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Original Article

Alterations in immunized antigens of *Anisakis pegreffii* by ampicillin-induced gut microbiome changes in mice

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Abstract

The gut microbiome plays an essential role in host immune responses, including allergic reactions. However, commensal gut microbiota is extremely sensitive to antibiotics and excessive usage can cause microbial dysbiosis. Herein, we investigated how changes in the gut microbiome induced by ampicillin affected the production of IgG1 and IgG2a antibodies in mice subsequently exposed to *Anisakis pegreffii* antigens. Ampicillin treatment caused a notable change in the gut microbiome as shown by changes in both alpha and beta diversity indexes. In a 1-dimensional immunoblot using *Anisakis*-specific anti-mouse IgG1, a 56-kDa band corresponding to an unnamed *Anisakis* protein was detected using mass spectrometry analysis only in ampicillin-treated mice. In the *Anisakis*-specific anti-mouse IgG2a-probed immunoblot, a 70-kDa band corresponding to heat shock protein 70 (HSP70) was only detected in ampicillin-treated and *Anisakis*-immunized mice. A 2-dimensional immunoblot against *Anisakis* extract with immunized mouse sera demonstrated altered spot patterns in both groups. Our results showed that ampicillin treatment altered the gut microbiome composition in mice, changing the immunization response to antigens from *A. pegreffii*. This research could serve as a basis for developing vaccines or allergy immunotherapies against parasitic infections.

Keywords: *Anisakis pegreffii*, immunoglobulin, immunoblot, ampicillin, microbiome, allergen

Introduction

Anisakis spp. are nematode parasites that infect a wide range of marine organisms. *Anisakis* also causes a human disease by consuming raw or undercooked fish infected with *Anisakis* spp., such as *Anisakis simplex*, *Anisakis pegreffii*, and *Anisakis physeteris* [1]. The severity of the disease varies from mild to severe and presents with distinct pathological symptoms [2]; however, parasite infection may cause adverse complications, such as abdominal pain, nausea, vomiting, allergy, anaphylactic shock, and even death [3]. *Anisakis* is the only parasite known to act as a food allergen and is a major cause of food allergy, causing allergies more often than seafood itself [3]. *A. simplex* and *A. pegreffii* show a worldwide distribution and are the major causes of anisakiasis [3]. Thus, *Anisakis* spp. are potential seafood-borne pathogens and allergens for humans, posing severe health and financial impacts on fisheries industry, where fishermen and handlers are at risk of developing occupational asthma caused by the inhalation of allergens produced by *Anisakis* spp. [4].

The World Health Organization (WHO) in collaboration with the International Union of Immunological Societies (IUIS) has entrusted the WHO/IUIS Allergen Nomenclature

Author contributions
Data curation: Choi JH
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Conflict of interest
The authors declare no competing interests.

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Subcommittee with formulating a systematic and unequivocal nomenclature for allergenic proteins. Within this framework, 14 *Anisakis* allergens have been registered as Ani s 1–14 (www.allergen.org). Ani s 1, 2, 3, 7, 12, and 13 are major allergens [3], with Ani s 1, 4–9, and 13 being parasite excretory/secretory molecules; Ani s 2, 3, and 12 are somatic antigens [5]; and Ani s 1, 4, 5, 8, and 9 are heat-stable allergens, and patients sensitized to these develop adverse allergic reactions to cooked or canned fish. Most of the heat-stable allergens are present in the excretory/secretory products of *Anisakis* [5]. Moreover, several allergens are resistant even to freezing or pepsin treatment [5]. Kim et al. investigated the molecular and immunological aspects of allergen-like entities from parasites, emphasizing their role in allergic reactions and their diagnostic and therapeutic significance [3].

The gut microbiome composition can be affected by age, diet, and environmental conditions [6]. The gastrointestinal tract commensal microbiota are essential for health as they contribute to the maturation of the mucosal and systemic immune systems and aid in resisting colonization by invading pathogens [6]. Furthermore, the gastrointestinal tract plays an important role in the development of either effector or tolerant responses to different antigens by balancing the activity of the T helper (Th) 1 and Th2 response [7]. Perturbations that disrupt the healthy composition of the gut microbiome may contribute to immune dysfunction in allergic diseases, such as allergic rhinitis and asthma [8]. Additionally, several studies have shown that antibiotics administered early in life significantly increase the risk of asthma in children [9]. Antibiotics cause dysbiosis of gut microbiota, disrupting the balance between the populations of the Th1 and Th2 cells, which can favor an increase in the frequency of allergen-based reactions and consequently decrease the quality of life of an individual [10]. Although gut microbial dysbiosis has been connected to the onset of food allergies, the intricate pathways involved remain under scrutiny. To decipher the comprehensive dynamics between gut dysbiosis and allergic manifestations, a more profound exploration is essential.

Herein, we investigated the effect of gut microbial dysbiosis on the synthesis of immunoglobulin (Ig) G1 and IgG2a antibodies on subsequent exposure to *A. pegreffii* protein extract. Importantly, IgG1 correlates with Th2 immune responses while IgG2a aligns with Th1 responses, both playing pivotal roles in allergic manifestations, although the production of IgE or other allergy indicators was not assessed in our study. To examine the changes in immunized allergen patterns caused by alterations in the composition of gut microbiota, we treated mice with ampicillin to eliminate gut microbiota. Proteomics on a 2-dimensional (2-D) immunoblot of *A. pegreffii* protein extract was performed using sera from mice immunized with *A. pegreffii*. Subsequently, we assessed whether each antigen was immunized with Th1-related IgG2a or Th2-related IgG1 and confirmed that changes in the microbiome could alter the immunization of individual proteins of *A. pegreffii*.

Materials and Methods

Ethics statement

All methodologies involving experimental animals received approval from the Department of Laboratory Animal Resources Committee of Yonsei University College of Medicine (no. 2020-0055).

Protein preparation

A single Chub mackerel (*Scomber japonicus*) was acquired from the Saruga shopping center, Seoul, Korea; *Anisakis* third-stage larvae were manually collected from the abdominal cavity, yielding a total of 150 larvae, which were washed before DNA and protein extraction processes. DNA from 15 *Anisakis* larvae was extracted using the NucleoSpin DNA Insect Kit (Macherey-Nagel, Düren, Germany). The presence of *A. pegreffii* was confirmed through polymerase chain reaction (PCR) using primers targeting the ITS region: Primer A (forward: 5′-GTCGAATTCGTAGGTGAACCTGCGGAAGGATCA-3′) and Primer B (reverse: 5′-GCCGGATCCGAATCCTGGTTAGTTTCTTTTCCT-3′) [11]. PCR amplifications were sequenced and aligned with *A. pegreffii* reference sequences obtained from GenBank, confirming that the 15 tested parasites were *A. pegreffii*. Protein extraction from the remaining 135 larvae was performed using sonication, followed by centrifugation at 10,000 × g for 30 min and filtration through a sterilized 0.22-μm filter (Merck Millipore, Darmstadt, Germany). The protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). The extract was maintained on ice throughout the extraction process and stored at -80°C.

Animals and antibiotic treatment

Female BALB/c mice ($n = 15$; 6 weeks old) were purchased from Orient Bio (Seongnam, Korea) and tested in 3 groups (5 mice per group): phosphate-buffered saline (PBS) group, *Anisakis*-immunized without ampicillin treatment group, and *Anisakis*-immunized with ampicillin group; the number of mice per group was consistent with that used in our previous *Anisakis* allergy research [12]. To ensure optimum conditions, mice were housed in a specific pathogen-free environment with a 12-h light/dark cycle and given a week to acclimatize before experimentation. A daily health assessment was systematically conducted per mouse.

For immunization, 100 μg of *A. pegreffii* protein extract was suspended in PBS while ampicillin was prepared at a concentration of 1 g/L in distilled water [13]. This concentration was then provided to specific groups as part of their daily water consumption. The ampicillin regimen was commenced a week before the primary sensitization and maintained throughout the experiment. Mice were intraperitoneally injected with the *A. pegreffii* extract (100 μg) once weekly for a total of 4 weeks [14]. Mice were euthanized a fortnight after the final dosage. This study was repeated 3 times.

Measurement of IgG levels via enzyme-linked immunosorbent assay

Mice were euthanized using CO_2 gas, and blood was collected from the inferior vena cava. For serum separation, blood samples were centrifuged for 30 min at 3,000 × g, and the supernatant was collected. The serum levels of *A. pegreffii*-specific IgG1 and IgG2a were measured using an enzyme-linked immunosorbent assay (ELISA). Briefly, 1 × 8 Stripwell 96-well plates (Corning, Corning, NY, USA) were coated with 100 μl/well coating buffer containing 0.3 μg *A. pegreffii* protein extract and incubated overnight at 4°C [12]. The plate was then washed with PBS+0.05% of Tween-20 (PBST) and blocked with 200 μl/well diluent buffer (PBST containing 1% of bovine serum albumin (BSA)) for 1 h at 25°C. Samples were diluted 1:100 with diluent buffer. After blocking, 100 μl of samples were dispensed

per well and incubated for 2 h. Biotinylated goat anti-mouse IgG1 or biotin goat anti-mouse IgG2a (both diluted 1:1,000; NBP1-69914B and NBP1-69915B, respectively; Novus Biologicals, Littleton, CO, USA) were used as secondary antibodies. Wells were then incubated with an avidin-horseradish peroxidase (HRP) conjugate (BioLegend, San Diego, CA, USA) for 30 min, followed by incubation with 3,3,5,5-tetramethylbenzidine (Sigma-Aldrich) substrate (50 μl) in the dark for 5 min. The reaction was inhibited by adding 0.5M H_2SO_4. Absorbance was measured at 450 nm using VersaMax (Molecular Devices, Seoul, Korea). Statistical analyses were performed using GraphPad Prism software (version 8.0, GraphPad Software, San Diego, CA, USA).

Measurement of IgG levels via western blotting

Western blotting was performed using *A. pegreffii* protein. Proteins were separated by gel electrophoresis. *A. pegreffii* protein (10 μg) was loaded into each well of a mini format 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel. Following gel electrophoresis, proteins were transferred from the gel onto a polyvinylidene difluoride membrane. After transfer, the membrane was blocked with 5 ml of Tris-buffered saline Tween-20 (TBST) containing 5% of BSA for 2 h at 25°C. Pooled samples were diluted 1:1,000 with TBST containing 1% of BSA and incubated overnight at 4°C. The goat anti-mouse IgG1 and goat anti-mouse IgG2a heavy chain antibodies (both diluted 1:1,000; ab97240 and ab97245, respectively; Abcam, Cambridge, UK) were used as secondary antibodies conjugated with HRP (1:10,000; ab2116, Abcam) for detection. Bands were developed using the ECL reaction (Bio-Rad), and images were captured using Chemidoc XRS (Bio-Rad).

Sample preparation and 2-D separation

Protein concentrations were determined using the Bradford method (Bio-Rad). For 2-D analysis, pH 3–10 IPG strips (GE Healthcare Life Sciences, Pittsburgh, PA, USA) were rehydrated in swelling buffer containing 7 M urea, 2 M thiourea, 2.5% (w/v) dithiothreitol, and 4% (w/v) CHAPS. Protein lysates (600 μg) were loaded into the rehydrated IPG strips using an IPGphor III (GE Healthcare Life Sciences), and the 2-D separation was performed on a 10% SDS-PAGE gel. Following fixation of the gels for 1 h in a solution of 40% (v/v) methanol containing 5% (v/v) phosphoric acid, gels were stained with colloidal Coomassie blue G-250 solution (ProteomeTech, Seoul, Korea). Gels were destained using deionized water, and images were acquired via an image scanner (Bio-Rad). Image analysis was performed using ImageMaster 2-D Platinum software (Amersham Biosciences, Hercules, CA, USA). To compare protein spots, > 25 spots in all gels were marked and normalized. Data for the normalized spot intensities are shown in Table 1.

In-gel digestion with trypsin and extraction of peptides

Spots from 2-D gels were excised and in-gel digested with trypsin [15]. Briefly, protein spots were excised from the stained gels and cut into pieces, which were washed for 1 h at 25°C in 25 mM ammonium bicarbonate buffer, pH 7.8, containing 50% (v/v) acetonitrile (ACN). Following dehydration of gel pieces in a centrifugal vacuum concentrator (Biotron, Inc., Incheon, Korea) for 10 min, they were rehydrated in 50 ng of sequencing-grade trypsin solution (Promega, Madison, WI, USA). After incubation in 25 mM ammonium bicar-

Table 1. Allergen identification using LC-MS in 2D western blot analysis

Spot no.	NCBI BLAST	Protein name	Score	Mass (*m/z*)
1	P00761.1	RecName: Full = Trypsin; Flags: Precursor (*Sus scrofa*)	54	25,078
2	VDK55667.1	78 kDa glucose-regulated protein (*Anisakis simplex*)	345	75,647
3	2B5L_A	Chain A, Crystal Structure of Ddb1 in Complex with Simian Virus 5 V Protein (*Homo sapines*)	64	128,162
4	VDK50697.1	Chaperonin homolog Hsp-60, mitochondrial (*Anisakis simplex*)	1,039	50,811
5	BAX39002.1	Hemoglobin (*Anisakis pegreffii*)	197	39,305
6		No identification		
7	CAD43170.1	Enolase (*Anisakis simplex*)	225	47,672
8	VDK47804.1	Unnamed protein product (*Anisakis simplex*)	141	67,191
9	VDK54672.1	Unnamed protein product (*Anisakis simplex*)	913	61,520
10	VDK54672.1	Unnamed protein product (*Anisakis simplex*)	601	61,520
11	VDK17787.1	Unnamed protein product, partial (*Anisakis simplex*)	61	32,612
12	VDK17787.1	Unnamed protein product, partial (*Anisakis simplex*)	176	32,612
13	AIU38247.1	Heat shock protein 70 (*Anisakis pegreffii*)	433	70,955
14	BAX39002.1	Hemoglobin (*Anisakis pegreffii*)	58	39,305
15	VDK54672.1	Unnamed protein product (*Anisakis simplex*)	687	61,520
16	VDK45319.1	Unnamed protein product (*Anisakis simplex*)	213	53,638
17	VDK58822.1	Unnamed protein product (*Anisakis simplex*)	61	52,879
18	VDK61442.1	Unnamed protein product (*Anisakis simplex*)	72	8,957
19		No identification		
20	VDK58822.1	Unnamed protein product (*Anisakis simplex*)	518	52,879
21	VDK58822.1	Unnamed protein product (*Anisakis simplex*)	301	52,879
22		No identification		
23	CAD43170.1	Enolase (*Anisakis simplex*)	240	47,672
24	CAD43170.1	Enolase (*Anisakis simplex*)	634	47,672
25	CAD43170.1	Enolase (*Anisakis simplex*)	885	47,672
26	CAD43170.1	Enolase (*Anisakis simplex*)	1,758	47,672
27	Q0PGG4.1	RecName: Full = Actin, cytoplasmic 1; AltName: Full = Beta-actin; Contains: RecName: Full = Actin, cytoplasmic 1, N-terminally processed (*Bos grunniens*)	60	42,064
28	BAX39002.1	Hemoglobin (*Anisakis pergeffii*)	181	39,305

bonate buffer, pH 7.8, at 37°C overnight, tryptic peptides were extracted in 100 μl of 1% formic acid (FA) containing 50% (v/v) ACN for 20 min with mild sonication. The extracted solution was then concentrated using a centrifugal vacuum concentrator. Before mass spectrometry (MS) analysis, the peptide solution was desalted using a reversed-phase column [16]. Briefly, after an equilibration step with 10 μl of 5% (v/v) FA, the peptide solution was loaded onto the column and washed with 10 μl of 5% (v/v) FA. Bound peptides were eluted in 8 μl of 70% ACN in 5% (v/v) FA.

Protein identification by liquid chromatography–tandem mass spectrometry

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis was performed using a nano ACQUITY UPLC and LTQ-orbitrap-mass spectrometer (Thermo Electron, San Jose, CA, USA) with a BEH C18 1.7 μm, 100 μm × 100 mm column (Waters, Milford, MA, USA). The mobile phase A for the LC separation was 0.1% FA in deionized water, and the mobile phase B was 0.1% FA in ACN. The chromatography gradient provided a linear increase from 10% B to 40% B for 16 min, from 40% B to 95% B for 8 min, and from 90% B to 10% B for 11 min. The flow rate was 0.5 μl/min. For tandem MS, mass spectra were acquired using data-dependent acquisition with a full mass scan (300–2,000 *m/z*), followed

by MS/MS scans. Each MS/MS scan acquired was the average of 1 microscan of the LTQ. The temperature of the ion transfer tube was controlled at 275°C, and the spray voltage was 2.3 kV. The normalized collision energy was set to 35% for MS/MS. Individual spectra from MS/MS were processed using the SEQUEST software (Thermo Quest, San Jose, CA, USA), and the generated peak lists were used to query the in-house database using the MASCOT program (Matrix Science, London, UK). The modifications of carbamidomethyl, deamidated, and oxidation were set for MS analysis, and the tolerance of the peptide mass was 10 ppm. The MS/MS ion mass tolerance was 0.8 Da, the allowance of missed cleavage was 2, and charge states +2 and +3 were considered for data analysis. We considered only significant hits as defined by the MASCOT probability analysis [17].

High-throughput sequencing of the 16S rRNA gene amplicon and bioinformatics

Mouse ceca were collected and stored at -80°C. Cecal DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Carlsbad, CA, USA). DNA corresponding to the V3–V4 region of the 16S rRNA gene was PCR amplified using the following bacterial universal primer pair: forward primer, 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′, and reverse primer, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′ [18]. Limited-cycle amplification was performed using the Nextera XT Index Kit (Illumina, San Diego, CA, USA) to add multiplexing indices and Illumina sequencing adapters to PCR products. Libraries were normalized, pooled, and sequenced using the MiSeq platform (600 cycles, Illumina MiSeq V3 cartridge; Illumina).

Bioinformatics analyses were performed as described by Kim et al. [18] Raw reads were processed through a quality check, and low-quality ($Q < 25$) reads were filtered using Trimmomatic 0.32 [19]. Paired-end sequence data were merged using PandaSeq [20]. Primers were trimmed using the ChunLab in-house program (ChunLab, Inc., Seoul, Korea), applying a similarity cutoff threshold of 0.8. Sequences were denoised using the Mothur preclustering program, which merges and extracts unique sequences, allowing up to 2 differences between them [21]. The EzBioCloud database [22] was used for taxonomic assignment using BLAST 2.2.22 [23], and pairwise alignments were generated to calculate the similarity [24]. The UCHIME algorithm and nonchimeric 16S rRNA database from EzBioCloud were used to detect chimeric sequences for reads with the best hit similarity rate of $< 97\%$ [25]. Sequence data were then clustered using CD-Hit and UCLUST [26,27]. All aforementioned analyses were performed using EzBioCloud, a commercially available ChunLab bioinformatics cloud platform for microbiome research (https://www.ezbiocloud.net/). Reads were normalized by random subsampling to 7,666 to perform the analyses. The Shannon index [28] and principal coordinate analysis (PCoA) [29] were computed based on the generalized UniFrac distance [30]. The Kruskal–Wallis test was used to compare differences between the number of operational taxonomic units (OTUs), and the Shannon index was used to compare microbiome diversity among the 3 groups. Linear discriminant analysis effect size (LEfSe) analysis was used to identify significantly different taxa [31].

Results

Metabarcoding of *Anisakis*-immunized mice

In the cecal microbiome study, the number of OTUs and Shannon index significantly decreased in the ampicillin-treated and *Anisakis*-immunized mice (Fig. 1A, B), indicating that the cecal microbiome diversity decreased after ampicillin treatment. In PCoA, the ampicillin-treated samples were distinctly clustered from untreated counterparts along the PCoA1 axis (Fig. 1C). Composition analysis suggested that the ampicillin-treated group exhibited the pronounced presence of a bacterial species resembling *Proteus* while the relative abundance of *Bacteroides* was markedly different across the groups (Fig. 2). We then performed LEfSe analysis to identify significant differences in bacterial abundance among the control and ampicillin-treated and untreated *Anisakis*-immunized groups. The LDA score for *Proteus vulgaritus* was 5.54, and the average relative abundance was 63.12%, 0.04%, and 0.02%, respectively in the ampicillin-treated *Anisakis*-immunized, control, and ampicillin-untreated *Anisakis*-immunized groups. The LDA score for *Bacteroides*_uc was 5.16, and its average relative abundance was 21.92%, 28.28%, and 0.08% in the control, ampicillin-untreated *Anisakis*-immunized, and ampicillin-treated *Anisakis*-immunized groups, respectively (Table 2).

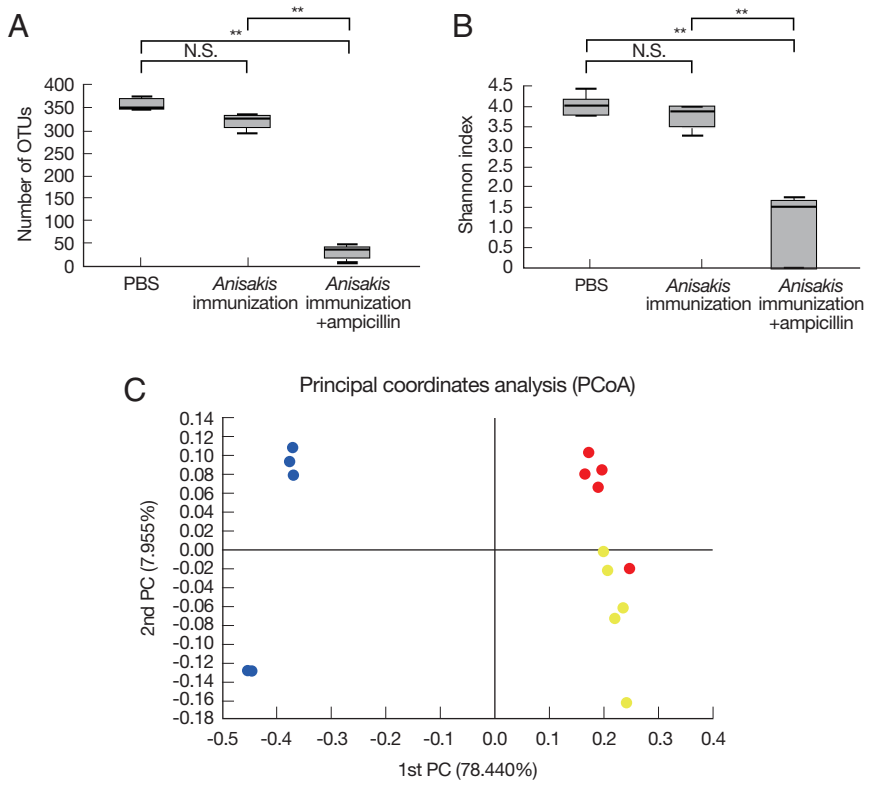

Fig. 1. Microbiome analysis of the mouse cecum. (A) Number of operational taxonomic units (OTUs). (B) Shannon index. (C) Principal coordinate analysis depicting differences in the taxonomic compositions of bacterial communities; control (yellow); ampicillin-untreated *Anisakis*-immunized group (red) and ampicillin-treated *Anisakis*-immunized group (blue). N.S., not significant. $^{**}P < 0.01$.

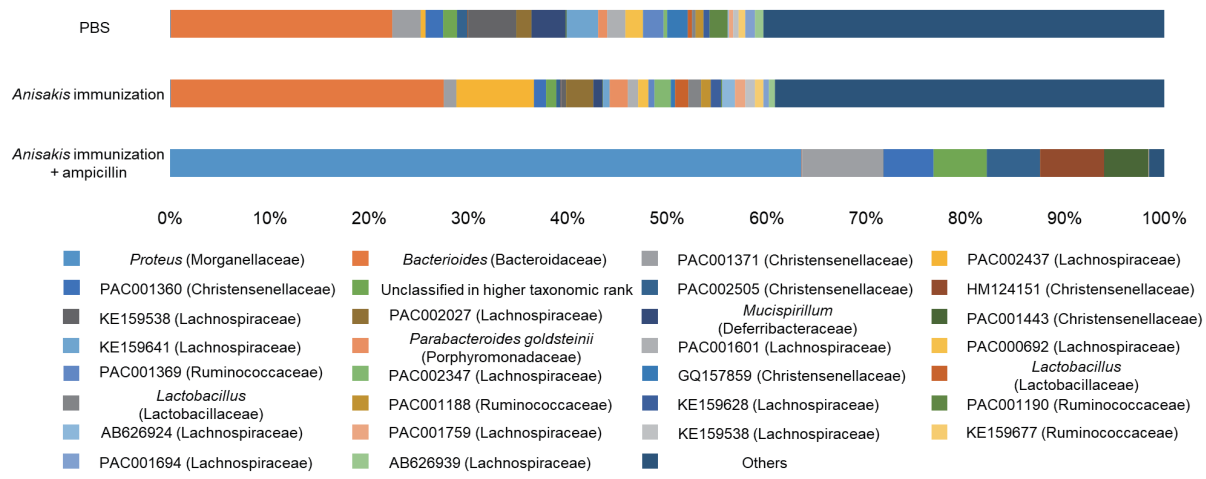

Fig. 2. Relative abundance in the mouse cecum at the genus level.

Table 2. Linear discriminant analysis effect size (LEfSe) analysis of differentially abundant bacterial taxa between control group, *Anisakis* immunization without ampicillin group and *Anisakis* immunization with ampicillin group. Only taxa meeting an LDA significant threshold of > 4.5 are shown

Taxon name	Taxon rank	LDA effect size	*P*-value	Relative abundance in control (%)	Relative abundance in *Anisakis* immunization without ampicillin (%)	Relative abundance in *Anisakis* immunization with ampicillin (%)
Proteus vulgaris	Species	5.54	0.00489	0.04	0.02	63.12
*Bacteroides*_uc	Species	5.16	0.00650	21.92	28.78	0.08
Protues	Genus	5.17	0.00688	22.48	29.08	0.10
Bacteroides	Genus	5.54	0.00489	0.04	0.02	63.12
Morganellaceae	Family	5.54	0.00489	0.04	0.02	63.14
Lachnospiraceae	Family	5.33	0.00888	43.70	43.90	0.10
Bacteroidaceae	Family	5.17	0.00688	22.48	29.08	0.10
Ruminococcaceae	Family	4.83	0.00628	14.16	12.62	0
Gammaproteobacteria	Class	5.54	0.00489	0.04	0.02	63.14
Bacteroidia	Class	5.20	0.00688	23.34	31.26	0.10
Enterobacterales	Order	5.54	0.00489	0.04	0.02	63.14
Bacteroidales	Order	5.20	0.00688	23.34	31.26	0.10

Proteomics analysis of Anisakis-immunized mice

ELISA analysis showed that the *Anisakis*-specific IgG1 and IgG2a levels increased in the *Anisakis*-immunized mice with or without ampicillin treatment, although the difference in the IgG1 and IgG2a levels between *Anisakis*-immunized ampicillin-treated and untreated groups was not significantly different (Fig. 3A, B).

To determine which *Anisakis* antigens were immunized in mice, immunoblotting was performed using pooled sera. In a 1-dimensional (1-D) immunoblot using *Anisakis*-specific anti-mouse IgG1, a 56-kDa band was detected only in the ampicillin treatment group and identified as an "unnamed protein product (VDK17787.1 and VDK17834.1)" in MS analysis (Fig. 4A; Table 3). In an immunoblot with *Anisakis*-specific anti-mouse IgG2a, a band at 70 kDa disappeared in the ampicillin treatment group and was identified as an un-

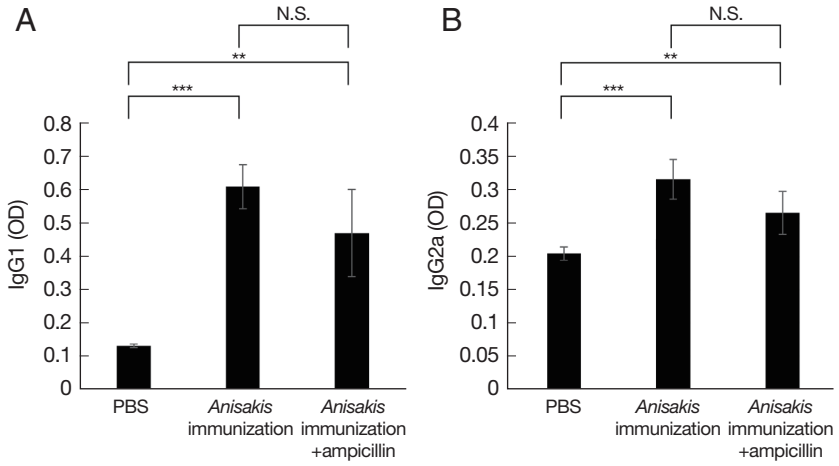

Fig. 3. Analysis of *Anisakis*-specific antibody responses in mice sera samples using enzyme-linked immunosorbent assay (ELISA). (A) *Anisakis*-specific IgG1, (B) *Anisakis*-specific IgG2a. N.S., not significant. $^{**}P < 0.01$ and $^{***}P < 0.001$.

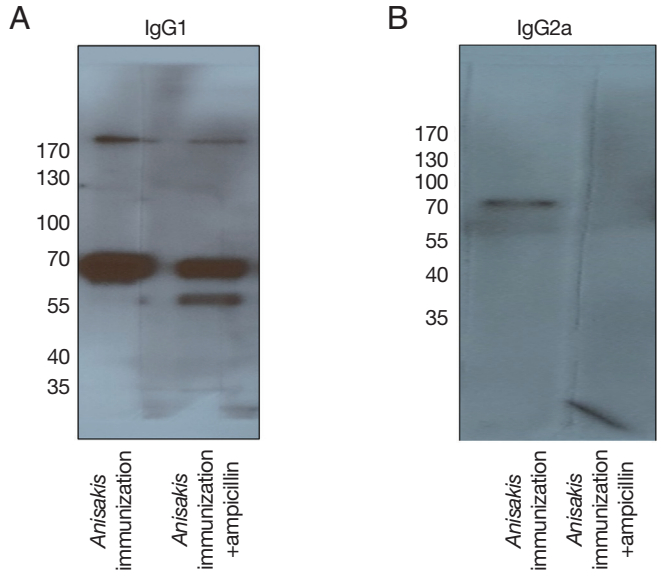

Fig. 4. Immunoblot analysis of *Anisakis pegreffii* protein extract with *Anisakis*-immunized mouse sera with or without ampicillin treatment. (A) *Anisakis*-specific IgG1 and (B) *Anisakis*-specific IgG2a.

named protein product (VDK17973.1) and heat shock protein 70 (HSP70) (AIU38247.1) (Fig. 4B; Table 3).

For improved resolution, we performed a 2-D immunoblot against *Anisakis* extract with immunized mouse sera. Using *Anisakis*-specific anti-mouse IgG1, spots 13 at 55 kDa and 19 at 46 kDa appeared only in the ampicillin-untreated *Anisakis*-immunized group while spots 4 at 70 kDa and 7 at 70 kDa and 14 kDa appeared only in the ampicillin-treated *Ani-*

Table 3. Allergen identification using MS analysis in 1D western blot analysis

Band size	Unused	Total	% Coverage	Accession no.	Name	Peptides (95%)
56 kDa	41.01	41.01	69.3	VDK17787.1	Unnamed protein product	22
	28.36	28.36	37.1	VDK17834.1	Unnamed protein product	15
70 kDa	69.05	69.05	63.7	VDK17973.1	Unnamed protein product	48
	64.53	64.53	70.1	AIU38247.1	Heat shock protein 70	46

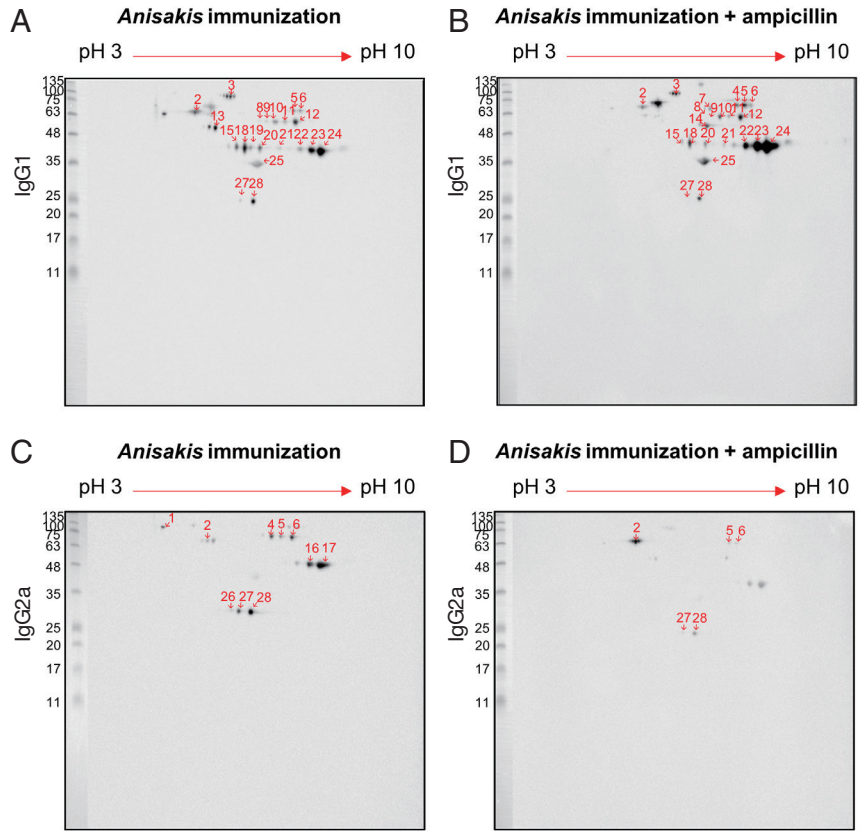

Fig. 5. Two-dimensional immunoblot analysis of *Anisakis pegreffii* extract with *Anisakis*-immunized mouse sera. *Anisakis* antigens reactive with IgG1 in *Anisakis*-immunized mouse sera without/with ampicillin treatment (A and B, respectively). *Anisakis* antigens reactive with IgG2a in *Anisakis*-immunized mouse sera without/with ampicillin treatment (C and D, respectively). Red shows the identified number, which can be checked in Table 1.

sakis-immunized group (Fig. 5A, B). Using *Anisakis*-specific anti-mouse IgG2a, spots 1, 16, 17, and 27 at 100, 48, 48, and 27 kDa, respectively, that were present in the ampicillin-untreated group disappeared in the ampicillin-treated group (Fig. 5C, D). Furthermore, spots 5 and 6 at 70 kDa were reduced in the ampicillin-treated group. MS was performed to identify these proteins of selected spots in the 2-D immunoblot assay (Table 1); spot 13 was identified at HSP70 (AIU38247.1) while spots 11 and 12 were identified as an unnamed protein product (VDK17787.1); these spots were detected in the 1-D western blotting MS analysis (Table 3). These observations suggest that ampicillin treatment altered the immu-

nized patterns of Th1- and Th2-inducing *Anisakis* antigens.

Discussion

In this study, we observed that antibiotic treatment altered the mouse microbiome with subsequent changes to Ig production patterns following *A. pegreffii* immunization. Antibiotic treatment reduced the number of commensal microbiota species and diversity in the mouse microbiome and significantly decreased the abundance of *Bacteroides*. Furthermore, ampicillin treatment altered the immunized antigen patterns, with numerous IgG1 spots but fewer IgG2a spots observed in the ampicillin group than in the untreated groups.

We hypothesize that gut microbiome dysbiosis causes a Th1/Th2 imbalance, which further alters the antigen-immunization pattern. Supporting this, previous studies suggest that antibiotic administration can cause the reorganization of bacterial flora, inhibiting signaling through the Toll-like receptor 4 (TLR4) pathway [32]. The inability to signal through TLR4 has been shown to contribute to the allergic immune reaction by markedly increasing the Th2 cytokine response to peanut-specific IgE [32]. Consistent with this finding, we observed that an additional band related to Th2-related IgG1 was detected using western blotting while several spots were increased in the 2-D immunoblot.

Interestingly, changes in the microbiome caused by *Anisakis*-immunization in mice without ampicillin treatment were also confirmed in the PCoA result. Samples from mice not treated with ampicillin after antigen injection exhibited a unique clustering pattern distinct from PBS-injected mice along the PCoA2 axis, hinting at potential sensitization-induced shifts in the gut microbiota composition. In a previous study, oral sensitization using OVA without any adjuvant produced marked allergic reactions and notable changes in the gut microbiome with disturbance of the gut flora [33]. Therefore, we propose that allergic inflammation itself can trigger changes in the microbiome.

In mice, systemic levels of IgG1 antibodies closely correlate with an overall Th2 immune response profile, whereas those of IgG2a antibodies indicate an overall Th1 profile [34]. Th1-dependent IFN-γ induces the production of IgG2a, whereas the Th2 cytokine, IL-4, induces the expression of IgG1 [34]. For *A. pegreffii*-specific IgG1, an additional band was detected in the ampicillin-treated group in the immunoblot assay but a respective band was not detected for *A. pegreffii*-specific IgG2a in the ampicillin-treated group. Bands were detected as an unnamed protein and HSP70. HSP70 is a major immunodominant antigen in many parasitic infections and plays a key role in host-parasite interactions. Allergens associated with the HSP70 family have been found in a heterogeneous range of sources. Among others, HSP70 is an inhalant allergen for house dust mites, storage mites, biting midges, black flies, and cockroaches [35].

Similarly, in the 2-D immunoblot, numerous IgG1 spots but fewer IgG2a spots were observed in the ampicillin-treated group than in the untreated groups. Enolase is a major cross-reacting allergen in plants, fungi, and fish and has also been recognized as an allergen in cockroaches [36]. Hemoglobin, a novel major allergen of *Anisakis*, is a ubiquitous protein found in prokaryotes, fungi, plants, and animals [37]. *Anisakis* hemoglobin has been recognized by serum IgE levels in > 50% of prospectively studied patients with cutaneous features after *Anisakis* parasitism [37].

In this study, we used *A. pegreffii* to immunize mice as this is the most prevalent species infecting humans and fish in Korea [38]. Although only *A. simplex* is currently registered as an allergen by the WHO, studies have suggested that *A. pegreffii* can also induce allergies. In a study comparing the major allergens of *A. pegreffii* and *A. simplex*, epitopes and motifs were found to be conserved in both species for Ani s 1, 2, and 9 [39]. The similarities in the amino acid sequences of these allergens suggest potential similar health risks to humans. In our previous research, *A. pegreffii* induced allergic asthma in a mouse model [12].

The study has some limitations. We did not examine how the restoration of the mouse microbiome may contribute to the recovery of the antigen-immunization pattern. We also used pooled samples as the amount of sera from 1 mouse was insufficient for protein analysis. Consequently, in future studies, we plan to use other animal models to obtain enough sera to perform all experiments using individual samples. In addition, despite collecting 150 *Anisakis* specimens from a single fish and molecularly identifying 15 as *A. pegreffii*, we proceeded with immunological experiments on the remaining 135 specimens under the assumption they were also *A. pegreffii*. This approach overlooks the possibility that a single fish could be infected by multiple *Anisakis* species.

In conclusion, we observed that ampicillin treatment altered the gut microbiome of mice, altering the immune response to antigens from *A. pegreffii*. Thus, changes in the gut microbiome after ampicillin treatment affected the immune response to *A. pegreffii* antigens in mice. The findings have implications for developing new treatment strategies or vaccines against parasitic allergies.

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