distribution of forward scatter versus side scatter. Further, CD4⁺ cells among the lymphocytes were gated, followed by measuring the percentage

of CD4⁺ cells positive for both FOXP3 and CD25. Data for 10,000 events were collected for each sample. The positive cell percentage was measured based on a cut-off set established by an isotype-matched non-specific control antibody. Results are presented as the percentage of regulatory T cells (Tregs) in CD4⁺ cells.

Statistical analysis

Data from each group are expressed as medians and interquartile ranges (IQRs). Chi-squared and Mann–Whitney U tests were used for comparisons between the two groups. *P*-values < 0.05 were considered statistically significant. BAPB were defined as the 61 bacterial species listed in Supplementary Table 4.¹ To verify the results, post-hoc power analysis with the significance level α set to 0.05 was performed using G*Power 3.1.9.4 (Heinrich-Heine University).²

Result

Post-hoc power analysis

A post-hoc power analysis was conducted to evaluate the primary outcome of the ratio of BAPB.² The data in this study had a large effect size (0.997) and the power was above 0.80 when the significance level α was set to 0.05 (0.830). Therefore, the analysis confirmed that the sample size was sufficiently large for statistical analysis.³

Discussion

Influence of age and mode of delivery on gut microbiota

Although not statistically significant, the HC group had a median age of 4.0 years, higher than that of the FA group (3.1 years). Notably, the gut microbiota in children stabilizes at around 2–3 years of age and is maintained throughout adulthood.^{4, 5} In this study, the participants were over two years of age and therefore, we consider that the influence of age difference is not extensive. Furthermore, the mode of delivery indeed significantly affects the gut microbiota in neonates. However, a few reports mention that this effect weakens during infancy and eventually dwindles by late infancy.⁶⁻⁸ Therefore, we consider that differences in mode of delivery do not exert substantial effects in the development of gut microbiota.

Influence of limited diet on gut microbiota

In this study, intake of egg was parental-restricted in some of the enrolled patients prior to the diagnosis of food allergy. Therefore, it is possible that the limited diet resulted in alteration of the gut microbiota. However, a randomized controlled clinical trial conducted by Bierut et al. in 277 9-month-old Malawian infants demonstrated that the

gut microbiota did not differ when the infants were fed with either bovine colostrum with egg powder or corn/soy flour for 3 months.⁹ Concomitant with this study, we consider that the intake of egg by itself does not significantly affect the gut microbiota in children.

Supplementary Table 1. Characteristics of the participants

	FA group (n = 18)	HC group (n = 22)	<i>p</i> -value
Sex, male (%)	13 (72%)	12 (55%)	0.380
Age (years)	3.1 (1.5–5.5)	4.0 (2.9–6.1)	0.280
Mode of delivery, cesarean section (%)	9 (50%)	4 (18%)	0.045
Use of antibiotics in the previous 3 months (%)	0 (0%)	0 (0%)	1.00
Total IgE level (IU/mL)	126.6 (59.1–393.8)	n.d.	
Egg white-specific IgE level (kU _A /L)	5.0 (2.9–20.2)	n.d.	
Ovomucoid-specific IgE level (kU _A /L)	4.0 (1.5–15.9)	n.d.	

Data are expressed as the number and percentage or median and interquartile range (IQR). FA: food allergy, HC: healthy control, IgE: Immunoglobulin E, n.d.: not determined.

Supplementary Table 2. Alpha diversity of gut microbiota

Alpha diversity index	FA Group	HC Group	<i>p</i> -value
Observed species	29 (19–31)	43 (32–61)	0.0011
Shannon index	3.37 (2.76–3.90)	3.84 (3.54–4.37)	0.025
Simpson index	0.87 (0.77–0.89)	0.90 (0.86–0.93)	0.013

Data are expressed as the median and interquartile range.

FA: food allergy, HC: healthy control.

Supplementary Table 3. Composition of gut microbiota of food allergy and control groups at the order level

	FA group	HC group	<i>p</i> -value
Clostridiales	34.0	39.3	0.19
Lactobacillales	7.1	11.5	0.012
Enterobacteriales	17.1	1.9	0.029
Bifidobacteriales	16.5	12.6	0.15
Bacteroidales	7.3	13.4	0.47
Others	1.2	6.4	NA

Data are expressed as the mean value.

FA: food allergy, HC: healthy control, NA: not applicable.

Supplementary Table 4. List of butyric acid-producing bacteria

<i>Acetoneema_longum</i>	<i>Clostridium_symbiosum</i>	<i>Lachnoanaerobaculum_saburreum</i>
<i>Acidaminococcus_fermentans</i>	<i>Coprococcus_catus</i>	<i>Megasphaera_micronuciformis</i>
<i>Alistipes_putredinis</i>	<i>Coprococcus_comes</i>	<i>Odoribacter_splanchnicus</i>
<i>Anaerococcus_hydrogenalis</i>	<i>Coprococcus_eutactus</i>	<i>Peptoclostridium_difficile</i>
<i>Anaerococcus_lactolyticus</i>	<i>Eubacterium_cellulosolvens</i>	<i>Peptoniphilus_duerdenii</i>
<i>Anaerococcus_prevotii</i>	<i>Eubacterium_desmolans</i>	<i>Peptoniphilus_hareii</i>
<i>Anaerococcus_tetradis</i>	<i>Eubacterium_dolichum</i>	<i>Peptoniphilus_lacrimalis</i>
<i>Anaerococcus_vaginalis</i>	<i>Eubacterium_hallii</i>	<i>Porphyromonas_asaccharolytica</i>
<i>Anaerofustis_stercorihominis</i>	<i>Eubacterium_limosum</i>	<i>Porphyromonas_endodontalis</i>
<i>Anaerostipes_caccae</i>	<i>Eubacterium_rectale</i>	<i>Porphyromonas_gingivalis</i>
<i>Anaerotruncus_colihominis</i>	<i>Eubacterium_saphenum</i>	<i>Porphyromonas_uenonis</i>
<i>Brachyspira_murdochii</i>	<i>Eubacterium_ventriosum</i>	<i>Propionibacterium_acidifaciens</i>
<i>Brachyspira_pilosicoli</i>	<i>Eubacterium_yurii</i>	<i>Pseudoramibacter_alactolyticus</i>
<i>Butyrivibrio_crossotus</i>	<i>Faecalibacterium_prausnitzii</i>	<i>Roseburia_hominis</i>
<i>Butyrivibrio_fibrisolvens</i>	<i>Fusobacterium_gonidiaformans</i>	<i>Roseburia_intestinalis</i>
<i>Butyrivibrio_proteoclasticus</i>	<i>Fusobacterium_mortiferum</i>	<i>Roseburia_inulinivorans</i>
<i>Clostridium_botulinum</i>	<i>Fusobacterium_nucleatum</i>	<i>Shuttleworthia_satelles</i>
<i>Clostridium_butyricum</i>	<i>Fusobacterium_ulcerans</i>	<i>Subdoligranulum_variabile</i>
<i>Clostridium_perfringens</i>	<i>Fusobacterium_varium</i>	<i>Treponema_phagedenis</i>
<i>Clostridium_saccharolyticum</i>	<i>Holdemanella_biformis</i>	<i>Treponema_vincentii</i>
<i>Clostridium_sporogenes</i>		

From Haak, et al.¹

References

1. Haak BW, Littmann ER, Chaubard JL, et al. Impact of gut colonization with butyrate-producing microbiota on respiratory viral infection following allo-HCT. *Blood*. 2018;131:2978-2986.
2. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods*. 2007;39:175-91.
3. Cohen J. A power primer. *Psychol Bull*. 1992;112:155-9.
4. Odamaki T, Kato K, Sugahara H, et al. Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC Microbiol*. 2016;16:90.
5. Robertson RC, Manges AR, Finlay BB, Prendergast AJ. The Human Microbiome and Child Growth - First 1000 Days and Beyond. *Trends Microbiol*. 2019;27:131-147.
6. Shao Y, Forster SC, Tsaliki E, et al. Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. *Nature*. 2019;574:117-121.
7. Stokholm J, Thorsen J, Chawes BL, et al. Cesarean section changes neonatal gut colonization. *J Allergy Clin Immunol*. 2016;138:881-889 e2.
8. Akagawa S, Tsuji S, Onuma C, et al. Effect of Delivery Mode and Nutrition on Gut Microbiota in Neonates. *Ann Nutr Metab*. 2019;74:132-139.
9. Bierut T, Duckworth L, Grabowsky M, et al. The effect of bovine colostrum/egg supplementation compared with corn/soy flour in young Malawian children: a randomized, controlled clinical trial. *Am J Clin Nutr*. 2020;