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# Dry Mouth, Focal Lymphocytic Sialadenitis, and Oral Dysbiosis in IκB-ζ-Deficient Mice

## IκB-ζ 결핍 쥐에서 구강건조증과 림프구성 타액선염의 발생 및 구강세균총의 변화

2019년 8월

서울대학교 대학원

치의과학과 면역 및 분자미생물치의학 전공

이 준 호

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지도교수 최 영 님

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치의과학과 면역 및 분자미생물치의학 전공

이 준 호

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## Abstract

# Dry Mouth, Focal Lymphocytic Sialadenitis, and Oral Dysbiosis in IκB-ζ-Deficient Mice

Jun Ho Lee

Deparment of Dental Science

Major in Immunology and Molecular Microbiology

The Graduate School, Seoul National University

(Supervised by Professor Youngnim Choi, D.D.S., Ph.D.)

## Background

Sjögren syndrome (SS) is a systemic autoimmune disease characterized by dryness of eyes and mouth. Etiopathogenetic mechanisms of SS remain elusive because numerous factors contribute to the progression of SS. It is believed that the cause of SS involves a combination of genetic and environmental factors. Mice lacking IκB-ζ, a protein encoded by

*Nfkbiz* gene, would be an appropriate animal model for SS because genetic and environmental factors are involved in the model. IκB-ζ-deficient epithelial cells cause changes in the transcriptional activity of NF-κB, leading to increased caspase-3 processing and apoptosis. Damaged epithelia fail to protect from the external environment. It is more accessible to encounter the pathogens and their substances. Moreover, a previous study demonstrated that IκB-ζ-deficient mice spontaneously develop SS-like autoimmune disease, resulting in increased autoantibody production, reduced tear secretion and lymphocyte-infiltrated dacryoadenitis. Although the previous study reported no symptom in the salivary glands of IκB-ζ-deficient mice, reduced salivary rates and sialadenitis were observed in my animal facility.

## Methods

IκB-ζ-deficient mice were bred and maintained under specific-pathogen-free condition in Laboratory Animal Facility at the School of Dentistry, Seoul National University. Five of each *Nfkbiz*$^{+/+}$, *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ mice were used in the experiment. Salivary glands and oral microbiota were obtained at 24 weeks. Salivary flow rate was measured for 10 min after pilocarpine injection. Salivary glands were harvested, fixed with 10% neural formalin solution, and embedded with paraffin. They were

subjected to staining with hematoxlin-eosin (H&E) or *in-situ* hybridization using a universal probe for bacterial 16s ribosomal RNA (rRNA). Focal lymphocytic sialadenitis (FLS) and bacterial invasion were examined under a microscope. The other pieces of salivary glands were embedded in OCT compound and then frozen in liquid nitrogen. The sections were stained with anti-mouse CD3-ζ and anti-mouse B220 antibodies. Immunofluorescence images were captured under a confocal microscope. Oral microbiota was collected with clean cotton swabs. Genomic DNA isolated from oral swab was subjected to microbiota analysis by high throughput sequencing.

## Results

Both *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ mice produced less saliva than *Nfkbiz*$^{+/+}$ mice in response to pilocarpine injection. FLS area and foci score tended to increase in *Nfkbiz*$^{-/-}$ mice compared to other genotypes, but statistical significance was not achieved. Importantly, three out of five IκB-ζ-deficient mice developed FLS with score ≥ 1. Immunohistofluorescent detection with anti-mouse CD3-ζ and anti-mouse B220 antibodies demonstrated that infiltrating lymphocytes were mostly T cells in the early stage. Afterwards, there was a large mass of B cells occupying salivary glands. In addition, *in-situ* hybridization using a universal probe for 16S rRNA

revealed that bacterial invasion of ductal cells occurs in submandibular glands. Oral microbiota was analyzed in *Nfkbiz*$^{+/+}$, *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ mice. In alpha diversity, Simpson (measure of evenness) and Shannon (measure of species richness and evenness) indexes showed significant differences between *Nfkbiz*$^{+/+}$ mice and *Nfkbiz*$^{-/-}$ mice. Beta diversity revealed that oral bacterial communities in *Nfkbiz*$^{+/+}$ mice and *Nfkbiz*$^{+/-}$ mice clustered closely; on the other hand, oral bacterial communities in *Nfkbiz*$^{-/-}$ mice were separated from those of other genotypes. While *Streptococcus danieliae* predominated the oral microbiota in *Nfkbiz*$^{+/+}$ and *Nfkbiz*$^{+/-}$ mice, it was substantially decreased in *Nfkbiz*$^{-/-}$ mice. Instead, *Staphylococcus sciuri group, Escherichia coli group, Enterococcus faecium group* and *Corynebacterium mastitidis group* were significantly increased in *Nfkbiz*$^{-/-}$ mice compared to *Nfkbiz*$^{+/+}$ mice. Surprisingly, those bacteria higher in *Nfkbiz*$^{-/-}$ mice significantly increased in FLS score of ≥ 1. *Nfkbiz*$^{+/-}$ mice had unique sets of oral bacteria, which were *Lactobacillus mudanjiangensis, Lactobacillus parabrevis group, Lactobacillus brevis*, *Lactobacillus plantarum group, Staphylococcus aureus group, Cupriavidu metallidurans* and *Lactobacillus_uc*. Those oral bacteria were also significantly higher in the groups that were below the salivary flow rate of 7.8 (mean).

## Conclusion

In conclusion, *Nfkbiz*$^{-/-}$ mice spontaneously developed dry mouth, FLS, and dysbiosis of oral microbiota. Oral microbiota analysis demonstrated that the groups *Staphylococcus sciuri, Escherichia coli, Enterococcus faecium,* and *Corynebacterium mastitidis* may be associated with FLS. These findings may provide valuable information pertaining to the combined effects of host genotype and oral microbiota acting in conjunction with environmental factors to develop dry mouth and FLS.

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**Keywords: Sjögren's syndrome (SS), focal lymphocytic sialadenitis (FLS), salivary flow rate, oral microbiota, IκB-ζ-deficient mice**

**Student Number: 2017-25212**

# CONTENTS

# 1. Introduction

Sjögren syndrome (SS) is a systemic autoimmune disease characterized by dryness of eyes and mouth [1]. Etiopathogenetic mechanisms of SS have been elusive because numerous factors contribute to the progression of SS. It is believed that the cause of SS involves a combination of genetic and environmental factors [2]. Mice lacking IκB-ζ, a protein encoded by *Nfkbiz* gene, would be an appropriate animal model for SS because genetic and environmental factors are involved in the model. IκB-ζ-deficient epithelial cells cause changes in the transcriptional activity of NF-κB, leading to increased caspase-3 processing and apoptosis [3]. Damaged epithelia fail to protect from the external environment. It is more accessible to encounter the pathogens and their substances.

Primary Sjögren syndrome (pSS) is diagnosed by specific diagnostic criteria. Systemic complications such as dry skin, fatigue and pain, which are present in 30% to 40% of patients, may provide the first clues to the disease [1]. However, the inclusion criteria are symptoms of ocular or oral dryness [4]. The patients who have at least one symptom are subjected

to diagnostic tests included in the 2016 ACR-EULAR Classification Criteria for pSS (Table 1). They include sialadenitis, dacryoadenitis, autoantibody production, decrease in tear and saliva production [4]. A previous study demonstrated that IκB-ζ-deficient mice spontaneously develop SS-like autoimmune disease, resulting in increased autoantibody production, reduced tear secretion and lymphocyte-infiltrated dacryoadenitis, thus satisfies the 2016 ACR-EULAR Criteria [5]. Although the previous study reported no symptom in the salivary glands of IκB-ζ-deficient mice, reduced salivary rates and sialadenitis were observed in my animal facility.

Focal lymphocytic sialadenitis (FLS), peri-ductal infiltration of mononuclear cells in salivary glands is an important diagnostic criterion for SS. Functional impairment of salivary glands is caused by an autoimmune attack of epithelial tissues by infiltrating lymphocytes [6]. Most infiltrating lymphocytes are activated T cells in early stage [7]. However, B cells dominate in late stages. B and T cells can form ectopic germinal center-like structures that function similar to secondary lymphoid organs [7]. The germinal center-like structures can initiate local T and B cell responses and serve as a reservoir of memory lymphocytes [8]. The

presence of GC-like structures is associated with increased autoantibody production [9].

The association between oral bacteria and autoimmune disease has not been fully elucidated. Oral cavity provides the portal of entry for bacteria in the environment to access the host. Previous studies also suggested that bacterial infection play a role in the induction autoimmune disease through molecular mimicry mechanism [10]. In this experiment, I aimed to determine the combined effects of host genotype and oral microbiota as an environmental factor on salivary flow rate and FLS.

**Table 1. 2016 ACR-EULAR Classification Criteria for Primary Sjögren's syndrome.**

| Item | Description | Score |
|---|---|---|
| Focus score of ≥1 | A score determined by the number of mononuclear-cell infiltrates containing ≥50 inflammatory cells per 4 mm2 of minor labial salivary gland obtained on biopsy | 3 |
| Presence of anti-SSA antibodies | Measured in serum; only anti-Ro60 antibodies have to be considered; isolated anti-Ro52 antibodies are not specific for Sjögren's syndrome | 3 |
| SICCA ocular staining score of ≥5 | A score determined by an ophthalmologist on the basis of examination with fluorescein and lissamine green staining; scores range from 0 to 12, with higher scores indicating greater severity | 1 |
| Schirmer test of ≤5 mm per 5 min | An assay for measuring tear production by inserting filter paper on conjunctiva in the lower eyelid and assessing the amount of moisture on the paper | 1 |
| Unstimulated whole salivary flow of ≤0.1 ml per min | An assay for measuring the rate of salivary flow by collecting saliva in a tube for at least 5 min after the patient has swallowed | 1 |
| **Total score** | | **9** |

Primery Sjögren's syndrome is defined as a score of 4 or more.

## 2. Materials and Methods

### 2.1 Mice and saliva collection

The experimental protocols and animal handling procedures were approved by the Seoul National University Animal Care and Use Committee (#SNU-180914-8) and conducted in accordance with the recommendations in the Guide from the Institute of Laboratory Animal Resources, Seoul National University under the Laboratory Animals Act 9025 of the Republic of Korea. *Nfkbiz*$^{-/-}$ mice were provided by Prof. Shizuo Akira (IFReC, Osaka University, Japan). All mice were maintained under specific pathogen-free conditions in Laboratory Animal Facility at School of Dentistry, Seoul National University. *Nfkbiz*$^{+/+}$, *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ mice were produced by mating wild type male and female mice (♂ *Nfkbiz*$^{+/+}$ **x** ♀ *Nfkbiz*$^{+/+}$); and heterozygous male and heterozygous (♂ *Nfkbiz*$^{+/-}$ **x** ♀ *Nfkbiz*$^{+/-}$) female mice; and homozygous male and heterozygous female mice (♂ *Nfkbiz*$^{-/-}$ **x** ♀ *Nfkbiz*$^{+/-}$). For genotyping, genomic DNA isolated from the tail of 2-week-old mice was used. PCR amplification was performed as previously instructed [11]. After weaning, *Nfkbiz*$^{+/+}$, *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ female mice were housed in separate cages. Five mice per group were evaluated at 24 weeks.

Mice anesthetized with isoflurane were intraperitoneally injected with 10 μg/g body weight of pilocarpine to stimulate saliva production. Saliva was collected for 10 min after pilocarpine injection.

**2.2** Immunofluorescence

The salivary glands were embedded in OCT compound (Sakura Finetek, Torrance, CA, U.S) and then frozen in liquid nitrogen. Sections (10 μm) were blocked with 10% horse serum in phosphate-buffered saline (PBS). The sections were stained with anti-mouse CD3-ζ monoclonal IgG clone 6B10.2 (Santa Cruz Biotechnology Inc, Dallas, Texas U.S), PE-conjugated rat anti-mouse B220 clone 553090 (BD Bioscience, San Jose, CA, U.S) and goat anti-mouse IgG (H+L) Alexa 488 clone A28175 (Thermoscience, Waltham, Massachusetts, U.S). Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI). Immunofluorescence images were captured under a confocal microscope (Leica, Wtzlar, Germany).

**2.3** Histological Analysis

The salivary glands were fixed with 10% neutral formalin solution (Sigma, St Louis, MO, U.S) and then embedded in paraffin. Sections were cut at 4-5 μm and were stained with hematoxylin and eosin (H&E).

## **2.4** *In situ* hybridization

All the protocols and procedures were performed according to the previous methods [12]. The paraffin-embedded sections (4-5 μm) were hybridized with a DIG-labeled universal probe targeting the 16S rRNA gene. The bound probes were detected with alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Science, Mannheim, Germany) and visualized with a nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) solution. The sections were counterstained with methyl green.

## **2.5** 16S rRNA gene sequencing and microbiota analysis

Oral microbiota was collected with cotton swabs. Genomic DNA was isolated using the Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, U.S). Further analysis was done at ChunLab, Inc. (South, Korea) according to their procedures. Briefly, the concentration of DNA was accessed using a Nanodrop (Thermo Scientific), and the quality was determined by agarose gel electrophoresis. Bacterial 16S rRNA gene sequences spanning the variable region V3-V4 were amplified using the primer 341F_805R. The amplicons were subjected to paired-end sequencing on an Illumina MiSeq platform. All of the figures were

constructed by the integrated database, called EzBioCloud [13].

### **2.5** Statistical analysis

Statistically significant differences between samples were analyzed with one-way ANOVA with Bonferroni post hoc, Kruskal Wallis with Bonferroni post-hoc and Mann-Whitney U test. The box-and-whisker plot presented median, lower and upper quartiles and minimum and maximum values. Data not included between the whiskers were plotted as outliers with dots.

## 3. Results

### 3.1 IκB-ζ-deficient mice have reduced salivary flow rate

*Nfkbiz*$^{+/+}$, *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ mice were raised under specific-pathogen-free conditions until they became 24 weeks old. As previously reported, dermatitis in IκB-ζ-deficient mice was observed primarily at the periocular region and progressively extended to the whole face, presenting as erosion and loss of hair (Figure 1A). No *Nfkbiz*$^{+/+}$ and *Nfkbiz*$^{+/-}$ mice exhibited symptoms of dermatitis throughout the experimental period until they reached the age of 24 weeks (Figure 1A). This coincides with the previous reports [11, 14].

However, both *Nfkbiz*$^{+/-}$ mice (~5.6 ± 1.1 mg/g) and *Nfkbiz*$^{-/-}$ mice (~6.8 ± 1.7 mg/g) produced less saliva than *Nfkbiz*$^{+/+}$ mice (~10.9 ± 1.5 mg/g) in response to pilocarpine injection (Figure 2B).

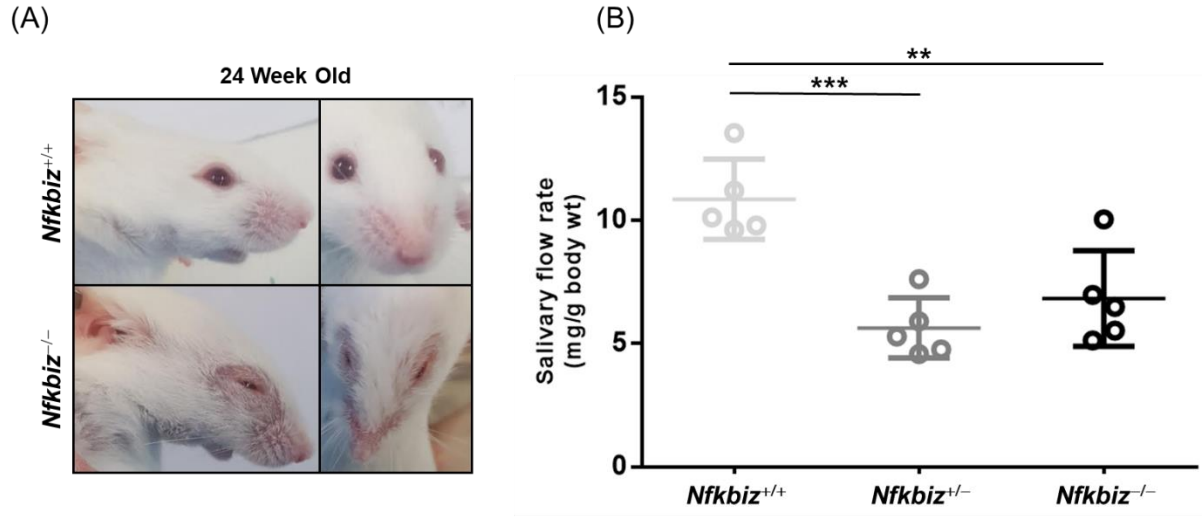


**Figure 1. Facial Phenotypes and Salivary Flow Rates**

(A) Representative images of the face of *Nfkbiz*$^{+/+}$ and *Nfkbiz*$^{-/-}$ mice at 24 weeks. (B) Saliva was collected for 10 min after pilocarpine injection. The salivary flow rate was calculated as the weight of saliva per the body weight. Values represent means ± SD, *** $p < 0.001$, ** $p < 0.01$, one-way ANOVA with Bonferroni post hoc.

**3.2** There is no significant difference in FLS

At week 24, FLS was found in all three genotypes (Figure 2A). FLS area and foci score tended to increase in *Nfkbiz*$^{-/-}$ mice compared to other genotypes, but statistical significance was not achieved. Importantly, three out of five IκB-ζ-deficient mice developed FLS with score ≥ 1 (Figure 2B).

In Figure 2C, immunofluorescent detection revealed that infiltrating lymphocytes were mostly T cells in early stage; in large foci, B cells predominated.

In Figure 3, *in-situ* hybridization using a universal probe for 16S rRNA demonstrated the bacterial invasion into ductal cells in submandibular glands among the mice.

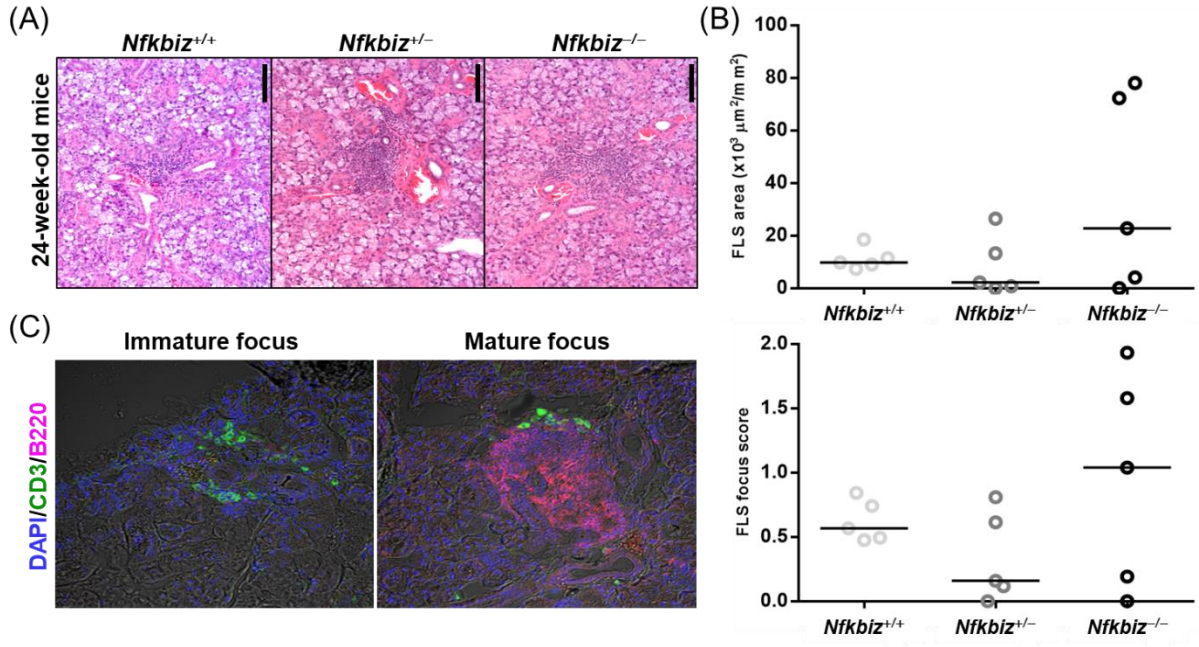


**Figure 2. Focal Lymphocytic Sialadenitis**

(A) Representative images of the hematoxlin and eosin stained sections of submandibular glands from *Nfkbiz*$^{+/+}$, *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ mice at 24 weeks. Bars, 100 μm. (B) Focal lymphocytic sialadentitis (FLS) area and focus scores were calculated from the areas of lymphocytic infiltrates divided by the total area of salivary glands, and the number of foci per 4 mm$^2$, respectively. Values represent median. (C) Representative images of immunohistofluorescent staining with anti-mouse CD3-ζ and anti-mouse B220 antibodies in submandibular glands from *Nfkbiz*$^{-/-}$ mice.

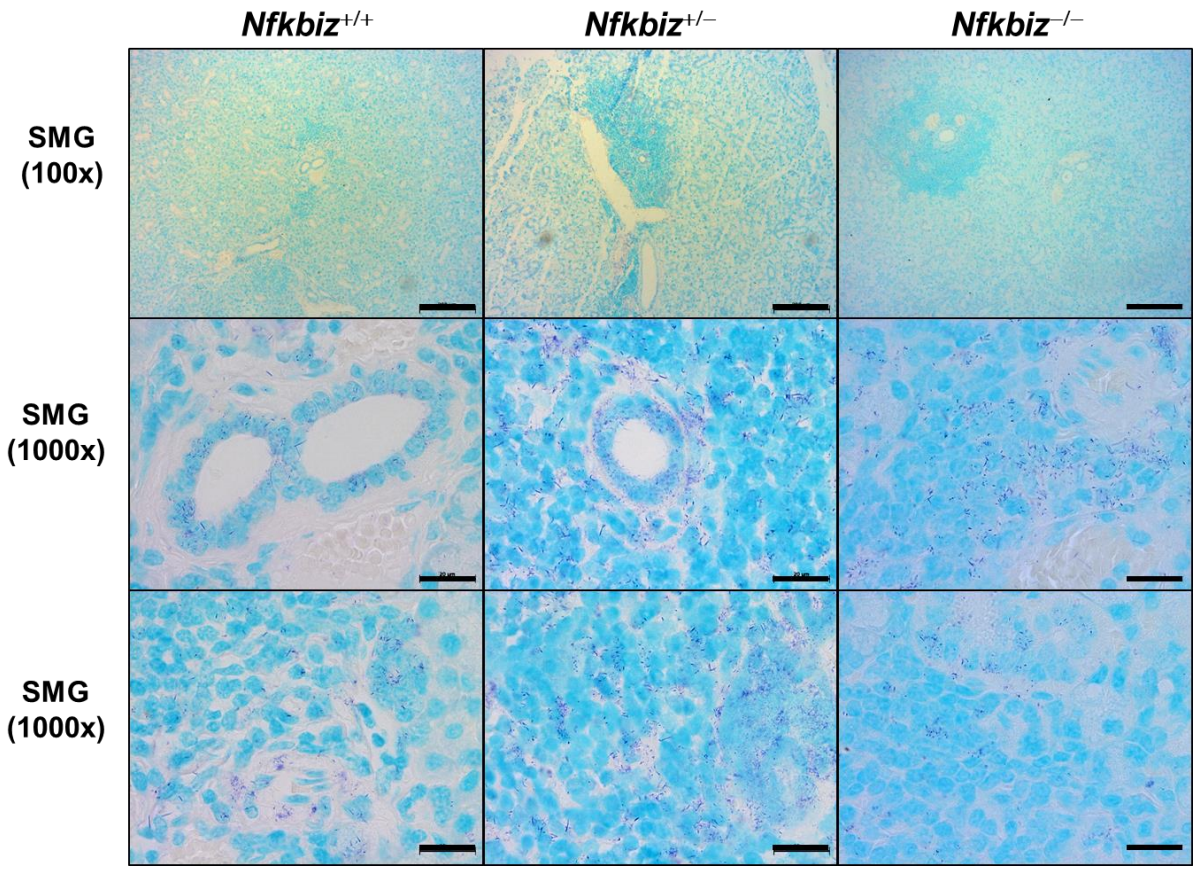


**Figure 3. Bacterial Invasion into Salivary Duct**

Representative *in situ* detection of bacteria in submandibular glands from *Nfkbiz*$^{+/+}$, *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ mice at 24 weeks. Magnification, 100x, 1000x. Bars, 20 μm, 200 μm.

### **3.3** IκB-ζ-deficient mice have oral dysbiosis

Oral microbiotas were analyzed from *Nfkbiz*$^{+/+}$, *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ mice by next generation sequencing. In alpha diversity, Simpson (measure of species evenness) and Shannon (measure of species richness and evenness) indexes showed significant differences between *Nfkbiz*$^{+/+}$ mice and *Nfkbiz*$^{-/-}$ mice (Figure 4). Beta diversity revealed that oral bacterial communities in *Nfkbiz*$^{+/+}$ mice and *Nfkbiz*$^{+/-}$ mice clustered closely; on the other hand, oral bacterial communities in *Nfkbiz*$^{-/-}$ mice were separated from those of other genotypes (Figure 5). While *Streptococcus danieliae* predominated the oral microbiota in *Nfkbiz*$^{+/+}$ and *Nfkbiz*$^{+/-}$ mice, it was substantially decreased in *Nfkbiz*$^{-/-}$ mice (Figure 6). Instead, *Staphylococcus sciuri group, Escherichia coli group, Enterococcus faecium group* and *Corynebacterium mastitidis group* were significantly increased in *Nfkbiz*$^{-/-}$ mice compared to *Nfkbiz*$^{+/+}$ mice (Figure 7). As the criteria suggested in Table 1, the mice were categorized into two groups: FLS score of < 1 and FLS score of ≥ 1. Surprisingly, those bacteria higher in *Nfkbiz*$^{-/-}$ mice significantly increased in FLS score of ≥ 1 (Figure 8). This was evident that the oral bacteria observed in *Nfkbiz*$^{-/-}$ mice may be

associated with FLS. Next, *Nfkbiz*$^{+/-}$ mice had unique sets of oral bacteria, which were *Lactobacillus mudanjiangensis, Lactobacillus parabrevis group, Lactobacillus brevis*, *Lactobacillus plantarum group, Staphylococcus aureus group, Cupriavidu metallidurans* and *Lactobacillus_uc* (Figure 9). Those oral bacteria were also significantly higher in the groups that were below the SFR of 7.8 (mean) (Figure 10). This indicated that those bacteria were related to dry mouth.

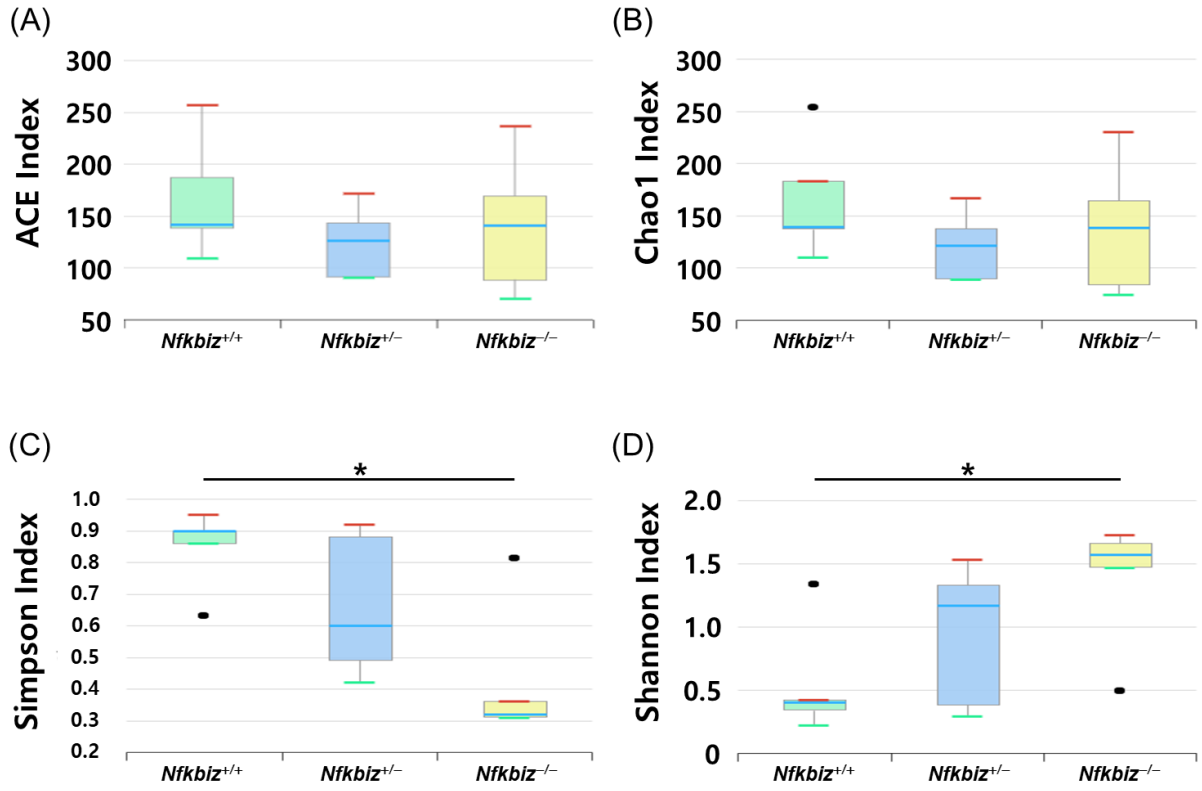


**Figure 4. Alpha-diversity of Oral Microbiota in *Nfkbiz* Mice**

The box-and-whisker diagrams represent species richness (A, B) and diversity index (C, D) of oral bacteria present in *Nfkbiz* mice. Values represent presented median, lower and upper quartiles and minimum and maximum values, * $p < 0.05$, Kruskal Wallis with Bonferroni post-hoc. Data not included between the whiskers were plotted as outliers with dots.

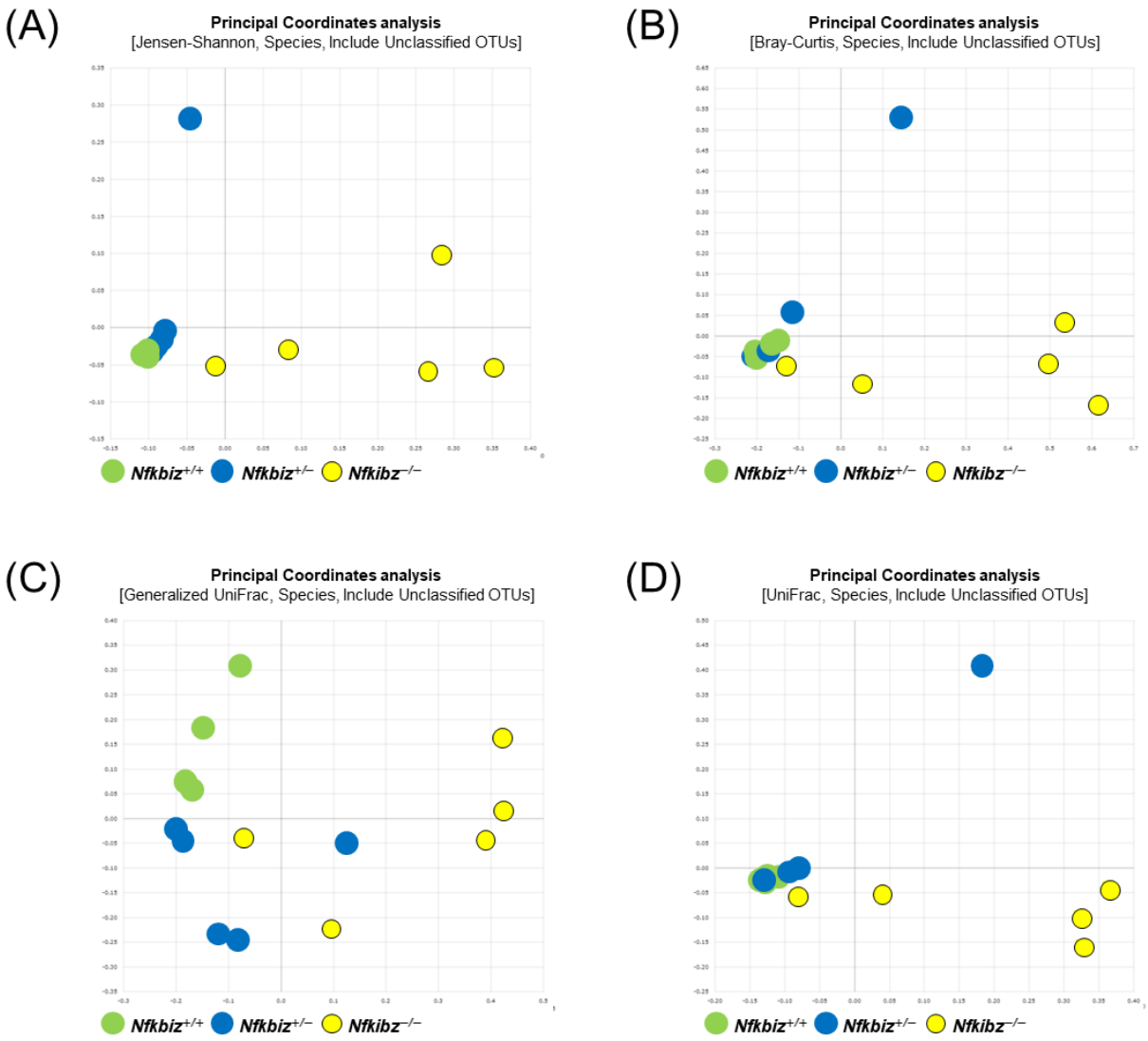


**Figure 5. Beta-diversity of Oral Microbiota in *Nfkbiz* Mice**

Principal coordinates analysis (PCoA) represents the oral bacteria in *Nfkbiz* mice with beta diversity distance of Jensen-Shannon (A), Bray-Curtis (B), Generalized UniFrac (C) and UniFrac (D).

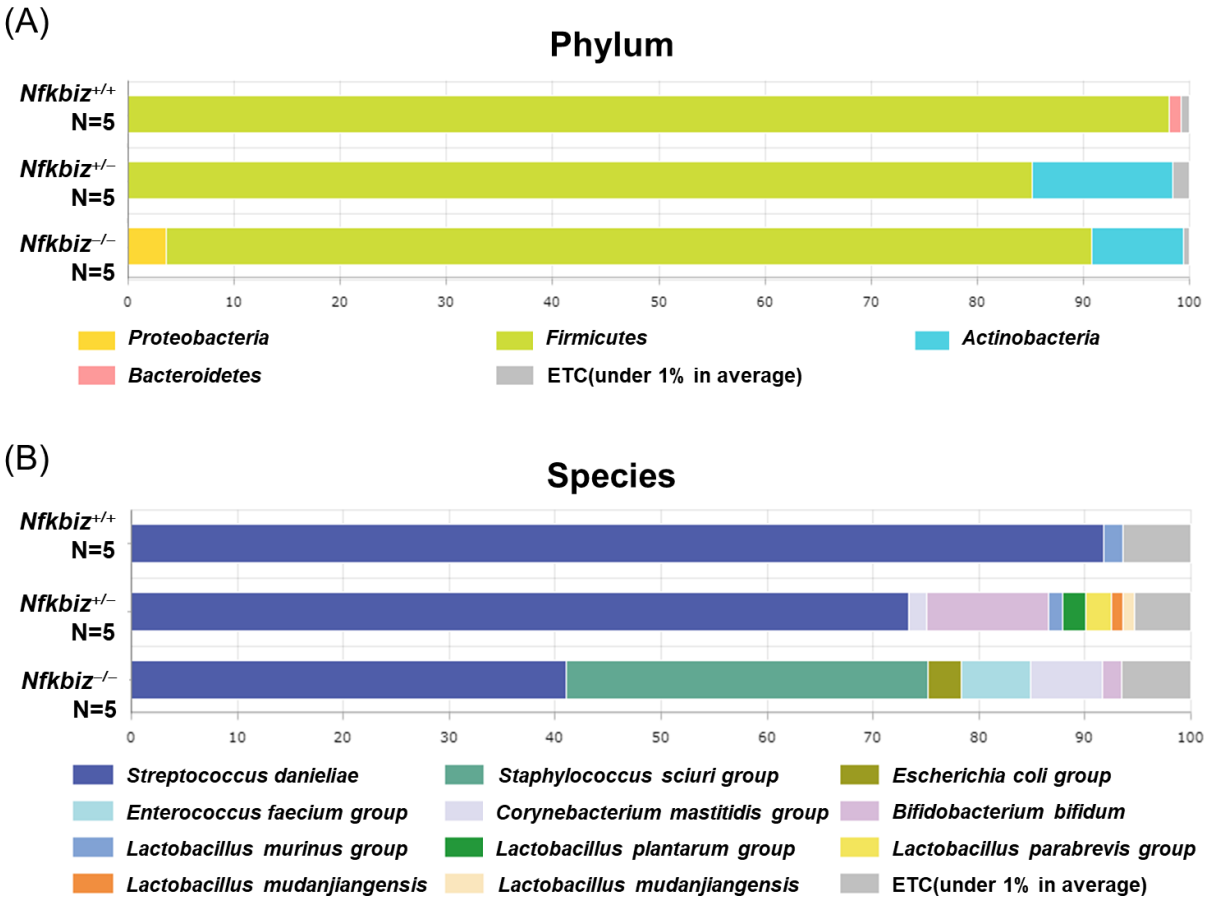


**Figure 6. Averaged Taxonomic Composition of MTP Set in *Nfkbiz* mice**

The bar graphs represent averaged composition of the oral microbiota present in *Nfkbiz*$^{+/+}$, *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ mice at 24 weeks. Phylum and species with mean abundance > 1% are shown. ETC: et cetera.

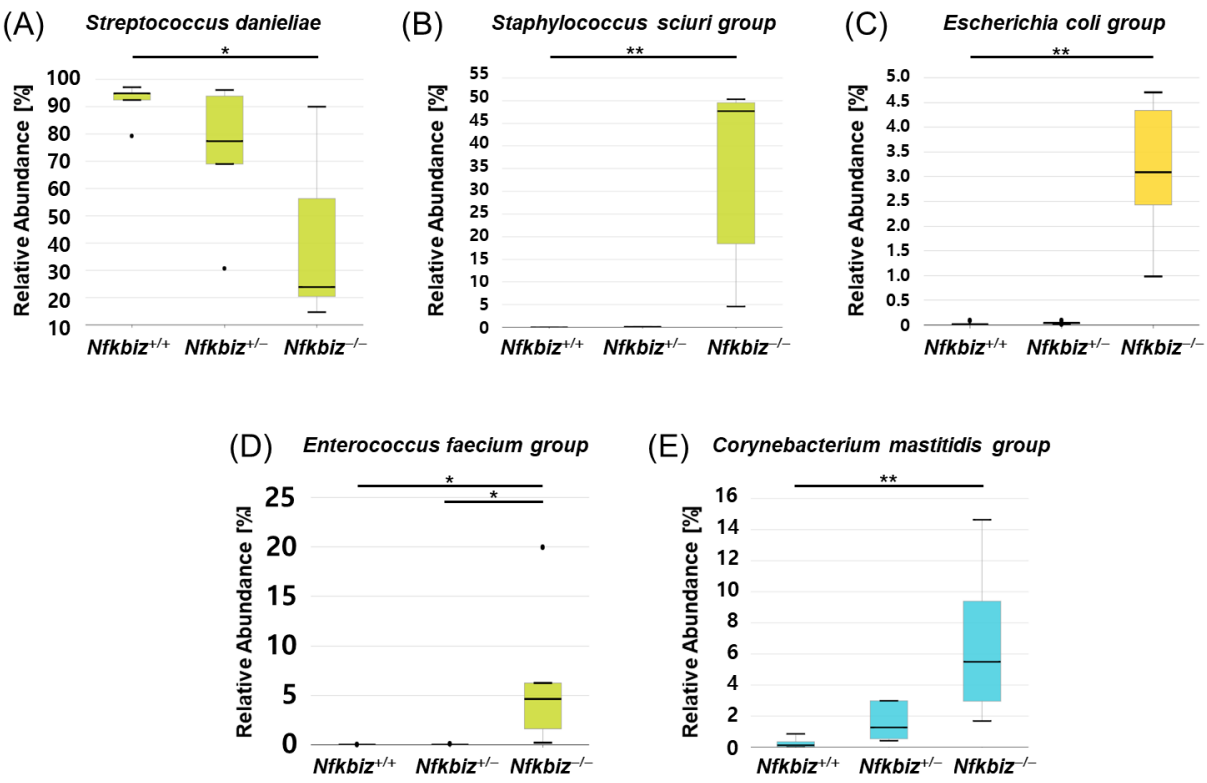


**Figure 7. Selected Species that were Decreased or Increased in *Nfkbiz*$^{-/-}$ Mice**

Values represent presented median, lower and upper quartiles and minimum and maximum values, ** $p < 0.01$, * $p < 0.05$, Kruskal Wallis with Bonferroni post-hoc. Data not included between the whiskers were plotted as outliers with dots.

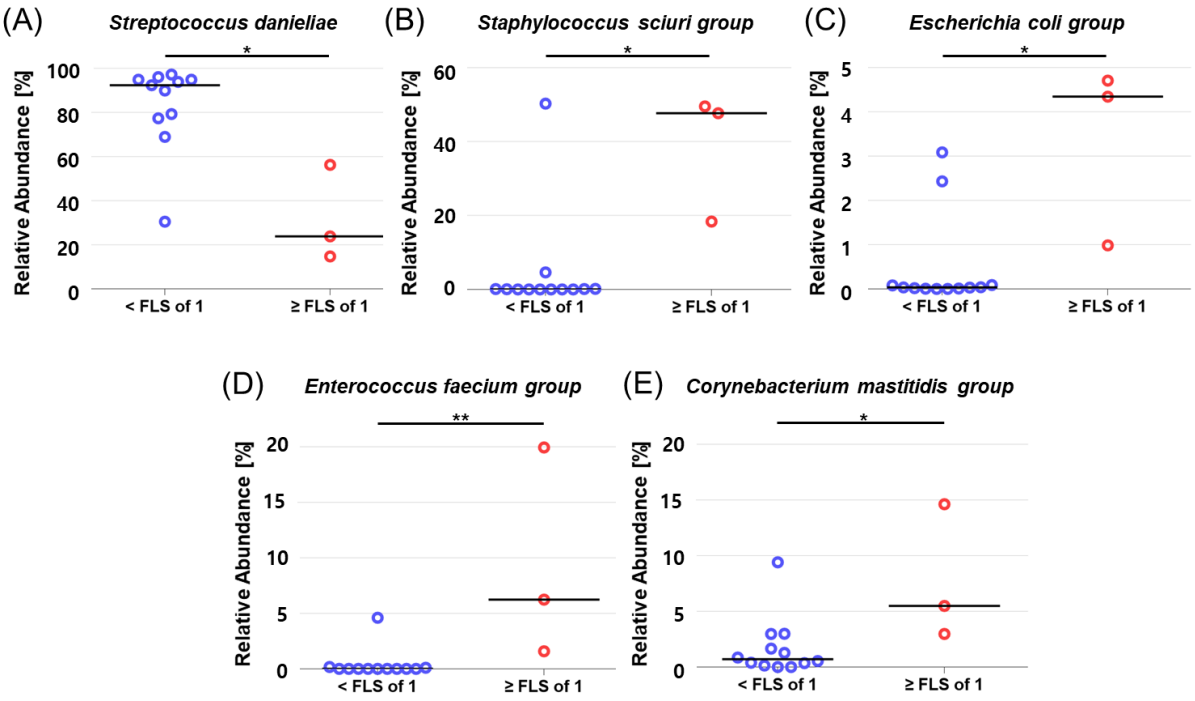


**Figure 8. Selected Species below or above FLS score of 1**

Values represent presented median, ** $p < 0.01$, * $p < 0.05$, Mann-Whitney U test.

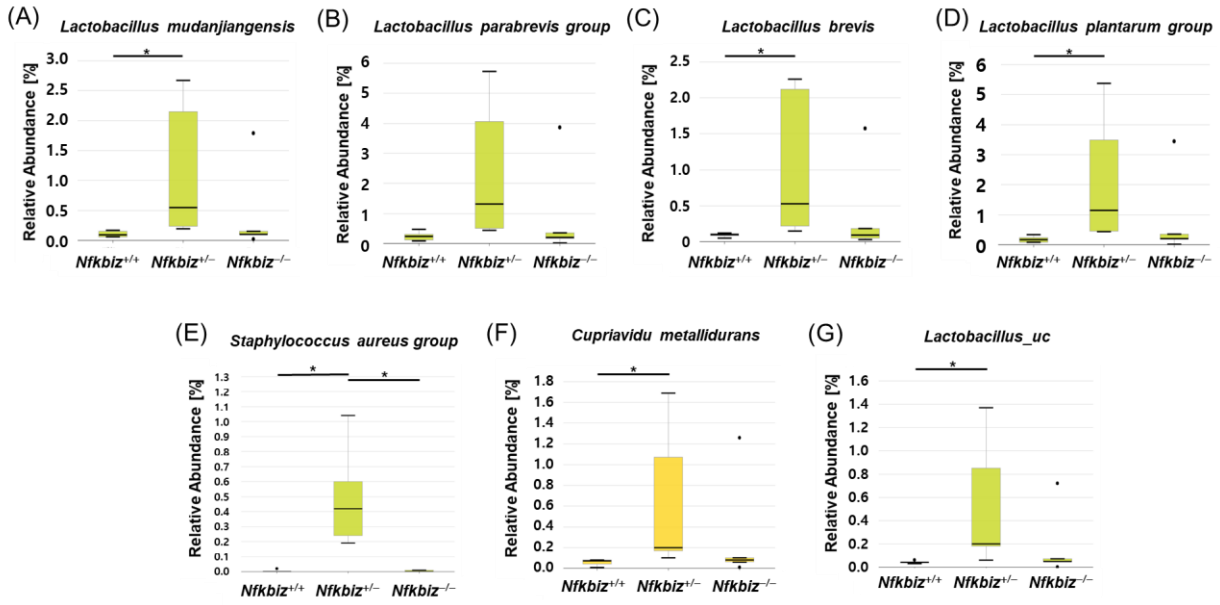


**Figure 9. Selected Species that were Increased in *Nfkbiz*$^{+/-}$ Mice**

Values represent presented median, lower and upper quartiles and minimum and maximum values, * $p < 0.05$, Kruskal Wallis with Bonferroni post-hoc. Data not included between the whiskers were plotted as outliers with dots.

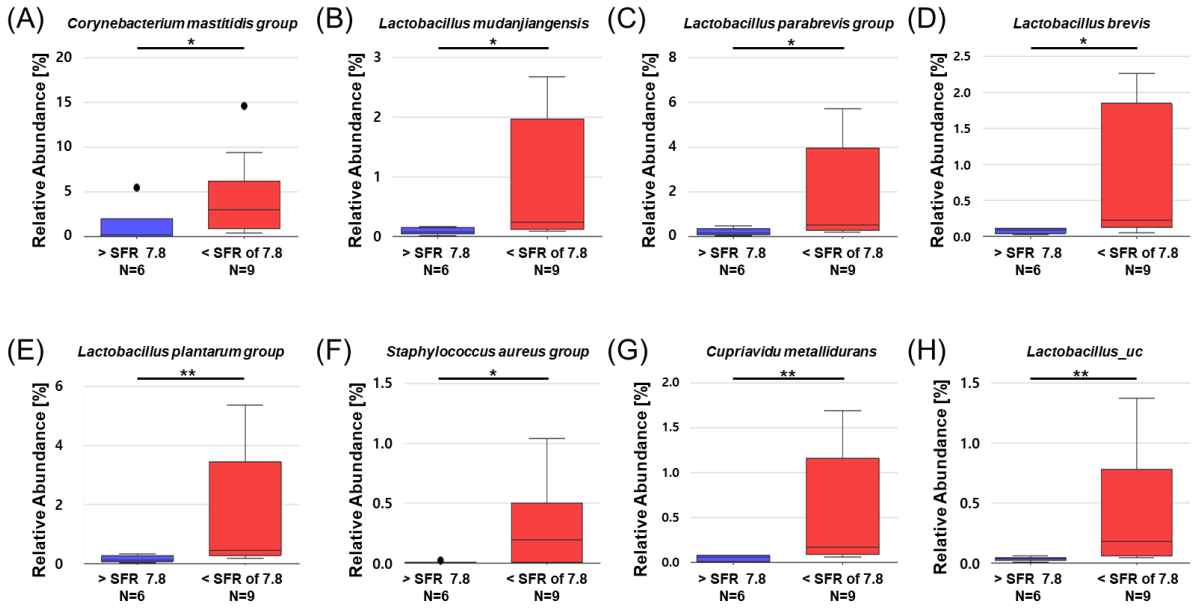


**Figure 10. Selected Species that were Increased in < SFR 7.8**

Values represent presented median, lower and upper quartiles and minimum and maximum values, ** $p < 0.01$, * $p < 0.05$, Mann-Whitney U test. Data not included between the whiskers were plotted as outliers with dots.

## Discussion

In summary, IκB-ζ-deficient mice spontaneously developed oral symptoms of SS-like diseases at 24 weeks. The mice showed decreased salivary flow rate. Three out of five mice exhibited an FLS score ≥ 1. In mild lesions, activated T cells were predominant, whereas B cells prevailed in severe histological lesions. These were the general symptoms shown by the patients with SS (Table 1).

The study provided evidence that the composition of oral microbiota was influenced by the host genetic background. Figure 3 and 5 show the presence of more diverse oral microbiota in *Nfkbiz*$^{-/-}$mice than *Nfkbiz*$^{+/+}$mice. It is possible that the damaged epithelia in *Nfkbiz*$^{-/-}$mice induce a favorable environment for increased proliferation of bacteria due to the destruction of the first line of defense. In addition, *Nfkbiz*$^{+/-}$ and *N*fkbiz$^{-/-}$ mice had dry mouth, causing a change in the composition of oral microbiota. For example, the number of *Lactobacillius* significantly increased in *Nfkbiz*$^{+/-}$mice compared to other genotypes (Figure 8). It was reported that salivary pH inclined to be acidic when saliva production decreased [15]. *Lactobacillius* were found in hyposalivation as well as in

pSS [16, 17]. In all likelihood, *Lactobacillius* could be one of the survivors in the acidic conditions induced in *Nfkbiz*$^{+/-}$mice. Although there is no evidence to resolve this proposition, the study demonstrated that the genetic background predetermined the selection of oral bacterial communities.

Sialadenitis is mostly caused by bacterial infections. It is widely accepted that accumulation of bacteria followed by neutrophil attacks results in acute sialadenitis [18]. *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus viridians,* and *Haemophilus influenza* are commonly known to affect submandibular glands [18]. Similarly, bacterial invasion into ductal cells was observed in submandibular glands among the mice (Figure 3). However, no such bacteria were found at FLS score ≥ 1. It is likely that their mechanisms are different, since FLS composed of mononuclear cell infiltrates, not neutrophils. Instead, those bacteria were higher in *Nfkbiz*$^{-/-}$ mice, which significantly increased the FLS score to ≥ 1. This finding may add to the increasing evidence that dysbiosis of oral microbiota promotes bacterial invasion into the submandibular glands.

*Nfkbiz*$^{+/-}$ mice showed unexpected results, when the salivary secretion

was decreased without an increase in FLS. In general, lymphocytic invasion into salivary glands leads to a malfunction in saliva production because of the following reasons. Primarily, cytotoxic T lymphocytes invade the salivary glands, and secrete toxic substances inducing the death of acinar cells that are responsible for saliva production [19]. Besides, T and B cells were involved in building ectopic germinal center-like structures that continuously produced autoantibodies and decreased salivary secretions [7, 20]. Unfortunately, the mechanisms of dry mouth and FLS remained elusive in *Nfkbiz*$^{+/-}$ mice. It is probable that haploid genotypes may drive decreased salivary flow rate without increase in FLS. There is a need to uncover the mechanisms behind this causal relationship in subsequent studies.

In conclusion, *Nfkbiz*$^{-/-}$ mice spontaneously developed dry mouth, FLS, and dysbiosis of oral microbiota. Oral microbiota analysis demonstrated that the groups *Staphylococcus sciuri, Escherichia coli, Enterococcus faecium,* and *Corynebacterium mastitidis* may be associated with FLS. These findings may provide valuable information pertaining to the combined effects of host genotype and oral microbiota acting in conjunction with environmental factors to develop dry mouth and FLS.

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**국문초록**

# IκB-ζ 결핍 쥐에서 구강건조증과 림프구성 타액선염의 발생 및 구강세균총의 변화

**이 준 호**

**서울대학교 치의학대학원**

**치의과학과 면역 및 분자미생물치의학전공**

**(지도교수 최 영 님)**

## 1. 배경

쇼그렌 증후군은 전신 염증성 자가면역질환으로서 외분비 샘의 기능장애와 건조 증상이 특징이다. 발명의 원인은 잘 알려져 있지 않지만 다양한 유적적, 환경적 요인이 관여한다. IκB-ζ 단백질이 결여된 상피세포는 NF-κB 의 전사 활동이 다르게 일어나, caspase-3

활성화와 세포 사멸이 증가한다. 상피의 손상은 외부 환경으로부터 내부조직을 보호해주지 못하고, 세균 감염이 자가 면역을 유도 할 수도 있다. 전 연구에서 IκB-ζ 가 결여된 쥐에서 눈물 분비 감소, 눈물샘염 그리고 자가 항체 생성 같은 쇼그렌 증후군의 증상을 나타냈지만, 타액선에는 아무런 증상을 보이지 않았다. 그러나, 저희 실험동물실에서는 IκB-ζ 단백질이 결여된 쥐에서 타액 감소가 관찰되었기에 IκB-ζ 결핍에 따른 분비율 변화, 타액선염, 구강 세균의 관계를 알아보고자 한다.

## 2. 방법

실험에 사용된 IκB-ζ 단백질이 결여된 쥐는 서울대학교 연건 치의학대학원 특정 병원체 부재 동물실에서 번식 그리고 유지시켰다. *Nfkbiz*$^{+/+}$, *Nfkbiz*$^{+/-}$, *Nfkbiz*$^{-/-}$ 5 마리씩 24 주 동안 사육한 후에 타액선 그리고 구강 세균을 채취했다. 타액은 필로카폰을 주입하여 10 분 동안 채취했다. 타액선은 파라핀 포매 후 hematoxlin-eosin (H&E) 로 염색해 림프구성 타액선 염 발생을 관찰하였다. 타액선의 세균의 침투 여부는 *in situ* hybridization 으로 관찰하였다. 또한 타액선의 일부를 급속 냉각하여 얼린 조직을 만들고, CD3-ζ 와 B220 형광 염색

관찰하였다. 구강 세균은 멸균된 면봉으로 채취 후 Power Soil DNA Isolation Kit 를 사용해 genomic DNA 를 얻었다. 구강 세균은 high throughput sequencing 으로 분석했다.

## 3. 결과

필로카폰에 의해 유도된 타액을 채취하여 각 그룹들끼리 비교했다. *Nfkbiz*$^{+/+}$ 쥐에 비해 *Nfkbiz*$^{+/-}$ 쥐와 *Nfkbiz*$^{-/-}$ 두 그룹에서 타액 분비량이 감소하였다. 헤마톡실-에오신에 염색된 타액을 각 그룹끼리 비교 했다. 그룹 간에 유의한 차이는 없었으나 *Nfkbiz*$^{-/-}$ 군 5마리 중 3마리에서 SS 진단 기준에 부합하는 1점 이상의 림프구성 타액선염이 관찰되었다. 형광 염색에서는 대부분의 T 세포가 타액선염 초기단계에 발견되었지만, 진행이 된 타액선염에서는 B 세포가 주로 관찰되었다. 그리고, *in situ* hybridization 결과에서 도관 세포를 통해 타액선으로 침투한 세균들이 모든 유전자형 쥐에서 관찰되었다. 구강 세균총 분석 결과에서는 *Nfkbiz*$^{+/+}$ 에 비해 *Nfkbiz*$^{-/-}$ 쥐에서 세균총의 균일성과 다양성이 증가하였음을 보여주었다. 또한, 베타 다양성 결과에서 *Nfkbiz*$^{+/+}$, *Nfkbiz*$^{+/-}$ 그룹에 비해 *Nfkbiz*$^{-/-}$ 가 따로 떨어져 있어, 매우 다른 구강세균총을 갖고 있음을 제시하였다.

*Streptococcus danieliae* 는 *Nfkbiz*$^{+/+}$, *Nfkbiz*$^{+/-}$ 에서 전체 구강 세균의 70% 이상을 차지하는데 반면 *Nfkbiz*$^{-/-}$ 쥐에는 크게 감소하였다. 그 대신, *Nfkbiz*$^{-/-}$ 쥐에서 *Staphylococcus sciuri group, Escherichia coli group, Enterococcus faecium group*, *Corynebacterium mastitidis group* 이 유의하게 증가하였다. *Nfkbiz*$^{-/-}$ 쥐에서 증가한 구강세균들은 FLS score of ≥ 1 그룹에서도 유의하게 증가하였다. *Lactobacillus mudanjiangensis, Lactobacillus parabrevis group, Lactobacillus brevis*, *Lactobacillus plantarum group, Staphylococcus aureus group, Cupriavidu metallidurans* and *Lactobacillus_uc* 세균종은 특이하게도 *Nfkbiz*$^{+/-}$ 쥐에서만 유의하게 증가하였다. 이 세균들은 salivary flow rate 평균 이하의 그룹에서도 유의하게 증가하였다.

## 4. 결론

*Nfkbiz*$^{-/-}$ 쥐에서 자발적으로 구강건조증, 림프구성 타액선염, 그리고 구강세균총의 변화가 일어났다. 구강세균 분석 결과, *Staphylococcus sciuri group, Escherichia coli group, Enterococcus faecium group*, *Corynebacterium mastitidis group* 이 FLS 와 관련이 있음을 제시하였다.

**주요어: 쇼그렌 증후군, 초점 림프구성 타액선 염, 침 분비량,**

**구강세균, IκB-ζ 결핍 쥐**

**학 번: 2017-25212**