의학석사 학위논문

Effect of IRT5 probiotics on dry eye in environmental dry eye mouse model

환경 건성안 쥐 실험 모델에서 IRT5 프로바이오틱스가 건성안에 미치는 영향에 대한 연구

2021년 8월

서울대학교 대학원

의학과 안과학 전공

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Abstract

Effect of IRT5 probiotics on dry eye in environmental dry eye mouse model

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Purpose : The purpose of this study was to investigate the clinical effects of IRT5 probiotics in the environmental dry eye mouse model.

Methods : 8-week-old male C57BL/6 mice were randomly divided into the following two groups; 1) control group (n = 16) was treated with oral gavage of 300 μL phosphate-buffered saline (PBS) alone once daily, 2) IRT5 group (n = 9) was treated with oral gavage of 1 x 10^9 CFU IRT5 probiotics powder mixed in 300 μL PBS once daily. Both groups were treated for 11 to 12 days with simultaneous dry eye induction of low humidity and intraperitoneal scopolamine injection (0.5 mg / 0.2 ml) thrice daily. Tear secretion, corneal fluorescein staining and conjunctival goblet cell density were evaluated. Quantitative real-time polymerase chain reaction for inflammation-related markers in cornea and conjunctiva, and extraorbital lacrimal gland was performed. 16S ribosomal RNA of fecal samples collected directly from

each mouse was analyzed for compositional differences, alpha and beta diversities.

Results: There was no difference in corneal fluorescein staining but a significant increase in tear secretion was observed in IRT5 group ($p < 0.001$). No significant difference in goblet cell density was observed. Cornea and conjunctiva exhibited increased TNF-α expression in IRT5 group ($p < 0.001$) whereas other inflammation related markers did not differ from control. IRT5 group possessed increased species diversity by Shannon index ($p = 0.041$). Beta diversity of genus by UniFrac principal coordinates analysis revealed significant distance ($p = 0.001$). Significant compositional differences were observed where several bacteria were associated with tear secretion. Multivariate linear regression analysis showed *Christensenellaceae* ($p = 0.009$), *Lactobacillus helveticus* ($p = 0.002$) and *PAC001797_s* ($p = 0.011$) to strongly influence tear secretion.

Conclusion: IRT5 probiotics supplementation increases tear secretion in the environmental dry eye mouse model. Tear secretion was found to be associated with and influenced by intestinal microbiome modification. These findings suggest that the intestinal microbiome may affect the lacrimal gland via mechanism other than inflammation regulation.

Keywords : Dry eye, Lacrimal gland, Microbiome, Probiotics, Tear secretion

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List of Abbreviations

BAFF, B cell activating factor

cDNA, complementary deoxyribonucleic acid

F/B ratio, *Firmicutes* / *Bacteroidetes* ratio

IFN, interferon

Ig, immunoglobulin

IL, interleukin

LDA, linear discriminant analysis

LEfSe, linear discriminant analysis effect size

MHC II, class II major histocompatibility complex

MMP9, matrix metallopeptidase-9

NEI score, National Eye Institute score

NOD, non-obese diabetic

OTU, operational taxonomic units

PBS, phosphate-buffered saline

PCR, polymerase chain reaction

RNA, ribonucleic acid

rRNA, ribosomal ribonucleic acid

SEM, standard error of mean

SFCA, short chain fatty acid

Th17, T helper 17

TNF, tumor necrosis factor

Chapter 1. Introduction

The dry eye disease is primarily aggravated by the evaporative water loss or decrement of tear production where both consequently lead to the hyperosmolar tissue damage of the ocular surface [1]. Several experimental dry eye disease studies have identified the dry eye disease association with the immune responses of the ocular surface, such as T helper 17 (Th17) cells, inflammation-related cytokines and chemokines, antigen presenting cells and inflammatory M1 phenotype macrophages [2–6]. Therefore, the main dry eye disease mechanism is the autoimmune based inflammation of the ocular surface [5, 7].

Over the past decade, eminent importance of the intestinal microbiome in possibly directly or indirectly affecting both local and systemic immunity has emerged and numerous studies have observed and identified their significance in human health and disease [8–10]. Particularly, intestinal dysbiosis has been found to be linked to affect several autoimmune diseases, such as Sjögren's syndrome and inflammatory bowel disease [11–14]. Furthermore, imbalance in intestinal microbiome has been observed to influence the ocular manifestations of

autoimmune diseases in both experimental models and clinical subjects [11, 15, 16]. Our previous clinical study observed significantly different intestinal microbiome of Sjögren's syndrome patients compared to that of normal subjects and that this intestinal dysbiosis was associated with clinical dry eye severity [17]. Interestingly, it was also noted that the environmental dry eye subjects' intestinal microbiome displayed features somewhere in between Sjögren's syndrome and normal subjects [17]. Therefore, the inflammatory immune reaction of dry eye disease may also be related to intestinal microbiome status [16].

Since the perception of intestinal microbiome's influence on immunity and relation to human health and disease, several studies have observed much promising clinical results from modification or normalization of intestinal microbiome by probiotics supplementations or fecal transplantation in various diseases [18–22]. IRT5 probiotics is a mixture of *Bifidobacterium bifidum*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri* and *Streptococcus thermophilus*, and was reported to have anti-inflammatory effects in several autoimmune models [20, 22, 23]. Recently, we found beneficial effects of IRT5 probiotics in attenuating the clinical manifestations in the autoimmune uveitis and autoimmune dry eye models

[24]. Moreover, we also observed that IRT5 probiotics influence immunity via downregulation of antigen-presenting related proteins [25].

A worldwide standardized management protocol for autoimmune or environmental dry eye syndrome is utilized and is constantly updated [26]. Still, there are patients who complain of insufficient symptom and/or sign relief despite extensive use of several medications and environmental modifications. Therefore, an establishment of a supplementary or additive management option which can aid in alleviating these residual needs may be beneficial. Herein, we report IRT5 probiotics' clinical effects in the environmental dry eye model.

Chapter 2. Materials and Methods

2.1. Animal

The protocol for this study was approved by the Institutional Animal Care and Use Committee of the Seoul National University Biomedical Research Institute (IACUC No. 18-0129-S1A0 and 19-0076-S1A0). All mice were managed in accordance with the Association for Research in Vision and Ophthalmology guidelines for the Use of Animals in Ophthalmic and Vision Research. All examination and sacrifice were performed under anesthesia by intramuscular injection of a mixture of xylazine (10 mg/kg) and tiletamine-zolazepam (30 mg/kg). All efforts to minimize suffering were conducted.

Male C57BL/6 mice 8-weeks of age (Koatech, Pyeongtaek, Republic of Korea) were used. All mice (n = 25) were bred under a specific pathogen-free environment and were maintained under an environment of 22–24℃ and relative humidity of 55% ± 5% with free access to water and food at the Mouse Facility at Biomedical Research Institute of Seoul National University Hospital (Seoul, Republic of Korea). Excretory feces from each cage were collected, minced, mixed together and re-

distributed to all cages in order for all mice to share excretory feces and simulate a co-housing environment. The overall health of all mice was monitored twice a week (weight and hair loss). The mice were randomly divided into two groups; the control group (n = 16) was treated with oral gavage of 300 μL phosphate-buffered saline (PBS) once daily, the IRT5 group (n = 9) was treated with oral gavage of 1×10^9 CFU IRT5 probiotics powder mixed in 300 μL PBS once daily. Both groups were treated for 11 to 12 days with simultaneous dry eye induction (Figure 1A). At the end of the study, all mice underwent euthanasia using compressed CO_2 gas, according to the American Veterinary Medical Association Guidelines for the Euthanasia of Animals: 2013 Edition.

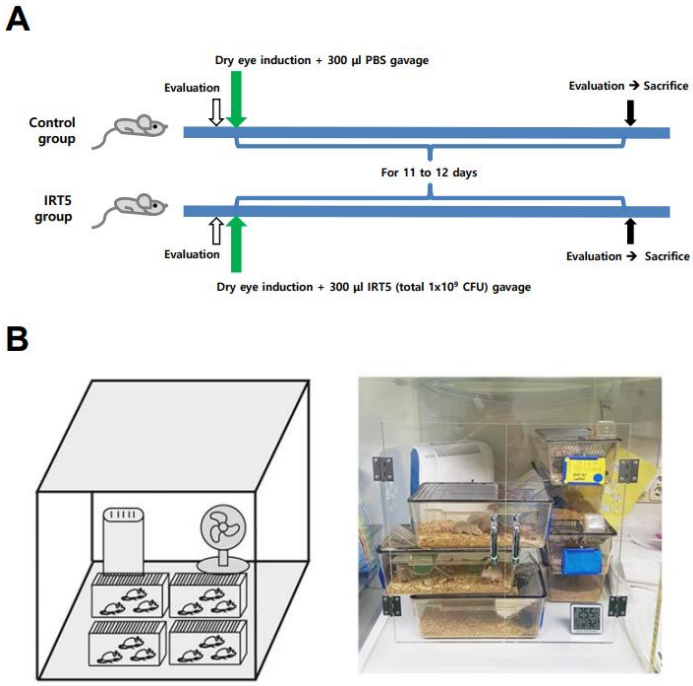

Figure 1. Environmental dry eye induction design. Eight-week-old male C57BL/6 mice were divided into control and IRT5 groups and underwent dry eye induction, which was composed of exposure to dry environment of the dry chamber and sterile intraperitoneal injection of scopolamine hydrobromide thrice daily, with oral gavage of either 300 μL PBS or 1 x 10^9 CFU IRT5 probiotics powder in 300 μL PBS for 11 to 12 days (A). All mice were placed in a dry chamber that had drafty low humidity (30–35%) all day during the 11 to 12 days' experiment (B). PBS, phosphate-buffered saline

2.2. Environmental dry eye induction

All 8-week-old male C57BL/6 mice underwent dry eye induction for 11 to 12

days (Figure 1A). The desiccating stress was induced by sterile intraperitoneal injection of scopolamine hydrobromide (Sigma, Saint Louis, USA) (0.5 mg / 0.2 ml) thrice daily with exposure to a drafty low humidity (30–35%) all day (Figure 1B).

2.3. Preparation of IRT5 probiotics mixture

IRT5 probiotics powder (1×10^9 CFU/g), which consists *L. casei, L. acidophilus, L. reuteri, B. bifidum,* and *S. thermophiles,* and contains 2×10^8 CFU/g of each strain, was kindly provided by doctor Young-Tae Ahn (Korea Yakult Co., Giheung, South Korea). IRT5 probiotics powder was mixed in PBS to contain 2×10^8 CFU of each five strains. The mixture was performed under the same method as previous past studies which was the most efficient way to sufficiently blend the powder in PBS and transport all strains to the intestine. [22-25]

2.4. Clinical evaluation

Tear secretion and corneal fluorescein staining evaluations were performed in all mice prior to dry eye induction and at the end of experiment before sacrifice (Figure 1A). Tear secretion determined with phenol red-impregnated cotton threads

(FCI Ophthalmics, Pembroke, USA) which were inserted into the lateral canthus of anesthetized (anesthesia with a mixture of xylazine and tiletamine-zolazepam at a ratio of 3: 1) mice for 60 seconds. The wet length of the thread was measured in millimeters.

Corneal fluorescein staining was evaluated after application of one drop of 0.5% fluorescein to the lower lateral conjunctival sac under cobalt light excitation. Corneal fluorescein staining was scored in a blind manner by one investigator (JM) using National Eye Institute score (NEI score) [27].

2.5 Conjunctival goblet cell assessment

The conjunctiva was excised and fixed in 10% formalin. The samples were sliced and stained using PAS staining kit according to the manufacturer's instruction. Mucin-filled goblets cells were counted in a blind manner by three investigators (JM, JSR and JYK). The results from three investigators were averaged and used for analysis based on the protocol from previous study [28, 29].

2.6 Quantitative real-time polymerase chain reaction

The cornea and conjunctiva, and extraorbital lacrimal gland were cut into small pieces and lysed in ribonucleic acid (RNA) isolation reagent. Since the immune response and dry eye-related changes occur simultaneously in both the cornea and the conjunctiva, the cornea and conjunctiva were mixed and analyzed together. After sonication with a probe sonicator (Ultrasonic Processor, Cole Parmer Instruments, Vernon Hills, USA), total RNA was extracted using RNeasy Mini kit (Qiagen, Venlo, Netherlands), and first-strand complementary deoxyribonucleic acid (cDNA) was synthesized by reverse transcription (High Capacity RNA-to-cDNA Kit, Applied Biosystems, Foster City, USA). Real-time amplification was performed by TaqMan Universal polymerase chain reaction (PCR) Master Mix (Applied Biosystems) in an automated instrument (ABI 7500 Real Time PCR System, Applied Biosystems) targeting tumor necrosis factor (TNF)-α (Mm00443258_m1, Thermo fisher, Waltham, USA), interferon (IFN)-γ (Mm01168134_m1, Thermo fisher, Waltham, USA), interleukin (IL)-1β (Mm00434228_m1, Thermo fisher, Waltham, USA), IL-6 (Mm00446190_m1, Thermo fisher, Waltham, USA), IL-17A (Mm00439618_m1, Thermo fisher, Waltham, USA), IL-8 (Mm04207460_m1, Thermo fisher, Waltham, USA), IL-10 (Mm00439614_m1, Thermo fisher, Waltham, USA), matrix

metallopeptidase-9 (MMP-9, Mm00442991_m1, Thermo fisher, Waltham, USA) for cornea and conjunctiva, and TNF-α (Mm00443258_m1, Thermo fisher, Waltham, USA), IFN-γ (Mm01168134_m1, Thermo fisher, Waltham, USA), IL-1β (Mm00434228_m1, Thermo fisher, Waltham, USA), IL-17A (Mm00439618_m1, Thermo fisher, Waltham, USA), class II major histocompatibility complex (MHC-II, Mm00439216_m1, Thermo fisher, Waltham, USA), B cell activating factor (BAFF, Mm00446347_m1, Thermo fisher, Waltham, USA) for extraorbital lacrimal gland.

2.7 Fecal microbiota 16S ribosomal RNA analysis

The fecal pellets from all mice were collected at the beginning and end of study. They were directly collected from the anus of each mouse by holding it and allowing defecation. The collected feces were immediately stored at -80℃ till analysis. Fecal samples were referred to Chunlab, Inc. (Seoul, Republic of Korea) for analysis. The ribosomal RNA (rRNA) analysis was performed at the V3 to V4 region of 16S rRNA in the same way as described in our previous study.[25] Compositional differences, alpha and beta diversities, and linear discriminant analysis (LDA) effect size (LEfSe) of intestinal microbiome were evaluated. Only those taxa that showed a

p value < 0.05 and a log LDA score ≥ 2 were ultimately considered for biomarker evaluation.

2.8 Statistical analysis

SPSS software version 22 (SPSS, Inc, Chicago, USA) and GraphPad software version 5 (GraphPad Software, San Diego, USA) were used. Outliers were excluded. Mann–Whitney U test was performed to compare clinical signs and inflammation-related markers between groups. Wilcoxon rank-sum test was performed to compare intestinal microbiome compositions between groups. Univariate and multivariate linear regression analysis were performed to determine the correlation between clinical signs and intestinal microbiome. The family and species variables with $p < 0.2$ observed in univariate linear regression analysis were included in multivariate linear regression analysis to assure all pertinent and potential predictive variables. P values less than 0.05 were considered statistically significant. The results are presented as mean ± standard error of mean (SEM) unless otherwise indicated.

Chapter 3. Results

3.1 IRT5 probiotics improves tear secretion

NEI score of the control group significantly increased indicating that dry eye induction was successful ($p < 0.001$, Figure 2A). Both groups exhibited increase in NEI score (Figure 2B). However, there was no difference in NEI score between groups (Figure 2C). Significant increase in tear secretion was observed in IRT5 group compared to control group ($p < 0.001$, Figure 2D). There was no significant difference regarding goblet cell density between groups ($p = 0.103$, Figure 2E and 2F).

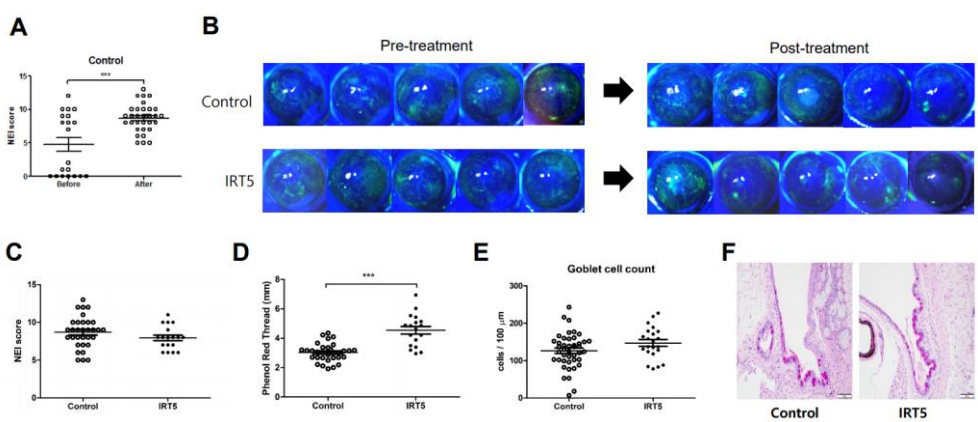

Figure 2. Clinical results. Significant NEI score increase in the control group indicated appropriate dry eye induction ($p < 0.001$) (A). Representative corneal fluorescein stain photos of 5 mice in each group are

shown (B). There was no difference in NEI score between groups (C). Significant increase in tear secretion was observed in IRT5 group ($p < 0.001$) (D). There was no difference in goblet cell density between groups ($p = 0.103$) (E and F). NEI score: National Eye Institute score. Statistical analysis with error bars indicating mean and SEM of data points by Mann–Whitney U test: *** $p < 0.001$.

3.2 IRT5 probiotics increases TNF-α expression in cornea and conjunctiva

Quantitative real-time PCR of cornea and conjunctiva showed an increased expression of TNF-α in IRT5 group ($p < 0.001$, Figure 3A). Other inflammation-related markers from cornea and conjunctiva did not show any difference (Figure 3A). Also, all inflammation-related markers from the extraorbital lacrimal gland were not different between groups (Figure 3B).

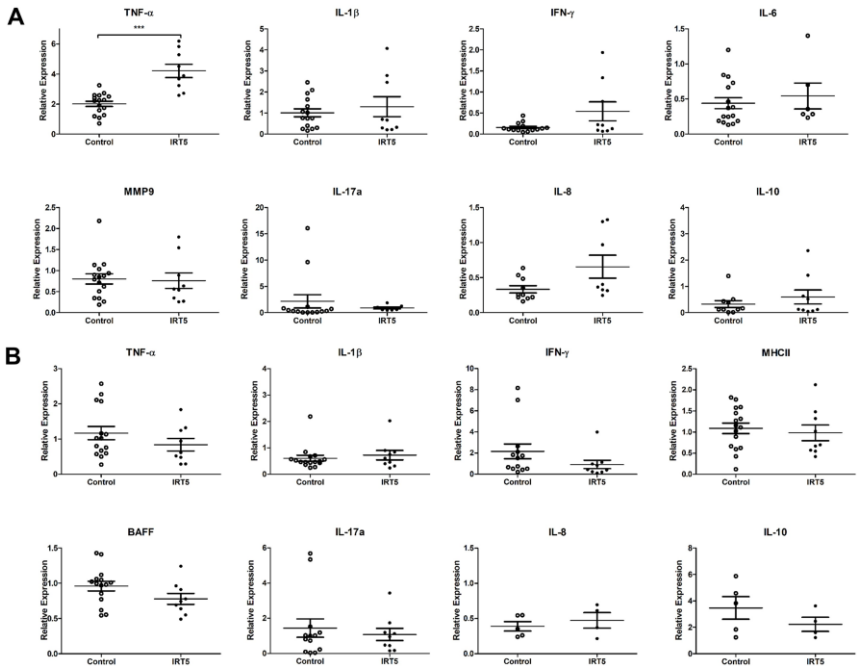

Figure 3. Quantitative real-time PCR of cornea and conjunctiva, and extraorbital lacrimal gland. Quantitative real-time PCR of inflammation-related markers' RNA transcripts was performed in cornea and conjunctiva (A), and extraorbital lacrimal gland (B). The results of quantitative real-time PCR of cornea and conjunctiva are shown in A. Increased expression of TNF-α in IRT5 group was observed ($p < 0.001$) whereas other markers did not differ from the control group (A). The results of quantitative real-time PCR of extraorbital lacrimal gland are shown in B. There was no difference in all inflammation-related markers between groups ($p > 0.05$) (B). Statistical analysis with error bars indicating mean and SEM of data points by Mann–Whitney U test: *** $p < 0.001$.

3.3 IRT5 probiotics increases intestinal microbiome diversity

The species richness according to Chao 1 index did not differ between groups (Figure 4A). There was significant increase in species diversity by Shannon index ($p = 0.041$, Figure 4B). Beta diversity of genus by UniFrac principal coordinates analysis revealed significant distance between groups ($p = 0.001$, Figure 4C).

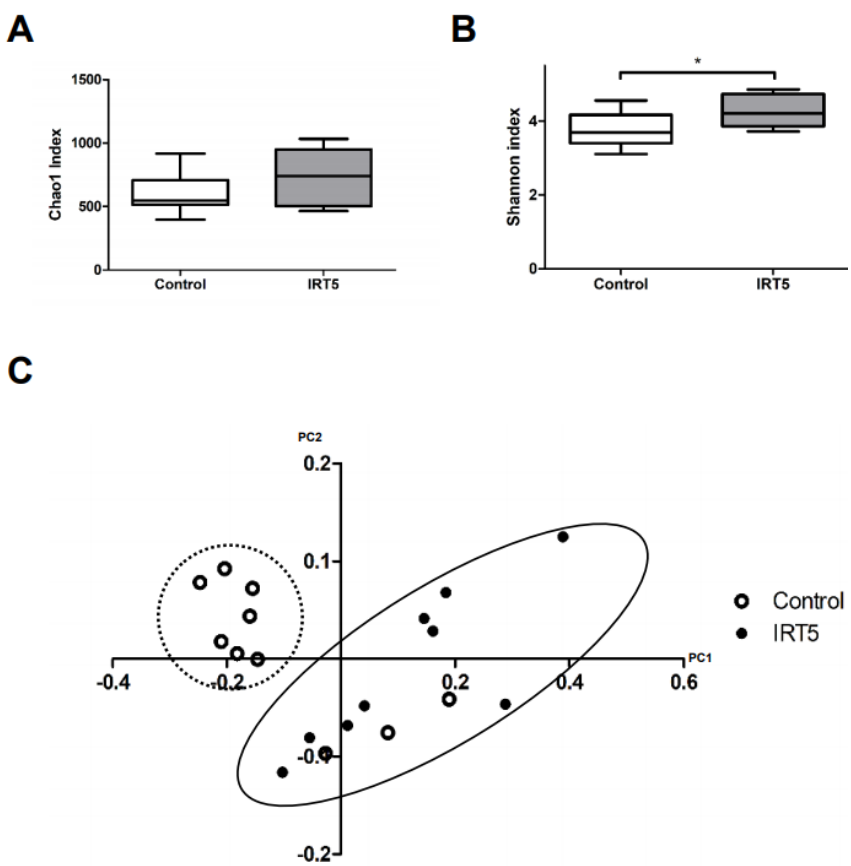

Figure 4. Alpha and beta diversity of intestinal microbiome. Species richness by Chao1 index was not different between groups ($p > 0.05$) (A). There was significant increase in Shannon diversity index in IRT5 group ($p = 0.041$) (B). Beta diversity of genus by UniFrac principal coordinates analysis revealed significant distance between IRT5 and control groups ($p = 0.001$) (C). Error bars indicate the minimum and maximum data points. Wilcoxon rank-sum test, * $p < 0.05$.

3.4 IRT5 probiotics modifies the intestinal microbiome composition

Significant compositional differences at the phylum level between groups were observed, such as *Verrucomicrobia*, *Bacteroidetes*, *Firmicutes* ($p < 0.05$, Figure 5A and 5B). Also, *Firmicutes* / *Bacteroidetes* (F/B) ratio was significantly increased in the IRT5 group ($p < 0.01$, Figure 5B). In the order level, *Clostridiales* was increased in the IRT5 group ($p = 0.009$, Figure 5C). In family, IRT5 group was observed to have reduced *Akkermansiaceae* ($p = 0.009$) and *Prevotellaceae* ($p = 0.014$), and increased *Christensenellaceae* ($p = 0.001$), *Ruminococcaceae* ($p = 0.018$), *Lachnospiraceae* ($p = 0.018$) (Figure 5C). In genus, IRT5 group showed a decrease in *Akkermansia* ($p = 0.009$), *Prevotella* ($p = 0.041$) and *Paraprevotella* ($p = 0.041$) (Figure 5C). *Lactobacillus* and *Bifidobacterium* were not different between groups (Figure 5C).

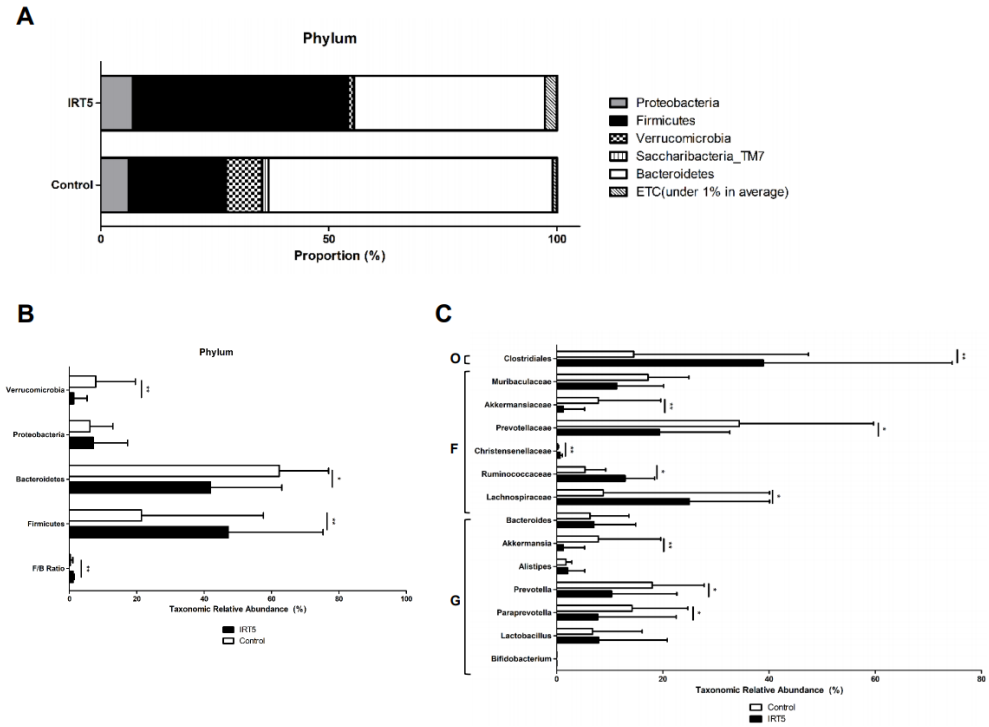

Figure 5. Compositional changes in intestinal microbiome. Compositional differences in phylum were observed (A). Decreased *Verrucomicrobia* (p = 0.009) and *Bacteroidetes* (p = 0.011), increased *Firmicutes* (p = 0.009) were observed in IRT5 group (A and B). The *Firmicutes* / *Bacteroidetes* (F/B) ratio was significantly increased in IRT5 group (p = 0.009) (B). In IRT5 group, the order *Clostridiales* was increased (p = 0.009) (C). In family level, IRT5 group had decreased *Akkermansiaceae* (p = 0.009) and *Prevotellaceae* (p = 0.014), and increased *Christensenellaceae* (p = 0.001), *Ruminococcaceae* (p = 0.018), *Lachnospiraceae* (p = 0.018) (C). In genus level, IRT5 group revealed to have decreased *Akkermansia* (p = 0.009), *Prevotella* (p = 0.041) and *Paraprevotella* (p = 0.041) (C). No significant

difference in *Lactobacillus* and *Bifidobacterium* between groups was observed (C). O: order, F: family, G: genus. Error bars indicate the minimum and maximum data points. Wilcoxon rank-sum test, * $p < 0.05$, ** $p < 0.01$.

LEfSE analysis revealed significant biological taxonomic differences between groups. 159 operational taxonomic units (OTUs) were different between groups (Table 1). Among them, 110 OTUs were more abundant and 49 OTUs were scarce in IRT5 group compared to control group (Table 1). Among the 86 OTUs at the species level, 61 OTUs were more abundant and 25 OTUs were scarce in IRT5 group (Table 1).

Table 1. Differences in abundance of microbials assessed by LEfSe

Taxon Name	Taxon Rank	Control	IRT5	LEfSE	P value
Increased					
Cyanobacteria	Phylum	0.21345	0.63812	3.32730	0.00192
Firmicutes	Phylum	21.41913	47.17320	5.10982	0.00898
Erysipelotrichi	Class	0.08382	0.24542	2.90799	0.01789
Vampirovibrio_c	Class	0.21293	0.63812	3.32783	0.00192
Clostridia	Class	14.50284	38.95078	5.08722	0.00898

Erysipelotrichales	Order	0.08382	0.24542	2.90799	0.01789
FR888536_o	Order	0.21293	0.63812	3.32783	0.00192
Clostridiales	Order	14.50284	38.95049	5.08721	0.00898
Clostridiaceae	Family	0.04628	0.10192	2.44869	0.02224
Mogibacterium_f	Family	0.06230	0.14836	2.63653	0.00705
Erysipelotrichaceae	Family	0.08382	0.24542	2.90799	0.01789
PAC000197_f	Family	0.03832	0.27374	3.07149	0.03345
FR888536_f	Family	0.21293	0.63812	3.32783	0.00192
Christensenellaceae	Family	0.17156	0.64033	3.37025	0.00082
Ruminococcaceae	Family	5.34587	12.88198	4.57613	0.01789
Lachnospiraceae	Family	8.79699	24.97943	4.90802	0.01789
PAC001525_g	Genus	0.00118	0.00345	2.02262	0.04711
PAC001377_g	Genus	0.00399	0.02251	2.00982	0.00547
PAC001270_g	Genus	0.00110	0.02541	2.11351	0.00620
PAC001609_g	Genus	0.00482	0.02614	2.04761	0.00302
PAC001219_g	Genus	0.01217	0.03305	2.04940	0.04451
PAC002042_g	Genus	0.01207	0.04280	2.18949	0.04804
JQ084194_g	Genus	0.00684	0.04304	2.26681	0.02730
PAC001524_g	Genus	0.00513	0.05026	2.35696	0.00142
PAC001440_g	Genus	0.01323	0.05139	2.29827	0.01784
PAC000672_g	Genus	0.01292	0.05205	2.29931	0.03345

Massilioclostridium	Genus	0.01635	0.06074	2.35459	0.01784
PAC001372_g	Genus	0.01690	0.06703	2.40319	0.02948
PAC001207_g	Genus	0.01195	0.07369	2.49419	0.04087
KE159797_g	Genus	0.01059	0.08051	2.54536	0.00796
Harryflintia	Genus	0.01362	0.08068	2.53086	0.00061
Arthromitus	Genus	0.04601	0.10068	2.44117	0.02224
PAC001402_g	Genus	0.04000	0.10705	2.53138	0.01789
PAC001386_g	Genus	0.00335	0.10960	2.72711	0.03393
Sporobacter	Genus	0.06899	0.14162	2.56508	0.01789
PAC001138_g	Genus	0.03238	0.15012	2.77136	0.01431
Acetatifactor	Genus	0.03244	0.17680	2.85915	0.02412
PAC000197_f_uc	Genus	0.00474	0.17708	2.93635	0.02307
PAC001360_g	Genus	0.05199	0.24266	2.97976	0.00082
Agathobaculum	Genus	0.04161	0.26270	3.04400	0.02468
PAC001199_g	Genus	0.08380	0.31337	3.06029	0.00548
Clostridium_g24	Genus	0.07475	0.31952	3.08810	0.00550
Alloprevotella	Genus	0.10236	0.33310	3.06269	0.03221
Anaerotruncus	Genus	0.11007	0.46174	3.24536	0.03376
FR888536_g	Genus	0.21293	0.63812	3.32783	0.00192
PAC001092_g	Genus	0.25754	0.99655	3.56777	0.02224
Pseudoflavonifractor	Genus	0.46942	1.43934	3.68581	0.01137

LLKB_g	Genus	0.23776	1.48360	3.79450	0.01784
PAC000664_g	Genus	0.56082	1.57273	3.70420	0.03376
PAC001525_s	Species	0.00118	0.00345	2.02262	0.04711
PAC001743_s	Species	0.00029	0.00411	2.07344	0.00093
PAC001070_s	Species	0.00739	0.01408	2.03992	0.03725
PAC001377_s	Species	0.00399	0.02251	2.00982	0.00547
EU772178_s	Species	0.00249	0.02580	2.09701	0.01812
EU511112_s	Species	0.00471	0.02587	2.08624	0.00338
PAC001713_s	Species	0.00371	0.02694	2.10685	0.00132
AB622833_s	Species	0.00279	0.02862	2.11894	0.00448
PAC001740_s	Species	0.00659	0.02929	2.08108	0.00796
PAC001369_s	Species	0.00607	0.02958	2.11277	0.00251
PAC001801_s	Species	0.00251	0.03075	2.15858	0.04698
PAC001785_s	Species	0.00000	0.03205	2.20867	0.00846
PAC001557_s	Species	0.00886	0.03299	2.12055	0.01442
JQ084476_s	Species	0.00426	0.03330	2.17125	0.01231
PAC002042_s	Species	0.00595	0.03365	2.14833	0.03052
PAC001360_g_uc	Species	0.00497	0.03482	2.19100	0.02797
FR888536_g_uc	Species	0.00862	0.03558	2.14653	0.02797
PAC001560_s	Species	0.00308	0.03586	2.22565	0.00077
PAC000183_s	Species	0.00371	0.03827	2.24671	0.00547

Neglecta timonensis	Species	0.00275	0.03861	2.26403	0.03830
PAC001547_s	Species	0.01328	0.03896	2.13674	0.02749
PAC001518_s	Species	0.01476	0.03902	2.10205	0.02218
EU455092_s	Species	0.00667	0.04078	2.24648	0.00142
PAC001574_s group	Species	0.00994	0.04176	2.21138	0.03650
JQ084194_s	Species	0.00478	0.04193	2.27752	0.01046
PAC001131_s	Species	0.01046	0.04471	2.25249	0.00695
PAC001742_s	Species	0.01176	0.04668	2.24596	0.02224
PAC002453_s	Species	0.00719	0.04827	2.31935	0.01346
PAC001524_s	Species	0.00485	0.04877	2.34531	0.00142
PAC001371_s	Species	0.01433	0.05328	2.29221	0.00427
Lactobacillus helveticus group	Species	0.00131	0.05526	2.44171	0.00019
PAC001222_s	Species	0.00349	0.05926	2.44848	0.00215
PAC001521_s	Species	0.01635	0.06043	2.35151	0.01784
PAC001372_s	Species	0.01377	0.06217	2.38781	0.03632
AB606300_s	Species	0.00167	0.06437	2.49928	0.01242
PAC001109_g_uc	Species	0.00384	0.06526	2.49545	0.04711
PAC001746_s	Species	0.01310	0.06602	2.42690	0.03299
PAC002505_s	Species	0.01136	0.07733	2.52039	0.00425
PAC001549_s	Species	0.00881	0.07825	2.54356	0.04451

PAC001366_s	Species	0.02072	0.08989	2.54016	0.03221
DQ777929_s	Species	0.01797	0.09289	2.57602	0.00703
AP012202_s group	Species	0.04601	0.10068	2.44117	0.02224
Pseudoflavonifractor_uc	Species	0.03337	0.10306	2.54478	0.04123
PAC002391_s	Species	0.02341	0.10610	2.61837	0.02681
PAC001386_s	Species	0.00335	0.10960	2.72711	0.03393
PAC002511_s group	Species	0.02425	0.12523	2.70505	0.04087
Flintibacter butyricus group	Species	0.03545	0.12924	2.67305	0.02749
PAC001501_s	Species	0.02083	0.15446	2.82579	0.01784
PAC001186_s group	Species	0.04174	0.15636	2.75908	0.02224
PAC001925_s	Species	0.01626	0.16201	2.86339	0.03041
PAC001083_s	Species	0.03858	0.16790	2.81137	0.00961
PAC001374_s	Species	0.02452	0.17386	2.87392	0.00542
KI535319_s	Species	0.05016	0.17931	2.81089	0.02749
PAC001540_s	Species	0.02586	0.18988	2.91482	0.04964
KE159628_s	Species	0.04247	0.24264	3.00089	0.01133
PAC001797_s	Species	0.07099	0.32413	3.10274	0.00656
PAC002479_s	Species	0.10236	0.33310	3.06269	0.03221
PAC002428_s	Species	0.00000	0.54749	3.43752	0.00846
KE159605_s	Species	0.28754	1.21172	3.66484	0.04904

AB606236_s	Species	0.09954	1.25150	3.76049	0.03658
PAC001120_s	Species	0.45587	4.02073	4.25104	0.03299
Decreased					
Saccharibacteria_TM7	Phylum	1.45117	0.52054	3.66785	0.03376
Verrucomicrobia	Phylum	7.83707	1.25239	4.51752	0.00898
Bacteroidetes	Phylum	62.26908	41.86771	5.00863	0.01137
Saccharimonas_c	Class	1.45117	0.52054	3.66785	0.03376
Verrucomicrobiae	Class	7.83707	1.25239	4.51752	0.00898
Bacteroidia	Class	62.23134	41.77948	5.00971	0.01137
Saccharimonas_o	Order	1.45117	0.52054	3.66785	0.03376
Verrucomicrobiales	Order	7.83707	1.25224	4.51753	0.00898
Bacteroidales	Order	62.23134	41.77948	5.00971	0.01137
Saccharimonas_f	Family	1.45117	0.52054	3.66785	0.03376
Akkermansiaceae	Family	7.83707	1.25224	4.51753	0.00898
Prevotellaceae	Family	34.37106	19.35734	4.87546	0.01431
PAC002448_g	Genus	0.02109	0.00086	2.12992	0.00016
PAC001097_g	Genus	0.20915	0.00090	3.01843	0.01093
Rikenella	Genus	0.37786	0.03747	3.23166	0.03345
Muribaculaceae_uc	Genus	0.27647	0.11905	2.89845	0.02749
PAC001066_g	Genus	0.41938	0.20502	3.03073	0.04123
PAC001692_g	Genus	0.62005	0.31384	3.18607	0.01137

PAC001112_g	Genus	0.72164	0.37758	3.23612	0.00550
PAC000677_g	Genus	1.45117	0.52054	3.66785	0.03376
Akkermansia	Genus	7.83707	1.25224	4.51753	0.00898
Prevotella	Genus	18.00512	10.35555	4.58262	0.04123
PAC000186_g	Genus	7.80951	3.71838	4.31084	0.01137
Paraprevotella	Genus	14.20985	7.74434	4.50959	0.04123
PAC001122_s	Species	0.21735	0.00000	3.03656	0.00754
PAC001097_s	Species	0.20888	0.00000	3.01973	0.00754
PAC001678_s	Species	0.00273	0.00000	2.02140	0.01779
PAC001127_g_uc	Species	0.00456	0.00000	2.12001	0.03935
PAC002009_s group	Species	0.05664	0.00016	2.45442	0.04779
EU791023_s	Species	0.03208	0.00024	2.21482	0.00055
AB606390_s	Species	0.01176	0.00086	2.17405	0.00081
PAC001063_g_uc	Species	0.00710	0.00103	2.14413	0.00745
AM265449_s	Species	0.03434	0.00962	2.11676	0.00656
Rikenella_uc	Species	0.13128	0.01287	2.77697	0.01346
PAC000670_s	Species	0.24658	0.02460	3.04673	0.04087
PAC001267_s	Species	0.07804	0.03255	2.36234	0.01789
PAC001359_s	Species	0.10696	0.04806	2.47640	0.01789
EU622763_s	Species	0.20781	0.05675	2.87883	0.01123
PAC002452_s	Species	0.18435	0.12669	2.46664	0.04123

PAC001075_s	Species	0.44461	0.24771	2.99619	0.02224
Prevotella_uc	Species	0.90398	0.25348	3.51242	0.01431
PAC002446_s	Species	0.57828	0.30332	3.13943	0.01789
Muribaculum intestinale	Species	0.60838	0.37439	3.06876	0.02749
EU474208_s	Species	3.53634	0.48182	4.18397	0.00033
PAC001192_s group	Species	1.44297	0.51294	3.66758	0.03376
PAC001064_s	Species	1.61391	0.75363	3.63374	0.03376
Akkermansia muciniphila	Species	7.83625	1.25224	4.51747	0.00898
AY239398_s	Species	12.73321	5.82063	4.53862	0.01137
FJ880724_s	Species	14.20353	7.74176	4.50934	0.04123

3.5 Tear secretion is associated with intestinal microbiome modification from IRT5 probiotics

Univariate linear regression analysis was performed with taxons at the level of family that were observed to have significant compositional and LEfSE differences. The taxons at the level of species with taxonomic relative abundance average of at least 5% or above (*Lactobacillus helveticus* was included despite low taxonomic relative abundance because it was the only *Lactobacillus* that significantly differed

between groups) and significant compositional differences between groups (Figure 6A) were also used for univariate linear regression analysis. At the family level, tear secretion showed significant positive association with *Mogibacterium*_f ($p = 0.007$), which belongs to the order *Clostridiales*, and *FR888536*_f ($p = 0.018$), which belongs to the phylum *Cyanobacteria* and class *Vampirovibrio*_c (Figure 6B). Also, at the species level, tear secretion was positively associated with PAC001797_s ($p = 0.035$), which belongs to the phylum *Cyanobacteria* and class *Vampirovibrio*_c, and inversely related to *EU474208*_s ($p = 0.008$), which belongs to the family *Muribaculaceae* (Figure 6C).

Multivariate linear regression analysis of those variables with $p < 0.2$ from univariate linear regression analysis was performed in a stepwise manner with group classification adjustment. As a result, family *Christensenellaceae* ($\beta = -0.608$, $p = 0.009$), and species *Lactobacillus helveticus* ($\beta = -0.676$, $p = 0.002$) and *PAC001797*_s ($\beta = 0.478$, $p = 0.011$), which belongs to the family *FR888536*_f, order *FR888536*_o and class *Vampirovibrio*_c, and phylum *Cyanobacteria*, were observed to have significant influence on tear secretion.

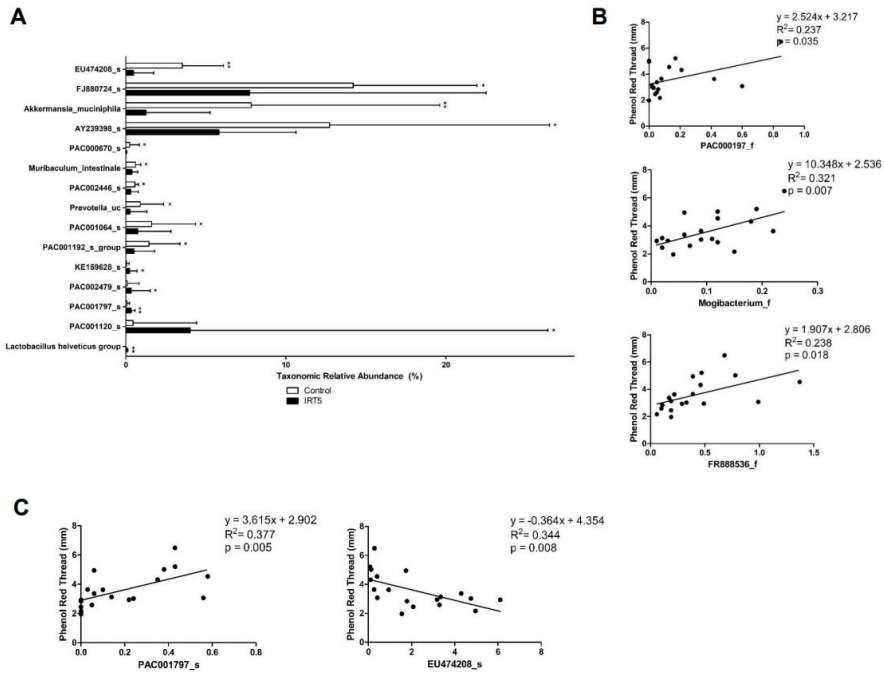

Figure 6. Univariate linear regression analysis between taxons of family and species, and tear secretion. Significant compositional difference in species was observed between groups (A). At the level of family, univariate linear regression analysis revealed tear secretion to have significant positive association with *Mogibacterium*_f (p = 0.007), which belongs to the order *Clostridiales*, and *FR888536*_f (p = 0.018), which belongs to the phylum *Cyanobacteria* and class *Vampirovibrio*_c (B). At the species level, tear secretion was positively associated with *PAC001797*_s (p = 0.035), which belongs to the phylum *Cyanobacteria* and class *Vampirovibrio*_c, and inversely related to *EU474208*_s (p = 0.008), which belongs to the family *Muribaculaceae* (C). Statistical analysis with error bars indicating the minimum and maximum data points by Wilcoxon rank-sum test: * $p < 0.05$, ** $p < 0.01$.

Chapter 4. Discussion

This study demonstrated that supplement with IRT5 probiotics may modify the intestinal microbiome and increase tear secretion in the experimental environmental dry eye model. This tear secretion increment did not show direct relation with inflammation regulation in extraorbital lacrimal gland nor cornea and conjunctiva. This suggests that IRT5 probiotics supplementation possesses only partial effects in environmental dry eye syndrome, whereas it exhibits more significant clinical and immunological effects in autoimmune related dry eye syndrome seen in previous studies.

IRT5 group had a significantly different intestinal microbiome compared to control group. Past studies suggest that reduction in intestinal microbiome diversity influence the ocular surface by promoting autoimmunity through the loss of short chain fatty acid (SFCA) producing commensal flora and inducing inflammation [15, 30]. Animal studies treated with antibiotics observed decrease in intestinal normal flora and diversity which were associated with impairments in the ocular surface that could be reversed with fecal transplantation or probiotics supplementation [19,

31, 32]. Allansmith et al., observed that the number of cells containing immunoglobulin (Ig)A and IgM in lacrimal glands were decreased in which tear IgA levels were also low in germ-free rats and these levels increased when these mice were relocated to a conventional environment [33]. Kudagas et al., found that gut supplementation with *B. acidifaciens* elevates IgA transcript levels in germ-free mice [31]. These findings suggest the existence of gut-eye-lacrimal gland-microbiome axis which indicates the indirect effects from intestinal microbiome to the eye and lacrimal gland [16]. In our study, IRT5 probiotics treated group displayed significantly increased intestinal microbiome diversity (Shannon Index, $p = 0.041$) with different intestinal microbiome compositions (Beta diversity, $p = 0.001$). Also, IRT5 group demonstrated better tear secretion ($p < 0.001$) with significant association with and influence from microbiome changes compared to control group.

IRT5 group exhibited relatively elevated SFCA-producing bacteria. Fecal analysis showed that IRT5 group had increased *Firmicutes* ($p < 0.01$) which also extends to the increase in F/B ratio compared to control group ($p < 0.01$). Increased F/B ratio is known to be strongly associated with augmented SFCA production because most SFCAs are made by bacteria from the phylum *Firmicutes* [34]. In family,

Ruminococcaceae, *Lachnospiraceae* and *Christensenellaceae*, which are families belonging to the phylum *Firmicutes*, were increased in the IRT5 group ($p < 0.05$). *Ruminococcaceae* and *Lachnospiraceae* are largely known important SFCA and lactic acid producing bacteria [34, 35]. Also, *Ruminococcaceae* is negatively associated with inflammation and is known to regulate lipid profile. Moreover, some species of *Lachnospiraceae* possess anti-inflammatory properties through butyrate production which is one of the main SFCA [36]. *Christensenellaceae*, a ubiquitous micro-organism among animals including human and also a SFCA-producing bacteria, is known to be related to the healthy gut status, longevity and normal body mass index [37]. Though the IRT5 probiotics is mainly composed of *Lactobacillus* species, they did not differ significantly between IRT5 and control groups, although it was slightly increased in IRT5 group. This result may indicate that the bacteria composing the IRT5 probiotics may not directly affect the gut-eye-lacrimal gland-microbiome axis but may more likely act as a coordinator to provide a better environment that encourages growth and function of beneficial bacteria. In addition, this minimal increase of *Lactobacilli* can be caused by desiccating stress the mice were under in which stress was reported to be associated with the reduction in *Lactobacilli* [38].

Though IRT5 probiotics treated environmental dry eye model has shown equally increased tear secretion similar to the autoimmune dry eye model (NOD.B10.*H2*^{b}), much incongruity in results between these two models after IRT5 probiotics supplement are observed [24, 25]. Additional comparison of intestinal microbiome between environmental dry eye model and NOD.B10.*H2*^{b} mice, the autoimmune dry eye model, was performed before and after IRT5 probiotics supplement (Figure 7). We have observed significant beta diversity difference before supplementation (Figure 7A, p = 0.001). Although NOD.B10.*H2*^{b} mice received IRT5 probiotics supplement for 3 weeks whereas the environmental dry eye model only received IRT5 probiotics for 11 to 12 days, beta diversity analysis revealed significant distance between the two groups after supplementation (Figure 7B, p = 0.001). Also, significant compositional differences were observed (Figure 7C). The phylum *Firmicutes* (p = 0.463), *Bacteroidetes* (p = 0.947) and their F/B ratio (p = 0.739) did not differ between groups. However, after IRT5 probiotics supplement, the environmental dry eye model exhibited increased phylum *Proteobacteria* (p = 0.003), family *Prevotellaceae* (p = 0.006) and *Christensenellaceae* (p = 0.006), and genus *Bacteroides* (p = 0.006) and *Prevotella* (p = 0.009) compared to NOD.B10.*H2*^{b} (Figure

7C). On the contrary, NOD.B10.*H2*^{b} revealed increased family *Muribaculaceae* (p = 0.003) and, genus *Bifidobacterium* (p = 0.003) and *Lactobacillus* (p = 0.004) (Figure 7C). NOD.B10.*H2*^{b} had increased *Lactobacillus reuteri* (p = 0.020), a composition of the IRT5 probiotics. Additionally, NOD.B10.*H2*^{b} exhibited increased species *Bifidobacterium pseudolongum* (p = 0.003), *Lactobacillus gasseri* (p = 0.003), *Lactobacillus hamster* (p = 0.003), *Lactobacillus helveticus* (p = 0.014) and *Lactobacillus paracasei* (p = 0.003).

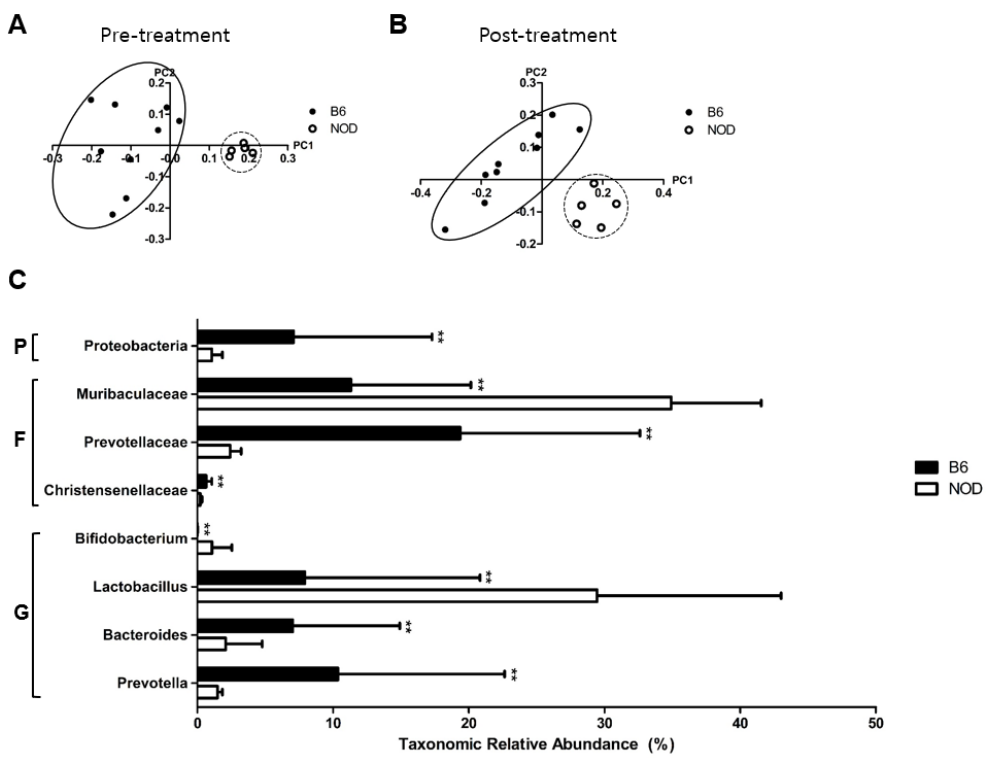

Figure 7. Beta diversity and compositional difference between environmental dry eye

C57BL/6 mouse model and NOD mouse model. Beta diversity of genus by UniFrac principal coordinates analysis revealed significant differences before (A) and after (B) IRT5 probiotics treatment (both p = 0.001). Compositional differences of intestinal microbiota after IRT5 probiotics between groups were observed (C). In phylum, *Proteobacteria* was increased in environmental dry eye model (p = 0.003) (C). In family, decreased *Muribaculaceae* (p = 0.003) and increased *Prevotellaceae* (p = 0.006) and *Christensenellaceae* (p = 0.006) were observed in environmental dry eye model (C). In genus, decreased proportions of *Bifidobacterium* (p = 0.003) and *Lactobacillus* (p = 0.004) were observed, while *Bacteroides* (p = 0.006) and *Prevotella* (p = 0.009) increased (C). B6: Experimental dry eye model C57BL/6, NOD: Sjögren's syndrome mouse model (NOD.B10.$H2^b$), P: phylum, F: family, G: genus. Error bars indicating the minimum and maximum data points by Wilcoxon rank-sum test: ** p < 0.01.

Although NOD.B10.$H2^b$ received IRT5 probiotics for 3 weeks, which is much longer than the current study of 11 to 12 days, this intestinal microbiome difference may be caused by the preexisting genetical difference that contributes to disparate intestinal environments allowing certain species to survive and proliferate while others cannot. Human clinical studies have also observed intestinal microbiome dissimilarity between Sjögren's syndrome and non-Sjögren's syndrome or

environmental dry eye subjects, which indicates this preexistence of distinct intestinal microbiome and environment before disease infliction [17, 39]. Another explanation for the different clinical response to IRT5 probiotics between environmental dry eye and Sjögren's syndrome autoimmune dry eye model may be that different immune cells are involved in each disease. While autoimmunity has substantial relation with B cells [40], the intestinal microbiome greatly affects the diversity of B cell clones and ultimately controlling B cell related chronic inflammations [41, 42]. On the other hand, environmental dry eye disease is an auto-inflammatory disease that is more associated with T cells, such as Th17 or CD4 or CD8 T cells, and therefore intestinal microbiome influence on B cells may be insufficient to produce significant clinical responses in this type of dry eye disease [2, 4]. Therefore, the presence of autoimmunity seems to affect the clinical response from IRT5 probiotics on dry eye disease.

Numerous studies have demonstrated the existence of a bidirectional microbiome-gut-brain axis [43–45]. The intestinal microbiome communicates with the central nervous system mainly through microbial-derived intermediates that can not only directly interact with enteroendocrine cells and mucosal immune system,

but also indirectly influence the nervous system by crossing the intestinal barrier and entering systemic circulation [38]. Several neurotransmitters and neuropeptides, such as neuropeptide Y and substance P, in relation to intestinal microbiome are reported to influence the central and vagal nervous systems [46–48]. Neuropeptide Y, one of the main factors in microbiome-gut-brain axis, may be related with the gut microbiota on inflammatory regulation and brain functions [48]. Also, SFCA produced by intestinal microbiota can directly induce the release of peptide YY from enteroendocrine cells [48]. The depletion of certain intestinal microbiomes alone can directly stimulate vagal neurons and cause firing of sympathetic neurons which was reported to be reversed with fecal transplantation or supplementation of specific microorganisms [45]. In the same concept, the lacrimal gland is innervated by both sympathetic and parasympathetic nerves, where the latter mainly controls tear secretion [49, 50]. In this study, only TNF-α in cornea and conjunctiva of IRT5 group increased, while other inflammation-related markers did not. TNF-α has been reported to increase in the intestine when dysbiosis or inflammation or infection is present [51, 52]. Also, depending on cellular conditions, TNF-α is known to be involved in both cell survival and cell death [52]. Therefore, this TNF-α increase response in IRT5 group compared to the control group may be associated with intestinal microbiome modifications toward inflammation of the ocular surface

rather than anti-inflammation, or may be an indication of cellular regulation in the cornea and conjunctiva of either survival or death. Further studies regarding this peculiar finding are necessary. Also, additional univariate and multivariate linear regression analysis between goblet cell density and gut microbiome performed in the same manner as this study revealed goblet cell density to have inverse correlation with only the species *PAC001064*_s ($p = 0.035$), *PAC002446*_s ($p = 0.039$) and *PAC000670*_s ($p = 0.041$), while no significance was observed from multivariate linear regression analysis. Altogether, these findings may indicate that the modified intestinal microbiome from IRT5 probiotics in environmental dry eye model indirectly or possibly directly affects the eye or lacrimal gland via different mechanisms other than the regulation of inflammation. Change in intestinal microbiome through IRT5 probiotics may subsequently alter the release of certain gut microbial-related neuropeptides, or the compositional change of certain microorganisms itself could affect the parasympathetic nerve innervating the lacrimal gland to increase tear secretion. While multivariate linear regression analysis revealed tear secretion to be strongly influenced by the family *Christensenellaceae* and species *Lactobacillus helveticus* and *PAC001797*_s, *Christensenellaceae* and *Lactobacillus helveticus* are known SFCA-producing bacteria. Though under insufficient discovery, species *PAC001797*_s belongs to the phylum

Cyanobacteria which is known to accumulate SCFAs under certain conditions through a yet unknown mechanism, and was observed to have significant impact on tear secretion confirmed by both univariate and multivariate linear regression analysis. Therefore, compositional changes of these certain bacteria may directly affect the parasympathetic nerve or may take part in facilitation of neuropeptides release, such as peptide YY, which subsequently affect the nervous system. Further investigations elucidating these possible mechanisms and future studies to discover the properties of specific bacteria are warranted.

There are some limitations to this study. Though several studies focus on the microbiome's influence through immunomodulating cells, we did not perform any proteomics study nor other cellular level studies. However, through previous studies have already observed that IRT5 probiotics reduces $CD8^+$ interferon-γ^{hi} cells and increases regulatory T cells [24]. In addition, IRT5 probiotics was found to induce downregulation of proteins associated with defense response and immune system process [25]. Although IRT5 probiotics was observed to regulate inflammation through immune cells, the environmental dry eye model from this study exhibited little relevance with inflammation control. Therefore, future studies with probiotics to elucidate the specific mechanism of action are necessary. In addition, this study was performed using only male mice in order to investigate the

probiotics' sole effects in environmental dry eye by excluding possible confounding factors such as hormonal effects from female mice. Indeed, dry eye is more common in female subjects. Therefore, future studies regarding probiotics and desiccating stress induced in female mice models may be clinically helpful. Also, the number of mice studied in the IRT5 group was relatively smaller than the control group, in which the effects of probiotics could have been more prominent had there been more mice in the IRT5 group. However, with ethical restriction in the number of mice that can be used and in consideration that nine mice is not too small, the IRT5 group in this study still applies as a relative representative of probiotics' effects. Nevertheless, future studies with a larger group may help illuminate the effects of probiotics that may have been subtle from this study. Another limitation is that this study did not include a negative control. Although a negative group was present at the beginning of this study, only clinical data comparison was performed without microbiome analysis due to the limited number for negative group of only 4 mice. However, a past study with female C57BL/6 mice aged 6–8 weeks under desiccating stress with drafty environment settings and scopolamine injection has already confirmed different intestinal microbiome in dry eye induced mice compared to negative controls [15]. Increased OTUs and Shannon diversity index, and significant beta diversity difference were observed in these dry eye induced mice compared to

negative controls [15]. Additionally, clinical data confirmed that adequate dry eye induction was present with significantly lower NEI score and better tear secretion in the negative group (Figure 8A and 8B). Goblet cell count did not differ among all groups (Figure 8C). Additionally, among all inflammation-related markers only TNF-α and IFN-γ in the cornea and conjunctiva were observed to significantly differ among groups (Figure 8D and 8E).

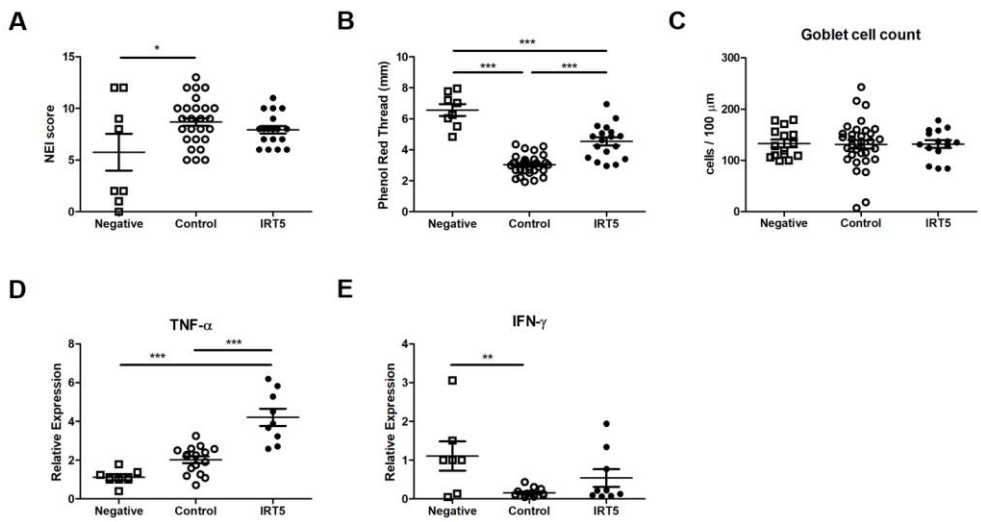

Figure 8. Clinical and inflammation-related marker comparison from negative group. The control group exhibited significantly higher corneal fluorescein staining scores compared to negative group ($p < 0.05$) (A). There were significant differences regarding tear secretion among groups where negative group had better phenol red thread test results ($p < 0.001$) (B). Goblet cell count did not differ among groups ($p > 0.05$) (C). The IRT5 group displayed increased TNF-α expression in the cornea and

conjunctiva compared to both negative and control groups ($p < 0.001$) (D). The expression of IFN-γ was increased in the cornea and conjunctiva of negative group compared to control group ($p < 0.01$) (E). Statistical analysis with error bars indicating the standard error of mean by Analysis of variance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

TNF-α was the lowest in the negative group which suggests that IRT5 probiotics treatment may be associated with possibly increasing ocular surface inflammation rather than reducing it. Also, fecal analysis alone lacks the ability to fully represent the whole intestinal microbiome. Microbiome can change according to location within the intestinal tract. Another limitation is that the OTUs' were analyzed at a cutoff value of 97%. There may be some microbials sharing more than 97% of the entire 16S rRNA. Also, several studies have seen that mice from laboratory bred and wild living have divergent microbiota which consequently may show different responses to treatments [53, 54]. Therefore, conventional laboratory bred mice may only have limited ability to predict complex physiological responses. Further studies including wild or wildling mice may be necessary. Lastly, we analyzed intestinal microbiome composition, alpha and beta diversities but not their functional properties. The microbiome creates and works inside a network where one function is not solely dependent on one type of microorganism but rather several

microorganisms together. Further studies regarding the functional properties of intestinal microbiome and their effects on ocular surface should be conducted.

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국문 초록

환경 건성안 쥐 실험 모델에서
IRT5 프로바이오틱스가 건성안에 미치는 영향에 대한 연구

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문자윤

목표: 본 연구를 통하여 환경 건성안 마우스 모델에서 IRT5 프로바이오틱스의 임상 효과를 조사하고자 하였다.

방법: 8 주령 수컷 C57BL/6 마우스를 무작위로 다음 두 그룹으로 나누었다; 1) 대조군 (n = 16)은 300 μL 인산 완충 식염수 단독으로 매일 1 회 경구 투여하고 2) IRT5 그룹 (n = 9)은 1 x 10^9 CFU IRT5 probiotics 분말을 인산 완충 식염수에 녹인 300 μL 혼합액 투여했다. 두 그룹 모두 11 ~ 12 일 동안 투여하였으며 낮은 습도의 환경과 복강 내 스코폴라민 주사 (0.5mg / 0.2ml)를 매일 3 회 처치하였다. 눈물 분비, 각막 플루오레세인 염색 및 결막 술잔 세포 밀도를 평가했다. 각막과 결막, 안와 외 눈물샘의 염증 관련 마커에 대한 정량적 실시간 중합 효소 연쇄 반응을 수행했다. 각 마우스에서 직접 수집 한 대변 샘플의 16S 리보솜 RNA를 분석하였으며 마이크로바이옴 구성 차이,

알파 및 베타 다양성에 대해 분석했다.

결과: 각막 플루오레세인 염색에는 차이가 없었으나 IRT5 군에서 눈물 분비의 유의 한 증가가 관찰되었다 ($p < 0.001$). 결막 술잔 세포 밀도에서 유의한 차이는 관찰되지 않았다. IRT5 그룹에서 각막과 결막의 증가된 TNF-α 발현을 보인 반면 ($p < 0.001$) 다른 염증 관련 마커는 대조군과 다르지 않았다. IRT5 그룹은 Shannon 지수에 의해 증가된 종 다양성이 관찰되었다 ($p = 0.041$). UniFrac 주 좌표 분석에 의한 속의 베타 다양성은 유의한 간격을 보였다 ($p = 0.001$). 두 군간 구성 차이가 관찰되었으며 여러 박테리아가 눈물 분비와 관련이 있는 것으로 나타났다. 다변량 선형 회귀 분석에서 Christensenellaceae ($p = 0.009$), Lactobacillus helveticus ($p = 0.002$) 및 PAC001797_s ($p = 0.011$)가 눈물 분비에 강한 영향을 미치는 것으로 나타났다.

결론: IRT5 프로바이오틱스 보충은 환경 건성안 마우스 모델에서 눈물 분비를 증가시키는 것으로 나타났다. 눈물 분비는 장내 마이크로바이옴의 변화와 연관되어 있으며 이로부터 영향을 받는 것으로 관찰되었다. 이러한 결과는 장내 마이크로바이옴이 염증 조절 이외의 메커니즘을 통해 눈물샘에 영향을 미칠 수 있음을 시사합니다.

주요어: 건성안, 눈물샘, 눈물 분비, 마이크로바이옴, 프로바이오틱스

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