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Original Article

Evaluation of toll-like receptors 2 and 4 polymorphism and intestinal microbiota in children with food allergies

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ABSTRACT

Background. Mutual regulation between immune system and gut microbiota is achieved through several mechanisms including the engagement of toll-like receptors (TLRs) which is expressed on numerous cell types.

In this study we aimed to explore the association between food allergies and TLR gene polymorphisms in association with gut microbiota.

Methods. Toll-like receptors polymorphism frequencies and some bacteria in the gut microbiota in 130 infants aged 1-24 months with egg and/or milk allergy in a prospective cohort were compared with 110 non-food allergic controls. Four candidate polymorphisms (*TLR2* rs1898830/rs5743708 and *TLR4* rs4986790/rs4986791) were genotyped by allelic discrimination polymerase chain rection (PCR) method. Gut microbiota analysis was achieved by using high-throughput sequencing.

Results. The *TLR4* rs4986790 (Asp299Gly) single nucleotide polymorphism (SNP) major/minor allele frequency was 0.788/0.212 in food allergy patients and 0.719/0.280 in controls (p=0.017). There was a statistically significant difference between groups in terms of genotype frequencies (AA, AG, GG). Gut microbiota analysis revealed increased Firmicutes phylum in stool of the patients with food allergy. Except for *TLR4* rs4986791 (Thr399lle) allele, the other TLR polymorphisms were not associated with food allergies in children. When the bacteria in the intestinal microbiota and *TLR2* and *TLR4* gene polymorphisms were compared; we determined a statistically significant increase in *Bifidobacterium* concentration in the intestinal microbiota in *TLR4* rs4986791 CT heterozygous genotype (p=0.004).

Conclusions. This study demonstrated a partial role of *TLR4* gene polymorphism and gut microbiota in the development of food allergies. Future work in this area will be required to clarify the roles of different microbial strains that modulate gut microbiota composition and function in conjunction with TLR transcription pathways.

Key words: food allergy, Toll-like receptor 2, Toll-like receptor 4, genetic polymorphism, gut microbiota, children.

Food allergies are immune reactions against food proteins and may cause life-threatening severe systemic reactions. The prevalence of food allergies is 6-8% in children up to three years of age, whereas this rate decreases to 2% over seven years of age.[1] The incidence of food allergies has increased in the last 10-20 years, and is considered to be related to numerous risk factors.[2] The factors that directly play roles in food sensitivity development are generally divided into two groups: host factors and antigenic features. Numerous host factors such as genetic predisposition, the age at the time of exposure to the antigen, a high socioeconomic status, urban living, breastfeeding, formula feeding, exposure to domestic animals, time of transition to complementary foods, the mother's diet, disruption of the intestinal barrier, and quantitative changes of the intestinal microbiota have been defined.[3]

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The human gut microbiota involves hundreds of different phylogenetic species classified into six primary microbial phyla: Firmicutes, Bacterioidetes, Actinobacteria, Proteobacteria, Verrucomicrobia, and Euryarchaeota. The first four primary phyla constitute 98% of the intestinal microbiota.[4] The microbiota's taxonomic combination also changes with age. For example, prior to maturing an adult-like microbiota dominated by Firmicutes and Bacteroidetes, the neonatal intestinal microbiota is initially dominated by Proteobacteria such as *Escherichia* and *Shigella*, and then by Actinobacteria such as *Bifidobacterium*. Such maturing is reflected in the ratio between *Enterobacteriaceae* (Proteobacteria and Actinobacteria) and *Bacterioidaceae* (Firmicutes and Bacteroidetes). The relative ratio of *Enterobacteriaceae* decreases, and the rate of *Bacteroidaceae* increases with age. Microbial colonization developing in the human gut during infancy plays a role in the maturation and epigenetic regulation of the immune system.[5] The factors affecting early-life intestinal microbial colonization and combination have been shown to impact the development of atopic disorders, including food allergies. Impairment of the original microbiota combination (dysbiosis) has been found to be associated with food allergy development.[6] Dysbiosis developing in children with food allergies might cause immune imbalances among effector T cells and regulatory T cells (Treg) cells. Microbiota deficiency has been found to be associated with Th2 development and IgE production against food allergens in germ-free mice.[7] On the other hand, the high concentration of some microorganisms in the intestinal microbiota may lead the immune system towards a T helper (Th) 1 phenotype that is protective against atopy rather than the Th2 cell phenotype associated with atopy in infancy/childhood.[5,8]

Toll-like receptors (TLRs) play essential roles in responses against microbial agents, inflammatory pathways, and the regulation of innate immune responses.[9] The innate immune system can control the adaptive immune system's developing response against food proteins. Dendritic cells and TLRs play essential roles in this task.[10] TLRs induce the development/differentiation of Th1, Th2, Th17, and $CD4^+$ Tregs. TLRs regulate the development and functions of Tregs by mediated signals and alter the development of atopic disorders. TLRs flawed expressions or genetic changes can contribute to imbalanced Th1 or Th2 immunity levels.[11] Besides, it was reported that TLRs could affect the intestinal microbiota's development and combination.[9] In some studies on mice have demonstrated that the Treg cells, stimulated by *TLR2* activation, were necessary for successful intestinal colonization.[12,13] In addition, it was reported in animal studies that the *TLR2* and *TLR4* gene expression levels changed due to alterations in the intestinal microbiota's combination.[9,14] Therefore, TLR gene polymorphisms (particularly *TLR2* and *TLR4*) and, accordingly, the TLR system's impaired signaling mechanisms have been reported to have associations with the risk of allergy development.[9] On the other hand, TLR4-related signals induced by intestinal flora were found to inhibit the development of reactions against food antigens. In recent years, several simple nucleotide polymorphisms (SNPs) have been described in the *TLR4* gene (*TLR4* rs4986790, *TLR4* rs4986791) and *TLR2* gene (*TLR2* rs1898830, *TLR2* rs5743708).

This study aimed to investigate the associations between food allergies, intestinal microbiota combination, the genetic polymorphisms of rs5743708 and rs1898830 in the *TLR2* gene and rs4986791 and rs4986790 in the *TLR4* gene.

Methods

Study design

A total of 130 children, aged between 1 and 24 months, followed up with the diagnosis of food allergy in our hospital's Pediatric Allergy and Immunology Department were included in the study. A total of 110 children with

similar age characteristics who had applied to the healthy child follow-up clinic of our hospital constituted the control group. In the healthy pediatric outpatient clinic; children were vaccinated, age-appropriate nutrition education was provided, and their growth and development were monitored. Children with chronic pulmonary and other respiratory system disorders, gastrointestinal system diseases such as inflammatory intestinal disorders, renal/urologic, hepatic, cardiovascular, metabolic, and neurologic diseases, children with cystic fibrosis, bone diseases, and history of immune deficiencies, children under continuous antibiotic prophylaxis, and those with the history of antibiotic use within the last six months were excluded from the study.

Food allergy was diagnosed by obtaining the patient's medical history, the skin prick test (SPT) with foods, the child food panel (fx5), food sIgE measurement, and oral provocation test with foods. Commercial allergen solutions were utilized for SPTs with foods (Allergopharma, Reinbek, Germany). Moreover, SPTs for milk and egg white were performed with commercial allergen solutions, and a prick-to-prick test was used with fresh food. Commercial allergen solutions were used for aeroallergens and food in children aged two years (Allergopharma, Reinbek, Germany). Patients with >95% predictive values in the enduration diameter of skin prick tests for milk or egg or sIgE levels and who had a medical history of anaphylaxis due to these foods within the last six months did not undergo a provocation test.[15] Diagnoses of these cases were directly considered food allergies; they went on an elimination diet and were included in the study. Besides, provocation tests were performed in patients with <95% predictive value for these foods. The diagnosis was considered a food allergy in patients who developed a reaction during the test, and they were included in the study. The Ethics Committee of Fırat University approved the study (date June 21, 2018, and decision # 11/04). Moreover, the participants' families were informed about the study, and their written consent was obtained.

Sample collection

In addition to routine tests of the cases in the patient and control groups, one ml of blood was obtained for genotyping. In addition, fresh stool (100 mg) was obtained for microbiota analysis; the samples were placed in sterile, sealed, covered stool sample containers within 0-2 hours and kept at -80°C until the time of analysis.

Molecular genetic analysis

The Real-time Polymerase Chain Reaction (RT-PCR) method was used to analyze rs5743708 and rs1898830 polymorphisms in the *TLR2* gene and rs4986791 and rs4986790 polymorphisms in the *TLR4* gene. Blood was thawed to room temperature before isolating Deoxyribonucleic acid (DNA). The Genomic DNA isolation WIZARD Genomic DNA Purification Kit (Catalog # A1125, Promega, MA, USA) was used for isolation. The samples' DNA concentrations were measured in a nanodrop (Maestrogen, Maestro NanoDrop, USA) device, and the concentrations were adjusted to 1-10 ng. The PCR reaction was prepared by adding 2.5 µl of DNA sample, 0.5 µl of polymorphism-specific Tag-Man probe, 2.5 µl of Taqman genotyping master mix (Catalog# 4371355), and 2.5 µl of ddH_2O to each well of a 96-well plate. The plate was placed in the ABI 7500 Fast Real-Time System (Applied Biosystems, Foster City, CA) device. The conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation (92°C for 15 s), annealing, and extension in one step (60°C for 1 min). After the Polymerase Chain Reaction, the homozygous mutant, heterozygous, and homozygous normal genotypes were determined using the device's software system and according to Allele1 / Allele2 differentiation.[16]

DNA extraction from stool

One hundred mg of stool sample was weighed, 3 ml of 0.9% saline was added to the sample, and vortexed. The supernatant was taken into

a new tube following centrifugation at 3.000 rpm for 2 minutes. It was centrifuged at 1.000 rpm for one minute in this new tube, and the supernatant was discarded following the centrifugation process. Two ml of phosphate-buffered saline (PBS) was added to the pellet that remained at the bottom of the tube and then vortexed. The supernatant was discarded following centrifugation at 10.000 rpm for two minutes., As previously described by Kumar et al.[17], bacterial 16S rRNA amplification was performed following the isolation of bacterial DNA in the obtained pellet samples. Table I shows the studied microbiota-specific bacteria and used primers. Microbiota analysis was performed by breaking down the formed amplicons according to their hypervariable regions.

Statistical analysis

Statistical Package for Social Sciences (SPSS) v.22 software was used for statistical analysis. The quantitative variables with a normal distribution were expressed as the mean ± the mean's standard error, and those not having a normal distribution as the median and interquartile range. The Kruskal Wallis test was used to compare groups, whereas the Mann Whitney-U test was used for paired-group comparisons. Fisher's exact chi-square and Pearson's chi-square tests were used for comparing qualitative data. The results of the analyzes were assessed within the 95% confidence interval, and $p<0.05$ was considered statistically significant.

Results

Of 130 children with food allergies, 89 (68.5%) were male, and 41 (31.5%) were female, whereas 61 (55.5%) were male and 49 (44.5%) were female in the control group. The mean age was 8.1 ± 5.9 months in the patient group and 8.8 ± 4.6 months in the control group. No significant difference was found between the groups regarding age ($p=0.847$). In addition, a comparison of the groups for type of delivery, gestational age, and birth weight revealed no statistically significant differences. The clinical and demographic data of the food allergy and control groups are presented in Table II.

No significant differences were determined between the patient and control groups regarding genotype allele distributions for rs5743708 and rs1898830 SNPs in the *TLR2* gene and rs4986791 SNP in the *TLR4* gene ($p>0.05$). For *TLR2* rs5743708, Ma/Mi AF was 0.884/0.116 in the patient group and 0.911/0.09 in the control group. For *TLR2* rs1898830, Ma/Mi AF was calculated as 0.60/0.40 in the patient group and 0.546/0.453 in the control group. For the *TLR4* gene's rs4986790 SNP (A/G), the Ma/Mi AF allele frequencies were determined as 0.788/0.212 and 0.719/0.280 in the patient and control groups, respectively. In addition, it was determined that the minor allele frequency of the *TLR4* gene's rs4986790 polymorphism was 0.07 in the patient group and 0.04 in the control group. The incidences of *TLR4* rs4986790 polymorphism heterozygote genotype (AG) and G allele significantly increased in the patient group ($p=0.017$ and $p=0.017$, respectively) (Table III).

Table I. The bacteria studied and primers used in stool samples in QRT-PCR.

Bacteria (16S rRNA specific)	Forward primer	Reverse primer
Bacteroides	5′-GGCGACCGGCGCACGGG-3′	5′-GRCCTTCCTCTCAGAACCC-3′
Bifidobacterium	5′-CCTGGTAGTCCACGCCGTAA -3′	5′-CAGGCGGGATGCTTAACG-3′
Firmicutes	5′-TGAAACTYAAAGGAATTGACG-3′	5′-ACCATGCACCACCTGTC-3′
Fusobacterium	5′-TGAAACTYAAAGGAATTGACG-3′	5′-ACCATGCACCACCTGTC-3′
Actinobacteria	5′-TACGGCCGCAAGGCTA-3′	5′-TCRTCCCCACCTTCCTCCG-3′
Lactobacillus	5′-AGCAGTAGGGAATCTTCCA-3′	5′-CATGGAGTTCCACTGTCCTC-3′
Universal	5′-AAACTCAAAKGAATTGACGG-3′	5′-CTCACRRCACGAGCTGAC-3′

Table II. Comparison of clinical and demographic data for food allergy and control groups.

Features	Patient (n=130)	Control (n=110)	p value
Gender, n (%)†			0.38
Boy	89 (68.5)	61 (55.4)	
Girl	41 (31.5)	49 (44.6)	
Age (months), (mean ± SD)†	8.1±5.9	8.8±4.6	0.84
Type of birth, n (%)			0.63
Cesarean section	78 (60)	68 (61.8)	
Normal delivery	52(40)	42 (38.2)	
Gestational age (weeks), n (%)			0.40
≥38 weeks	82 (63.1)	76 (69.0)	
<38 weeks	48 (36.9)	34 (31.0)	
Birth weight (grams), n (%)			0.31
≥3500	33 (25.4)	19 (17.3)	
3000-3500	45 (34.6)	44 (40)	
<3000	52 (40.0)	47 (42.7)	
Feeding style, n (%)			0.21
Breast milk	64 (49.2)	49 (44.5)	
Breast milk + formula	22 (16.9)	15 (13.6)	
Breast milk + formula + cow's milk	21 (16.2)	19 (17.2)	
Breast milk + cow's milk	20 (15.4)	17 (15.4)	
Formula	3 (2.3)	10 (9.3)	
Antibiotic use, n (%)			0.54
Yes	73 (56.2)	66 (60)	
No	57 (43.8)	44 (40)	

†Data were presented as mean ± standard deviation (SD) and percentage.

No significant correlations of patients' characteristics participating in the study, such as gender, admission complaints, allergy types, feeding characteristics, and history of antibiotic use, with polymorphisms of *TLR2* rs5743708, *TLR2* rs1898830 and *TLR4* rs4986791 were determined (p>0.05). On the other hand, a significant relationship was present between the *TLR4* rs4986790 gene polymorphism and the type of food allergy, and the *TLR4* rs4986790 variant significantly increased in the group with isolated milk allergy compared to the control group (p=0.025). This difference was determined to be more significant between the group with isolated milk allergy and the group with multiple food allergies (p=0.036) (Table IV).

When the relationships of the patients' SPTs with the *TLR2* and *TLR4* gene polymorphisms were investigated, it was determined that in the patients with SPT positivity for milk only, the *TLR4* rs4986790 and *TLR4* rs4986791 heterozygote genotypes were significantly increased compared to the other SPT positivities (p=0.02, and p=0.003, respectively). However, no significant correlations of skin prick tests and oral provocation tests with the other analyzed polymorphisms were determined (p>0.05) (Table IV).

Microbiota analysis revealed that the partial concentration of Firmicutes increased 4.14-fold in the patient group compared to the control group, and this increase was statistically significant (p<0.001). On the other hand, even though Actinobacteria were 2.31-fold higher in the patient group than in the control group, this increase was not statistically significant (p>0.05) (Table V). In addition, there was a positive

Table III. Genotype and allele frequencies of TLR2 and TLR4 gene polymorphisms in patients with food allergies and the healthy control group.

TLR	SNP	Major/ Minor allele	Patient group (n=130)		Control group (n=110)		Minör OR (%95 CI)	p value for minor allele	OR (95% CI) for genotype	p value for genotype
			Major/ Minor allele frequency	Genotype number (n)	Major/ Minor allele frequency	Genotype number (n)				
TLR2	Rs5743708 (Arg753Gln)	A/G	0.88/ 0.12	127/3/0	0.91/ 0.09	108/2/0	0,79 (0.13-4.75)	0.578	0.78 (0.13-4.78)	0.578
	Rs1898830	A/G	0.60/ 0.40	43/59/ 28	0.55/ 0.45	40/48/ 22	0.91 (0.63-1.30)	0.643	0.91 (0.49-1.70)	0,860
TLR4	Rs4986791 (Thr399Ile)	C/T	0.68/ 0.32	122/8/0	0.62/ 0.38	102/8/0	1.18 (0.44-3.22)	0.463	1.10 (0.43-3.23)	0,463
	Rs4986790 (Asp299Gly)	A/G	0.93/ 0.07	111/19/0	0.96/ 0.04	102/8/0	0.35 (0.14-0.81)	**0.017***	0,34 (0.13-0.88)	**0.017***

CI: confidence interval, OR: odds ratio, SNP: single nucleotide polymorphism, TLR: toll like receptor.

correlation between egg-specific IgE level and *Bacteroides* concentration in the gut microbiota in the patient group (p=0.009, r=0.270).

When quantitative values of bacteria within the gut microbiota were compared to the *TLR2* and *TLR4* gene polymorphisms, it was determined that the concentration of *Bifidobacterium* significantly increased in the *TLR4* rs4986791 CT heterozygote genotype (p=0.004) (Table VI).

Discussion

In our study, we found that the concentration of Firmicutes bacteria increased in children with food allergies compared to healthy children. There was a positive correlation between egg-specific IgE level and Bacteroides concentration in the gut microbiota in the patient group. Also, the incidences of *TLR4* rs4986790 polymorphism heterozygote genotype (A/G) significantly increased in children with food allergies. In addition, the *TLR4* rs4986790 variant significantly increased in the group with isolated milk allergy compared to the control group.

Information about the roles of gut microbiota in the development and course of food allergies has been progressively increasing day by day.[18,19] Evidence from studies shows that dysbiosis starts long before the onset of food allergy symptoms and signs. However, a specific type of microorganism responsible for food allergies has not yet been clearly isolated.[5,19] Numerous studies evaluating the relationship between food allergy development and the content of gut microbiota have been published in the literature. In some of these studies comparing children with eczema and developing a food allergy with healthy children, the concentrations of *Streptococcaceae, Lachnospiraceae, Leuconostocaceae, Ruminococcaceae, Clostridium*, Clostridia, *Enterococcus, Lactobacillus, Staphylococcus, Faecalibacterium* (Firmicutes phylum), *Escherichia, Shigella, Enterobacter* (Proteobacteria phylum), Firmicutes, and Bacteroidetes microorganisms have been

Table IV. The relationships of *TLR4* gene polymorphism with patients' demographic, clinical, and laboratory characteristics.

Features	The frequency and distribution for *TLR4* genotype n (%)							
	Rs4986790 (Asp299Gly)				Rs4986791(Thr399lle)			
	AA	AG	GG	p value	CC	CT	TT	p value
Gender								0.23
Boy	76 (58.5)	13 (10)	0	0.98	82 (63.24)	7 (5.3)	0	
Girl	35 (26.9)	6 (4.6)	0		40 (30.7)	1 (0.76)	0	
Complaints								0.36
Eczema	77 (59.2)	10 (7.6)	0		82 (63)	5 (3.8)	0	
Vomiting	11 (8.6)	2 (1.5)	0		13 (10)	0	0	
Cough	7 (5.6)	2 (1.5)	0	0.32	7 (5.3)	2 (1.5)	0	
Infantile asthma	6 (4.6)	2 (1.5)	0		8 (6.44)	0	0	
Other	10 (7.69)	3 (2.30)	0		12 (9.2)	1 (0.76)	0	
Allergy type								0.47
Isolated egg allergy	53 (40.7)	9(6.9)	0		59 (45.3)	3 (2.3)	0	
Isolated milk allergy	22 (16.9)	9(6.9)	0	0.025	26 (20)	5 (3.8)	0	
Multiple food allergy	35 (26.9)	1(0.76)	0		36 (27.6)	0	0	
Type of birth								0.26
Cesarean section	67 (51.5)	12 (9.2)	0	0.87	72 (55.46)	7 (5.38)	0	
Normal delivery	44 (33.8)	7 (5.5)	0		50 (38.4)	1 (0.76)	0	
Gestational age (weeks), n (%)								0.79
≥38 weeks	71 (54.6)	11 (8.4)	0	0.81	78 (60.07)	4 (3.07)	0	
<38 weeks	40 (30.85)	8 (6.15)	0		44 (33.79)	4 (3.07)	0	
Birth weight (grams), n (%)								0.18
≥3500	29 (22.3)	3 (2.3)	0		33 (25.3)	0	0	
3000-3500	41 (31.6)	5 (3.8)	0	0.45	43 (33.26)	2 (1.53)	0	
<3000	41 (31.6)	11 (8.4)	0		46 (35.3)	6 (4.61)	0	
Feeding style, n (%)								0.97
Breast milk	54 (41.5)	9 (6.9)	0		60 (46.1)	4 (3.07)	0	
Breast milk + formula	20 (15.3)	3 (2.3)	0		21 (16.1)	1 (0.76)	0	
Breast milk + formula + cow's milk	15 (11.64)	6 (4.6)	0	0.34	19 (14.6)	2 (1.53)	0	
Breast milk + cow's milk	19 (14.6)	1 (0.76)	0		19 (14.6)	1 (0.76)	0	
Formula	3 (2.4)	0	0		3 (2.48)	0	0	

Table V. Gut microbiota for patients with food allergies, expressed as partial fold increase in comparison to the control group

Microbiota	Partial fold increase in patients with food allergies	p value
Bacteroides	1.11	0.788
Bifidobacterium	0.64	0.171
Firmicutes	4.14	**<0.001***
Fusobacteria	0.99	0.626
Actinobacteria	2.31	0.204
Lactobacillus	1.36	0.985

Table VI. The relationships between gut microbiota and *TLR2*/*TLR4* gene polymorphisms in the patient group with food allergies.

Microbiota	*TLR2*						
	Rs1898830				Rs5743708 (Arg753Gln)		
	Partial fold increase				Partial fold increase		
	AA	AG	GG	p value	AA	AG	p value
Bacteroides	1.04	1.17	1	0.973	1.69	1	0.389
Bifidobacterium	2.87	1.19	1	0.140	1.15	1	0.169
Firmicutes	1.73	1.68	1	0.499	1.10	1	0.937
Fusobacteria	0.20	0.57	1	0.344	2.64	1	0.676
Actinobacteria	0.71	1.40	1	0.871	0.15	1	0.431
Lactobacillus	0.46	0.43	1	0.696	0.12	1	0.335
Microbiota	*TLR4*						
	Rs4986790 (Asp299Gly)			Rs4986791 (Thr399lle)			
	Partial fold increase			Partial fold increase			
	AA	AG	p value	CC	CT	p value	
Bacteroides	1.76	1	0.459	0.25	1	0.229	
Bifidobacterium	1.98	1	0.318	0.05	1	0.004	
Firmicutes	1.02	1	0.961	2.42	1	0.276	
Fusobacteria	2.48	1	0.197	1.70	1	0.781	
Actinobacteria	2.78	1	0.265	0.83	1	0.926	
Lactobacillus	1.76	1	0.459	0.25	1	0.229	

determined to increase.[8,20-24] However, in some other studies comparing children with eczema to healthy children, the concentrations of bacteria such as *Streptococcaceae*, *Oscillibacter*, *Lactococcus*, *Dorea* (Firmicutes phylum); *Enterobacter*, *Citrobacter* (Proteobacteria phylum), *Bifidobacterium* (Actinobacteria phylum), and *Bacteroides* (Bacteroidetes phylum) have been found to decrease in intestinal microbiota of children with eczema.[8,23,25] On the other hand, there are also studies in the literature showing that a statistical relationship was not present between food allergies and gut microbiota.[26,27] Our case-control study analyzed bacteria within the intestinal microbiota at three phylum and three genus levels. We determined that Firmicutes bacteria were increased in children with food allergies compared to the control group. Nylund et al.[25] reported higher levels of Firmicutes species such as *Clostridium* and lower levels of *Bacteroides* species within the gut microbiota in 6-month and 18-month old infants with atopic eczema than in healthy infants. The study of Ling et al.[24] showed that the amounts of *Bacteroides*, Proteobacteria, and Actinobacteria decreased, whereas Firmicutes filum significantly increased in the gut microbiota of children with food allergies. Firmicutes, one of the anaerobic bacteria species, play roles in stimulating the immune system and the inflammatory response. Furthermore, our study determined that *Bifidobacterium* and *Fusobacteria* microorganisms were relatively low, and Actinobacteria and *Lactobacillus* bacteria were relatively high in children with food allergies compared to the control group. However, no statistically significant difference was present between the groups.

In children with food allergies, the intestinal microbiota's composition might be specific to an isolated food allergen. Fazlollahi et al.[22], in their case-control study, reported that *Lachnospiraceae*, *Streptococcaceae*, and *Leuconostocaceae* species significantly increased in amount in the intestinal microbiota of

children with an egg allergy. Berni Canani et al.[23] determined increased concentrations of *Lachnospiraceae* and *Ruminococcaceae* in the gut microbiota of infants with cow's milk allergy. On the other hand, the same study reported a decreased level of Streptococcaceae within the intestinal microbiota. In our study, there was a positive correlation between egg-specific IgE level and *Bacteroides* concentration in the gut microbiota in the patient group. Obtaining different results in microbiome studies in children with food allergies; we think that it may be due to factors such as geographical and racial differences, the age of the child at the time of collection of stool samples, differences in collection and analysis of stool samples, and heterogeneity of food allergies.

The roles of *TLR2* and *TLR4* polymorphisms in food allergies have not yet been clearly investigated. Conducted studies reported that impaired signaling of *TLR2* and *TLR4* in mice caused allergic sensitivity to food proteins.[28,29] Bashir et al.[28] determined that the absence of *TLR4* expression in mice was associated with sensitivity development to food allergy. In a *TLR4* receptor-negative mouse model, intragastric administration of food allergens triggered the allergen-specific IgE release and a high histamine discharge. Moreover, it was shown that when sufficient time was provided for the flora's regeneration, antigen-specific IgE response and allergic symptoms decreased in these mice. Berin et al.[29] evaluated the relationship between TLR4 signal and food allergy, intestinal microbiota. The authors showed that TLR4 could negatively or positively affect allergic reactions according to individual genetic differences and the food antigens' nature and types. Conversely, Galli et al.[30] reported that there was no association between cow's milk allergy and *TLR2*/*TLR4* polymorphisms in children with food allergies. Our study determined that the AG heterozygote genotype and allele prevalence for the *TLR4* gene's rs4986790 SNP of the patient group were significantly increased. Moreover, there were significant relationships of the *TLR4* rs4986790 gene polymorphism in the food allergy group with the number and type of foods diagnosed as food allergy with the oral provocation test. Besides, our study found that, when compared to the other food allergy positivities, the heterozygote genotypes of the *TLR4* rs4986790 and *TLR4* rs4986791 gene polymorphisms were significantly increased in the patients with only milk-positivity in the skin prick test. When *TLR2* and *TLR4* gene polymorphisms were compared regarding quantitative ratios of bacteria in the intestinal microbiota, we determined that only the *Bifidobacterium* ratio significantly increased in the *TLR4* rs4986791 heterozygote variant genotype compared to the wild genotype. In the light of these results, we suggest that the *TLR2* and *TLR4* polymorphisms may lead to a predisposition to food allergy development by affecting TLR expressions and functions. We think that the probable mechanism for the *TLR4* rs4986790 polymorphism's contribution to the pathophysiology of food allergies is stimulating the pro-inflammatory process. Ferwerda et al.[31] demonstrated that the production of TNF-alpha, a potent pro-inflammatory cytokine, increased after stimulating with LPS in mice with the *TLR4* rs4986790 SNP.

Our study has various limitations. First, there was a relatively small number of cases in the study and control groups in our study. In addition, there was a small number of bacteria investigated in the intestinal microbiota and half of the analyzed bacteria were at the phylum level. The studies revealed that performing the analysis at the genus and species level was more critical in food-allergy-related microbiota analysis.[19,32] Secondly, the cases in the study group constituted a heterogeneous group. Some children had allergies to more than one food. The gut microbiota composition and TLR polymorphisms might be specific to an isolated food allergen. Different microorganism species in the gut microbiota might be associated with each food allergy's development.[19] For this reason, investigating and comparing microbial compositions in children with different food allergies continues to be a field deserving

further study. Thirdly, because our study was cross-sectional, we underline that definitive causality cannot be retrieved from the results of our study.

In conclusion, we believe that our study provided some data about potential relationships between food allergies and the bacteria within the intestinal microbiota, TLR2 and TLR4 receptors. When children with food allergies were compared to healthy children, we determined that the concentration of *Firmicutes* bacteria increased, and the A/G genotype of the *TLR4* rs4986790 polymorphism was statistically significantly increased. Because the intestinal microbiota has the potential to be modulated, it provides new research fields to develop innovative strategies for the prevention and treatment of food allergies. Furthermore, studies investigating the composition and functions of the intestinal microbiota and disclosing their relationships with TLR might also enable the evolution of novel treatment models.

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Ethical approval

The Ethics Committee of Fırat University approved the study (date June 21, 2018, and decision # 11/04). Moreover, the participants' families were informed about the study, and their written consent was obtained.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: MK, EB; data collection: EB, TK; analysis and interpretation of results: MK, EB, ET, EEO; draft manuscript preparation: MK, EB, ET. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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