이학석사학위논문

Effect of gut microbiota on the development of atopic dermatitis in NC/Nga mice

장내 미생물이 NC/Nga의 아토피성 피부염에 미치는 영향

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Abstract

Recent researches on gut microbiome provided better understanding of relationship between gut microbiota and host pathology, including atopic dermatitis, of which occurrence and development are known to be associated with gut microbiota but not characterized so far. To identify intestinal microorganisms related with atopic dermatitis, specific antibiotic cocktails were developed to change the two major phylotypes of gut microbiota, of which composition was examined by pyrosequencing using 16S rRNA gene tags. NC/Nga mice, model animal for atopic dermatitis, were administered two different antibiotic cocktails targeting either Gram negative (GNA) or Gram positive (GPA) bacteria by either *ad libitum* drinking water or oral gavage for 6 weeks.

The GPA group showed an increased serum IgE level accompanied with more severe skin damage compared to the control and GNA groups. Bacterial community analysis data also revealed dramatic changes in the gut microbiota composition of the GPA group with a significant increase in abundance of the species *Enterobacter cowanii* and *Enterobacter hormaechei* belonging to the Proteobacteria. The involvement of the two bacteria in the pathology of atopic dermatitis was tested by oral administration to NC/Nga mice. Only the mouse group colonized with *Enterobacter hormaechei* showed an aggravation of skin damage and an increased serum IgE level. Our data suggest that enterobacteria, *Enterobacter hormaechei*, may play an important role in development and severity of atopic dermatitis in NC/Nga mice

Key word: Atopic dermatitis, NC/Nga, pyrosequencing, antibiotics.

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I. Introduction

1. Human and microbiome

After surprising finding of relatively small genome size of human being, scientists shifted their focus to genetic material outside of human origin. The focus was now on what was known to be "forgotten organ" as it has functions beyond host ability: the microorganism living in human beings. Microbiome project launched by NIH sparked up the research on microbiota, and many physiological phenomena in human body including regulation of immune system, and metabolism of otherwise indigestive energy source were revealed to be closely related to the microbiota. Considering the magnitude of human physiology affected by microbiota, undersized human genome could be explained by compensatory effect of symbiotic microbiota (1).

Human microbiota is distributed along human body; skin, oral cavity, conjunctiva, vagina, and digestive tract. Unlike other areas of the body, intestinal tract is a very hospitable place for microbiota as temperature, pH, and available energy sources are suitable for microorganisms to live in. Because of this environmental favor, intestine especially small and large intestine harbor majority of microbiota.

Recent studies showed that seemingly unrelated human pathologies such as autism, allergic diseases, and immune deficiency are closely related with the gut microbiota. Thus much more research is currently being conducted to

discover better understanding of the interaction between human host and its complex microbiota (2).

One of the key research areas on gut microbiota is its relation to obesity due to their ability to utilize energy sources not used by host. Many mouse model experiments and human cohort studies have been conducted, linking gut microbiota with increase in weight and body fat. Also it has been reported that administration of gut microbiota of obese human led to an increase in the body fat contents in lean mouse. However exact intestinal bacteria responsible for obesity are not identified yet (3).

2. Gut microbiota and atopic dermatitis

It is reasonable to assume that the microbiota in intestinal tract play important functions in physiology of host gut, but some of the researches showed that the gut microbiota have effect on host physiology even outside of intestinal tract. (1). One of the major host pathologies that are suspected to be affected by gut microbiota is atopic dermatitis. Even though symptoms of the atopic dermatitis are presented on the skin, it is suspected that the reason behind this allergic disease is more systemic than local effect (4, 5).

Gut microbiota is also known to play a key role in immune development. Recent studies showed that commensal bacteria have a major role in balance of T cell proliferation (4). It also has been reported that immune response after decrease in diversity of gut microbiota may trigger allergic response, making the host vulnerable to atopic dermatitis (5).

Atopic dermatitis is a fairly common disease prevalent in infants and toddlers. It has been reported that the number of people with atopic dermatitis is increasing, causing a serious health problem. About 19% of infants and toddlers showed atopic dermatitis symptoms in South Korea alone in 2012 (6). Apart from being allergic disease, the cause and pathogenesis of atopic dermatitis were never clearly revealed.

Nevertheless, numerous cohort studies showed possible connections between gut microbiota and atopic dermatitis (7). On top of that, there was a report that the administration of heat-treated *Lactobacillus* may delay the onset of atopic dermatitis in the model mouse, NC/Nga (8). This study however did not demonstrate how a single bacterium or complex microbiota may influence host pathology.

Many researches on the atopic dermatitis use a model mouse, NC/Nga. NC/Nga mouse was discovered by a group of Japanese scientists. According to their research, NC/Nga mouse develops atopic dermatitis spontaneously when raised in conventional condition. NC/Nga mouse showed a severe skin lesion and an elevated IgE level corresponding to typical atopic dermatitis symptom (9).Thus, in this study NC/Nga has been used to study association between gut microbiota and atopic dermatitis.

3. Aim of this study

Even though much research showed that there were some linkages between gut microbiota and atopic dermatitis whether change in gut microbiota causes

atopic dermatitis or atopic dermatitis causes change in gut microbiota, and whether prevalence of critical bacteria is important or immune response is critical are not clarified.

While many studies have proposed the involvement of human gut microbiota in atopic dermatitis, they could not conclusively identify which member or members of gut microbiota are responsible for the development and severity of atopic dermatitis. Thus more recent researches are focused on finding the responsible bacteria for the development of atopic dermatitis. Based on the notion that altering gut microbiota can change the disease state of the atopic dermatitis, comparing severity of atopic dermatitis after altering gut microbiota composition may lead to finding microorganisms responsible for atopic dermatitis.

This study is focused on the alteration of gut microbiota composition by administering specific antibiotic cocktails which were designed to make perturbation of gut homeostasis, and induce atopic dermatitis to observe difference in severity of atopic dermatitis. The antibiotics used in the antibiotic cocktails developed in this study were carefully selected.

This study was conducted to identify the bacteria from gut microbiota which are responsible for development of atopic dermatitis. The experiment was designed to observe effect of only selected group of bacteria on atopic dermatitis through alteration of gut microbiota composition with specific antibiotic cocktail. By eliminating specific bacteria in intestine of atopic dermatitis induced mouse, this study would help narrow down bacteria responsible for atopic dermatitis.

II. Materials and Methods

1. Mouse strains

The NC/Nga mice used in this study were 3 weeks old male and purchased through Central Lab Animal Inc. (Seoul, Korea). All experiments were conducted with 5 to 10 mice per group, 5 mice per cage, in temperature, and humidity controlled facility with 12 hours light and dark cycle. Three weeks old mice were allowed to adapt to the facility for one week before experiment. All mice were fed with normal chaw diet (Purina, Sungnam, Korea) and autoclaved tap water throughout the experiment. All experimental procedure was approved by Seoul National University Institutional Animal Care and Use Committees.

2. Antibiotic cocktail and atopic dermatitis inducing agent

Antibiotics treatment was done on each experiment group to alter gut microbiota. All antibiotics were purchased from Sigma-Aldrich Korea (Seoul, Korea). Total of 5 antibiotics were used in this study; Bacitracin, Vancomycin, Nalidixic acid, Polymyxin B and Cefoxitin. Bacitracin and Vancomycin were selected to eradicating Gram positive bacteria in gut microbiota, and used as Gram positive antibiotics cocktail (GPA). Nalidixic acid, Polymyxin B and Cefoxitin were selected to eradicating Gram negative bacteria and used as

antibiotics cocktail (GNA). The antibiotics in the cocktails were selected taking their ability to not be absorbed in intestine, and metabolized in liver into account.

Each antibiotic have different solubility and median lethal dosage. Based on the approved median lethal dosage and solubility in water, appropriate amount of antibiotics were administered to each mouse based on their weight. When added to drinking water, 1 g of Bacitracin and 0.5 g of Vancomycin was added to 1 L of water for GPA. For GNA, 1 g of Nalidixic acid, 1 g of Polymyxin B and 0.25 g of Cefoxitin was added to 1 L of water. All drinking water was autoclaved.

Administration of antibiotics *ad libitum* by drinking water had its limitation such as inability to control amount of antibiotics given to each mouse as some mice may consume more drinking water than others. Thus, different method of administering antibiotics was needed, which was oral administration. Based on the ratio of antibiotics concentration used for *ad libitum* administration, 50 mg/kg of Vancomycin, 100 mg/kg of bacitracin, Nalidixic acid and Polymyxin B and 10 mg/kg of Cefoxitin was administered to each mouse (10-12).

Even though NC/Nga mouse is a model mouse for atopic dermatitis, spontaneous development of atopic dermatitis is difficult to achieve due to low probability and requires long time. To develop atopic dermatitis in timely manner among all experiment groups, chemical inducer of atopic dermatitis was used. 2,4-dinitrochlorobenzene (DNCB) was applied to shaved ventral side of each mouse. To ensure proper sensitization, 200 μl of 1% DNCB

mixed in acetone, olive oil 3:1 mixture was applied and then 100 μl of 0.2% of DNCB was applied three times per week. All mice were shaved with electronic shaver two days prior to initial sensitization. Once lichenfication became evident on the mouse skin, it was no longer possible to shave their hair, thus shaving dorsal side was done only when possible (13).

3. Bacterial strains

For bacterial administration experiment, *Enterobacter cowanii* and *Enterobacter hormaechei* were obtained from professor Chi Nam Seong, Sunchon National University (Sunchon, Korea). The bacteria was cultured in Tryptic soy broth (TSB) and 1×10^8 cells, calculated by optical density, were resuspended in PBS and administered to the mice via oral gavage.

4. Evaluation of the experimental treatment.

To confirm antibiotics treatment and colonization of administered bacteria, cell counting through quantitative PCR method was used. In order to achieve non-invasive confirmation of intestinal bacteria, bacteria DNA was extracted from stool samples using QIAamp DNA stool mini kit (Qiagen, Hilden, Germany). Using primers for 16s rDNA cell number of total stool bacteria and two major phyla was calculated to determine effect of antibiotics and administered bacteria. Quantification of stool bacteria was done by Pfaffl

method, using amplification efficiency of specific primers (14)

5. Pathology of atopic dermatitis: skin condition

Skin condition is a key phenotypic measure to evaluate the degree of severity in atopic dermatitis. Observing skin condition and scoring them in the mouse is very difficult as surface area of mouse skin is significantly smaller than that of human's. Thus modified method to score skin of mouse with atopic dermatitis was used. Based on Eczema Area and Severity Index (EASI), simple 0 to 3 scoring method disregarding area factor used in EASI was used. By observing four symptoms of atopic dermatitis symptom, erythema, induration, excoriations, and lichenfication, each symptom was scored 0 to 3 (15).

6. Serum IgE level detection.

Besides interleukins, one of the major biomarker for atopic dermatitis is immunoglobulin E (IgE). The atopic model mouse NC/Nga presents elevated IgE with increased severity of skin lesion (8). Thus serum IgE concentration is an easily detectable biomarker to confirm whether the mice present atopic dermatitis or not. Using the enzyme-linked immunosorbent assay (ELISA), serum IgE concentration was detected. Mouse IgE ELISA kit was purchased from Bethyl laboratory Inc. (Montgomery, USA) and followed instruction as

described. Mouse serum was collected each week via retro-orbital blood collection method. Collected blood sample was centrifuged for 15 minuets at 2500 rpm to separate out serum, which was stored at -80℃ until use.

7. Comparison of host gene expression

To evaluate host gene expression change, the expression of genes known to be associated with inflammation and immune response were measured with qRT-PCR. During sacrifice, host intestine tissue was harvested and frozen immediately with liquid nitrogen. Mouse RNA was extracted form 50 mg of mouse intestinal tissue using Qiagen RNA mini kit (Hilden, Germany). , Mouse RNA was immediately used to obtain cDNA, and stored at -80℃ until use. Specific primers for each gene were used to quantify host gene expression (Table 1). Internal control for the comparison was GAPDH and analysis was done with comparative Ct method (16).

8. Sequencing Gut microbiota

Gut microbiota of all mice were processed with highly parallel Roche 454 GS FLX Titanium pyrosequencer. Cecal contents of each mouse were collected when sacrificed, and frozen immediately with liquid nitrogen. 100 mg of cecal content were completely homogenized with mini-beadbeater with 50mg of two different sized beads. Bacteria DNA from completely homogenized cecal

content was extracted using QIAamp DNA stool mini kit (Hilden, Germany). Extracted DNA then amplified with primer proved by Chun Lab (Seoul, Korea). With barcoded primers provided by Chunlab, PCR of 16s rDNA was preformed and the PCR products were combined and sequenced by Chunlab.

Table 1. Primers used in this study.

Target	Name, sequence	Reference
Bacteroidetes	Bact934F, GGARCATGTGGTTTAATTCGATGAT Bact1060R, AGCTGACGACAACCATGCAG	19
Firmicutes	Firm934F, GGAGYATGTGGTTTAATTCGAAGCA Firm1060R, AGCTGACGACAACCATGCAC	19
Total bacteria	Universal 340F, ACTCCTACGGGAGGCAGCAGT Universal 514 R, ATTACCGCGGCTGCTGGC	20
Enterobacter	Enterobacter F, GGCTATTAAAAAGAAG	This study
E. cowanii	Cowanii R, ATCAGCAGTGGGATAACTAG	
E. hormaechei	Hormaechei R, GCGATCAGATGTGGATAACTC	
Nod 2	Nod2 F, CGACATCTCCCACAGAGTTGTAATCC Nod2 R, GGCACCTGAAGTTGACATTTTGC	21
Myd88	Myd88 F, TGCCGTCCTGTCTACATCTTTG Myd88 R, GTTGCTCAGGCCAGTCATCA	22
IRAK1	IRAK1 F, CGCCAAGCACTTCTTGTACGA IRAK1 R, GATCAAGGCCGCGAACT	23
IkappaB	IkBa F, CCAGAACAACCTGCAGCAGAC IkBa R, GCTCAGGATCACAGCCAGCTT	24
B-deffensin	B-def3 F, CTCTTTGCATTTCTCCTGGTGCTGCTG B-def3 R, CATCTTCATGGAGGAGCAAATTCTG	25
SAA1	SAA1 F, CATTTGTTCACGAGGCTTTCC SAA 1 R, GTTTTTCCAGTTAGCTTCCTTCATGT	26
SAA2	SAA2 F, TGTGTATCCCACAAGGTTTCAGA SAA2 R, TTATTACCCTCTCCTCCTCAAGCA	26
SAA3	SAA3 F, CGCAGCACGAGCAGGAT SAA3 R, CCAGGATCAAGATGCAAAGAATG	26
RegIII beta	*REG3β F,* TCCCAGGCTTATGGCTCCTA *REG3β R,* GCAGGCCAGTTCTGCATCA	27

RegIII gamma	*REG3γ F,* CATCAACTGGGAGACGAATCC *REG3γ R,* CAGAAATCCTGAGGCTCTTGACA3’	27
GAPDH	GAPDH F, CCTCGTCCCGTAGACAAAATG GAPDH R, TCTCCACTTTGCCACTGCAA	28
CCL28	CCL28 F, CAGCCCGCACAATCGTACT CCL28 R, ACGTTTTCTCTGCCATTCTTCTTT	28
CXCL9	CXCL9 F, AATGCACGATGCTCCTGCA CXCL9 R, AGGTCTTTGAGGGATTTGTAGTGG	28
CCL20	CCL20 F, TTTTGGGATGGAATTGGACAC CCL20 R, TGCAGGTGAAGCCTTCAACC	28
TLR 2	TLR2 F, GCTGGAGGACTCCTAGGCT TLR2 R GTCAGAAGGAAACAGTCCGC	29
TLR 4	TLR4 F, ACCAGGAAGCTTGAATCCCT TLR4 R, TCCAGCCACTGAAGTTCTGA	29
TLR 5	TLR5 F, ACCACACTTCAGCAGGATCA TLR5 R, AGTTGAAGCTGAGCAGGAGC	29
TLR 9	TLR9 F, AGGCTGTCAATGGCTCTCAGTT TLR9 R, TGAACGATTTCCAGTGGTACAAGT	30

III. Results

1. Alteration of gut microbiota through *ad libitum* drinking of antibiotic cocktails may affect pathology of atopic dermatitis.

1-1 Symptoms of atopic dermatitis differ between mice treated with different antibiotic cocktails.

The mice were divided into three groups, negative control group with only DNCB treatment (Ctrl, n=8) Gram Positive Antibiotics fed group (GPA, n=8) and Gram negative antibiotics fed group (GNA, n=8). There were no significant changes in water consumption differences among groups (data not shown). By the second week of DNCB treatment, some of the mouse started to show symptoms of atopic dermatitis. Even though severities of skin damage by atopic dermatitis are different, overall symptomatic phenotype such as worsening of skin damage over time manifested. At the fourth week of DNCB treatment, 10 weeks of age, skin damage of all mice was extremely severe. Skin damage of each group did not seem to be evident in the second week of treatment. However, from third week and by the fourth week of experiment, 9 and 10 weeks of age respectively, Ctrl and GPA group showed more severe skin damage than GNA group (Fig. 1). Using Eczema Severity Index (ESI, modified ESAI) skin scoring method, skin score from the first

week of DNCB treatment, 7 weeks of age, was measured. Both Ctrl and GPA group showed sharp increase in skin score and GNA showed relatively low skin score throughout the experiment (Fig. 2).

To confirm whether the difference in skin damage corresponds to difference in atopic dermatitis or not, serum IgE concentration was measured. Blood samples were collected and serum IgE concentration was measured to compare severity of atopic dermatitis. Serum IgE concentration measured showed definite elevation at fourth week of DNCB treatment. Elevated serum IgE and severe skin damage in all mice indicated successful atopic dermatitis induction. Similar to skin damage scores, IgE level of GPA is significantly elevated compare to GNA. Ctrl group, however, showed similar IgE level to GNA (Fig 3).

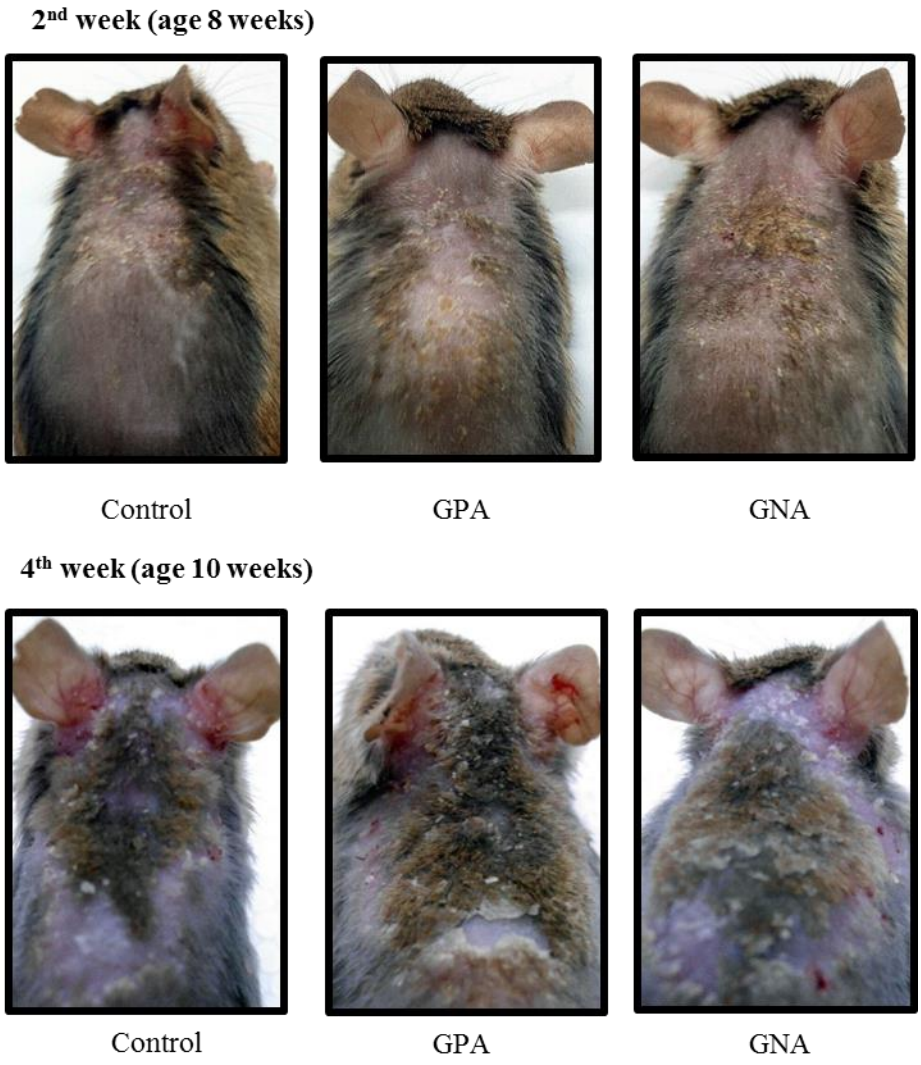


Figure 1. Skin condition of NC/Nga mouse after DNCB treatment.

Skin condition of NC/Nga mouse after 2 weeks and 4 weeks of 2,4-dinitrochlorobenzen treatment are shown. Skin score was based on modified Eczema Severity and Area Index. Photographs were taken from representative samples within each group at 8 weeks and 10 weeks of age. DNCB was applied 3 times a week, initial sensitization with 1% DNCB and then 0.2% DNCB in 3:1 acetone to olive oil mixture. DNCB treatment started 2 weeks after antibiotic cocktail administration, at 6 weeks of age, until the end of experiment at 10 weeks of age (n = 8).

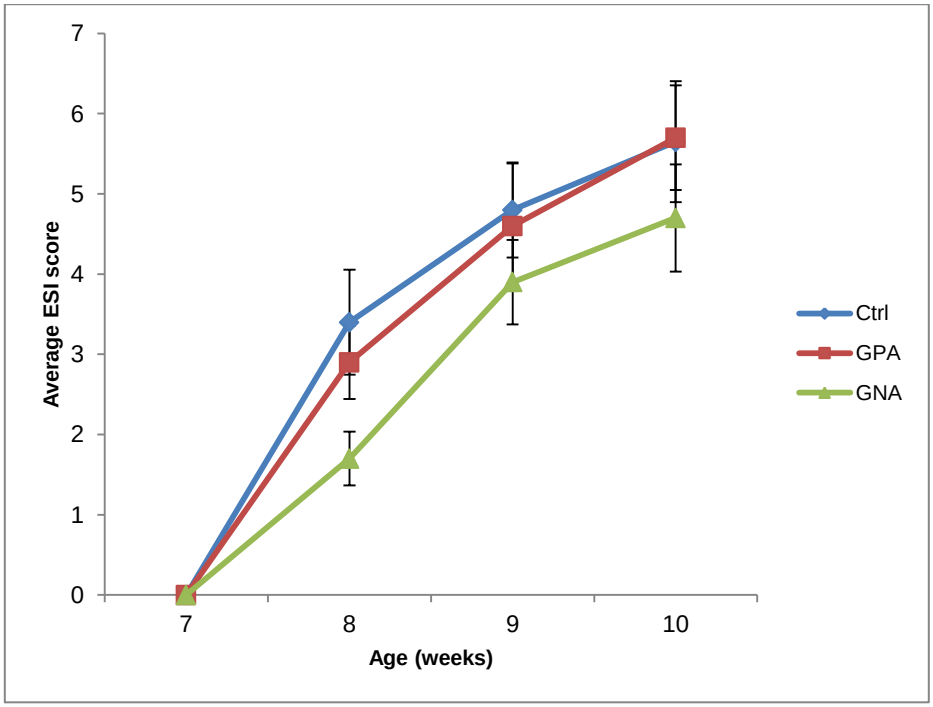


Figure 2. Change in Atopic Dermatitis Score during Antibiotics treatment

Atopic dermatitis skin damage score measured with ESI (modified ESAI) scoring method. It was scored considering 4 factors of skin damage in atopic dermatitis, erythema, induration, excoriations, and lichenfication (from score 0 for no sign of the symptom to 3 for severely affected). Average scores with standard errors are presented (n = 8).

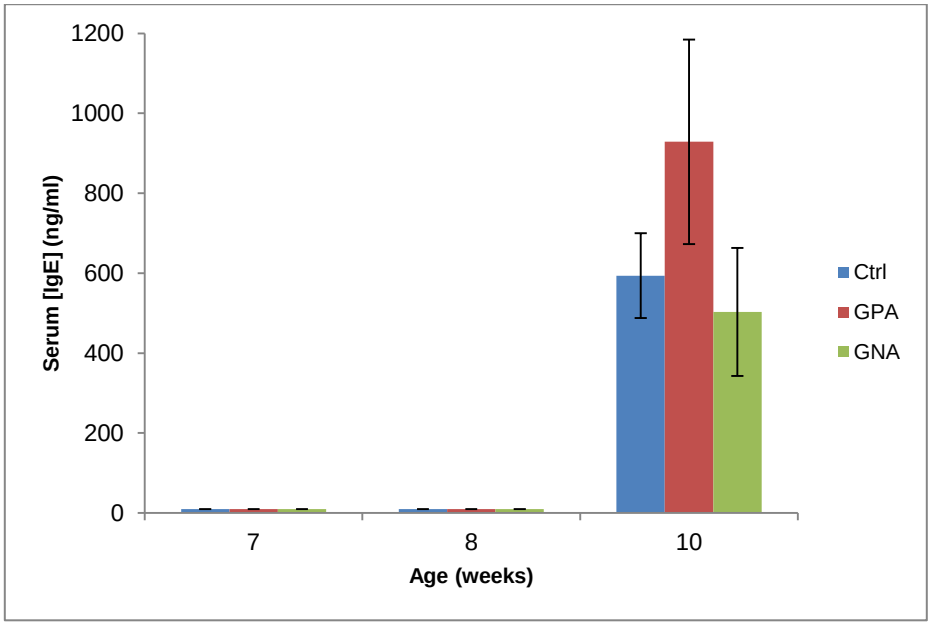


Figure 3. Serum IgE level during Antibiotics treatment

Serum IgE concentration of NC/Nga during experiment was measured. Blood from each mouse was collected every week, and serum was harvested by centrifugation. Average serum IgE concentrations are presented with standard errors (n = 8).

1-2 Antibiotic cocktail induced gut microbiota shift is extremely biased rise in two bacteria species.

With cecal bacteria DNA extracted from each sample, pyrosequencing was preformed to analyze gut microbiota composition. As most mammals do, gut microbiota of NC/Nga mouse were composed mostly of phylum Bacteroidetes and Firmicutes. For GPA and. GNA mouse, gut microbiota composition was significantly shifted as represented in figure 4. For Ctrl mouse, 90% of gut microbiota were Firmicutes, 8% were Bacteroidetes. Gut microbiota of GNA mouse were 99% Firmicutes. For GPA, 86% of gut microbiota were Proteobacteria, and 14% were Deferribacteres (Fig. 4).

Close analysis of GPA gut microbiota revealed that composition of two specific bacteria increased significantly. *Enterobacter cowanii* and *Enterobacter hormaechei* were the two species identified in the gut microbiota composition data analysis. Majority of gut microbiota present in GPA mouse was composed of the two species, from 48% to 77% of total gut microbiota was made up with the two bacteria. Other two groups, Ctrl and GNA did not show this type of identifiable significant change in species level (Fig 5).

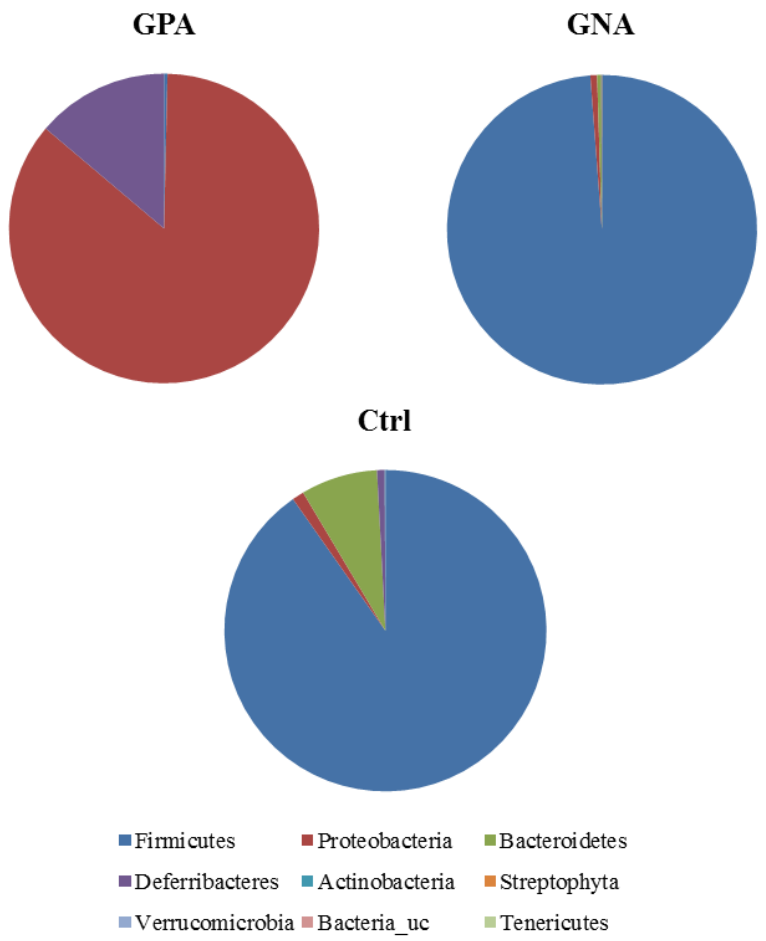


Figure 4. Relative abundances of different bacterial phyla in cecal microbiome of NC/Nga mice.

Genomic DNA was extracted from the cecal samples taken from mice treated with only DNCB for 4 weeks or with antibiotic cocktails for 2 weeks followed by antibiotic cocktails and DNCB for 4 weeks. Ctrl represents mouse with only DNCB treatment, GPA for Gram-positive targeting antibiotics and GNA for Gram-negative targeting antibiotics. Samples were analyzed for the bacterial composition by pyrosequencing V1 to V3 region of bacterial 16S rDNA (n = 8).

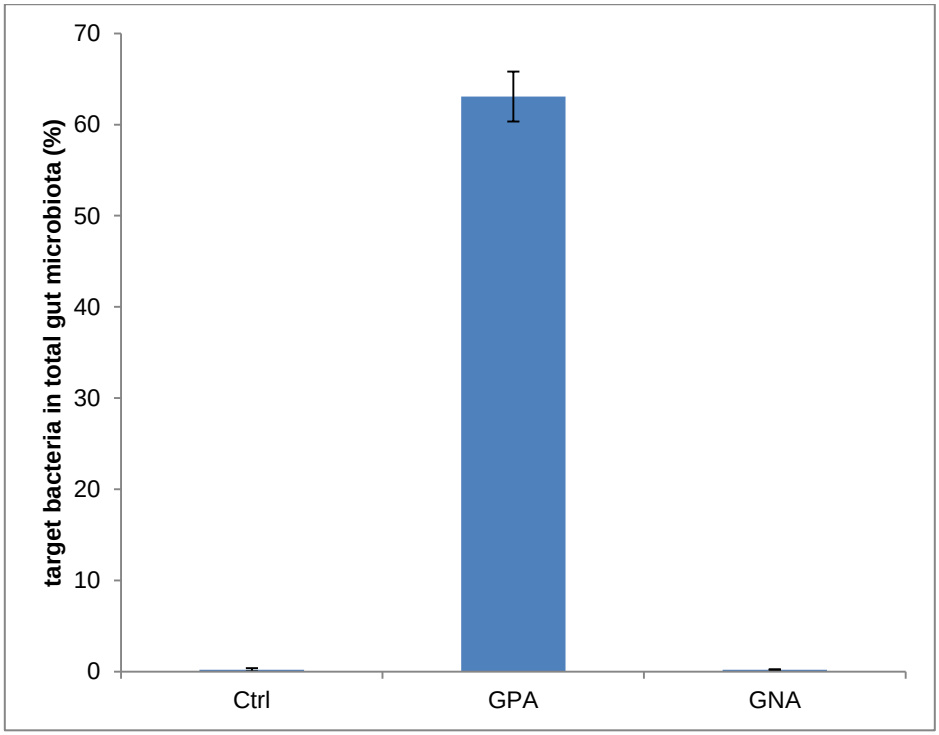


Figure 5. Comparison of percentage of *E.cowanii* and *E. hormaechei* in total gut microbiota among Control, GPA- and GNA- treated mice

GPA-treated mouse showed extremely elevated levels of the two bacterial species. The data were acquired from bacterial composition analysis, and present average reads with standard errors (n = 8).

1-3 Specific antibiotics treatment also shows host gene expression change in intestinal tissue.

Host gene expression was measured to identify possible host reaction antibiotics. As antibiotics may affect directly to intestine, gene expression from intestinal tissue was measured. Out of 17 genes tested, only 4 genes showed significantly altered gene expression. CXCL9 and SAA1 were the two genes which expression was increased in GNA mouse relative to GPA mouse. Expression of CXCL9 in GNA was within normal range, but expression CXCL9 in GPA mouse was significantly lower than Ctrl group. TLR2 and 4 were the other two genes with altered gene expression. Both genes were upregulated in both experimental groups by factor of 3 to 4 (Table 2).

Table 2. Comparison of the expression levels of host genes related to innate immunity (?) among control and GPA- and GNA-treated mice.

Gene	GPA vs control ($2^{-\Delta\Delta Ct}$)	GNA vs control ($2^{-\Delta\Delta Ct}$)
REG3g	1.175	1.481
CCL28	1.310	0.889
CXCL9	0.523	1.209
CCL20	0.912	0.833
SAA1	1.204	3.274
SAA2	1.456	1.492
SAA3	0.953	1.653
Nod2	0.710	1.136
IkB	0.936	1.357
B-def	0.591	0.519
Myd88	0.813	0.920
IRAK1	1.747	1.329
REG3b	1.384	2.054
TLR2	3.125	3.264
TLR4	4.427	3.984
TLR5	0.997	1.731
TLR9	0.602	1.105

Host gene expression levels in intestinal tissue were measured using qRT-PCR. Comparison of gene expression levels was done with the comparative Ct value method described in “Materials and Methods.” All values were normalized with the GAPDH expression level and compared with values from the negative control group (n = 5).

2. Gut microbiota shift caused by either antibiotic cocktails or bacterial colonization causes change in severity of atopic dermatitis.

2-1 Oral gavage of antibiotics or bacteria can alter severity of atopic dermatitis

Second set of experiment was set up to be confirmation experiment of the first one. According to Reikvam, administering antibiotics with drinking water could be unhealthy to the mouse and could cause ineffective antibiotic activity (11). Thus oral gavage was chosen as method for antibiotics delivery. Result from the first experiment suggested two bacteria could be closely related to atopic dermatitis. Thus administrating bacteria to NC/Nga was included in this set of experiment. NC/Nga mouse purchased from Central Animal Lab were divided into 6 groups. First 3 groups are similar to the first experiment, except that the number of mouse is 5 for each group (EXP 2 Antibiotics groups). The other 3 experimental groups were administered with both antibiotic cocktails, GPA and GNA, to simulate gut clearing effect described in Reikvam 2010. After gut clearing, bacteria were administered to each group *Enterobacter cowanii, Enterobacter hormaechei* and both Enterobacter (EXP 2 Bacteria group).

For antibiotics group, skin damage phenotype was evident from second week of DNCB treatment. The skin damage, which corresponds to severity of atopic dermatitis, started to vary between groups at second week treatment as

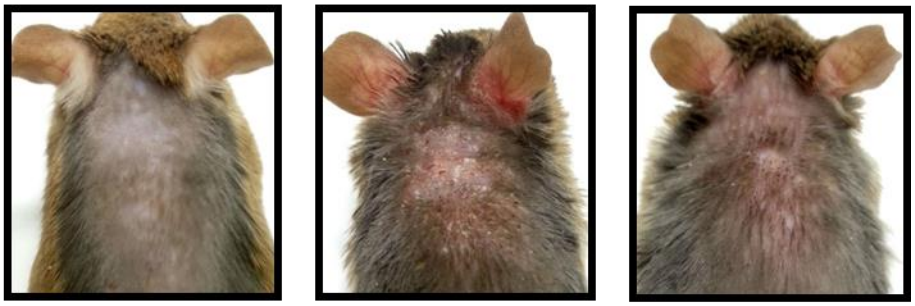
well. By fourth week of DNCB treatment, GPA mouse had severity damaged skin and even ear deformation was observed. Other two groups, Ctrl and GNA showed relatively low signs of skin damage. Skin score also corresponded with the observed state of the mouse. From third week of DNCB treatment, the difference between GPA and GNA was statistically significant (Fig. 6 and 8a).

Serum IgE concentration was similar to the first experiment. Increase in IgE level of GPA group was evident and statistically significant ($P < 0.012$) (Fig. 9a).

For bacteria group, *Enterobacter cowanii* (Ec), *Enterobacter hormaechei* (Eh) and both bacteria (Ech) were orally administered. The progression of skin damage could be observed, with more rapid and severe damage in Eh group. Skin score corresponds with the observation; however none of the sample was statistically significant (Fig. 7 and 8b).

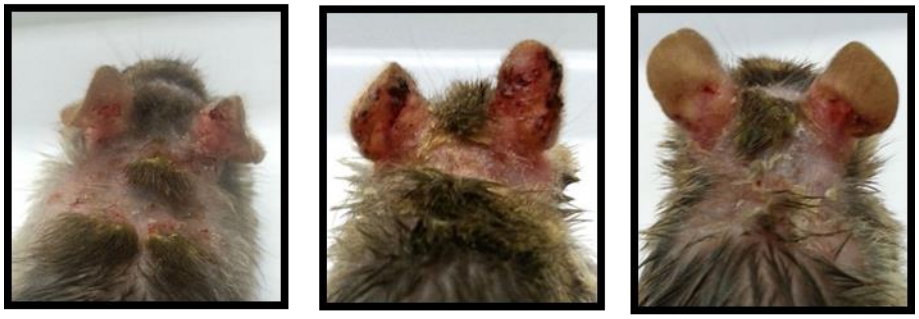
Serum IgE, however, did not correspond to the skin score. Compared to the serum IgE level of control group, all of the mice administered with bacteria had less or equal amount of serum IgE (Fig 9b).

2nd week (age 8 weeks)



Control GPA GNA

4th week (age 10 weeks)

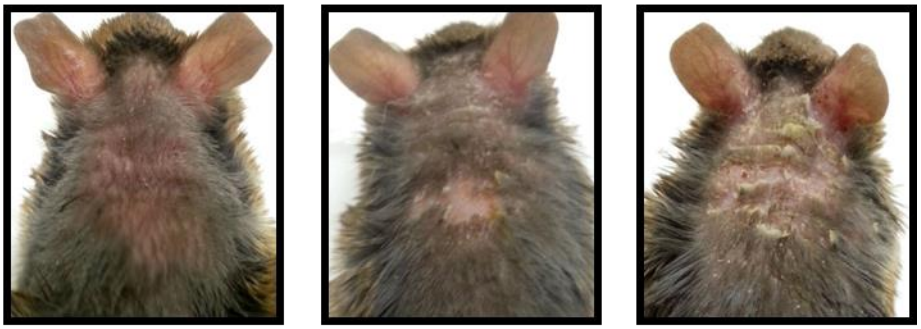


Control GPA GNA

Figure 6. Skin condition of NC/Nga mouse administered with antibiotics during DNCB treatment.

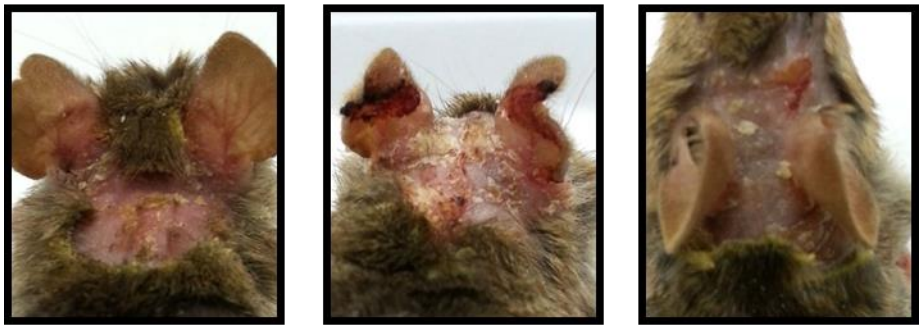
Antibiotics administration started 2 weeks prior to initial sensitization of DNCB. Antibiotics were administered through oral gavage three times a week. DNCB was applied 3 times a week, initial sensitization with 1% DNCB and then 0.2% DNCB in 3:1 acetone to olive oil mixture. The control group showed decreased severity compared to the GPA group which administered GPA orally (n = 5).

2nd week (age 8 weeks)



Ec Eh Ech

4th week (age 10 weeks)



Ec Eh Ech

Figure 7. Skin condition of NC/Nga mouse administered with bacteria during DNCB application.

Bacterial administration started 2 weeks prior to initial sensitization of DNCB. 1 × 10^8 cells of each bacterium were administered through oral gavage DNCB was applied 3 times a week, initial sensitization with 1% DNCB and then 0.2% DNCB in 3:1 acetone to olive oil mixture. Bacteria were administered 3 times a week before DNCB application (n = 4). Ec, *Enterobacter cowanii*; Eh, *Enterobacter hormaechei*; Ech, both *E. cowanii* and *E. hormaechei*.

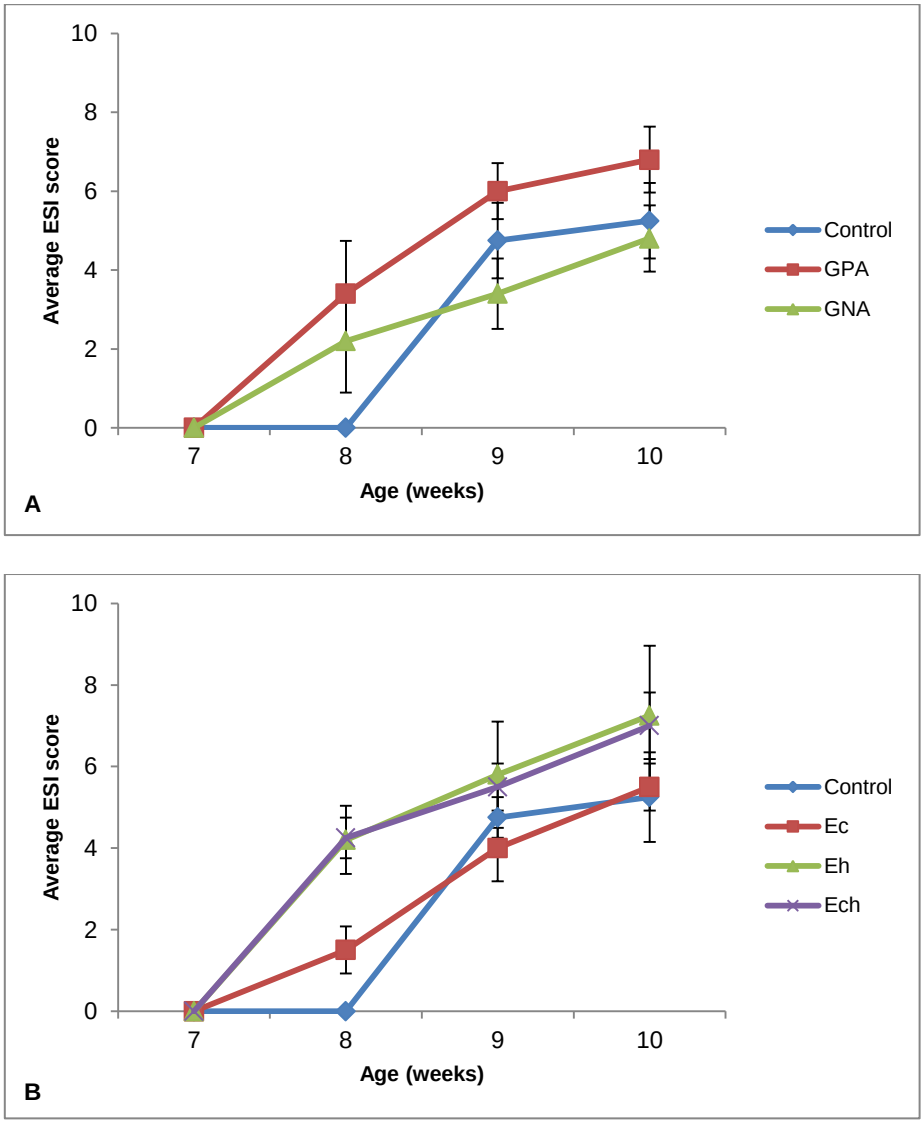


Figure 8. Skin score of NC/Nga mouse during antibiotics treatment and bacterial administration

Atopic dermatitis skin damage score measured with ESI (modified ESAI) scoring method. Score represented is average score with standard error for error bar. **A.** skin score for NC/Nga mouse administered with antibiotics (n=5). **B.** skin score for NC/Nga mouse administered with bacteria (n = 4).

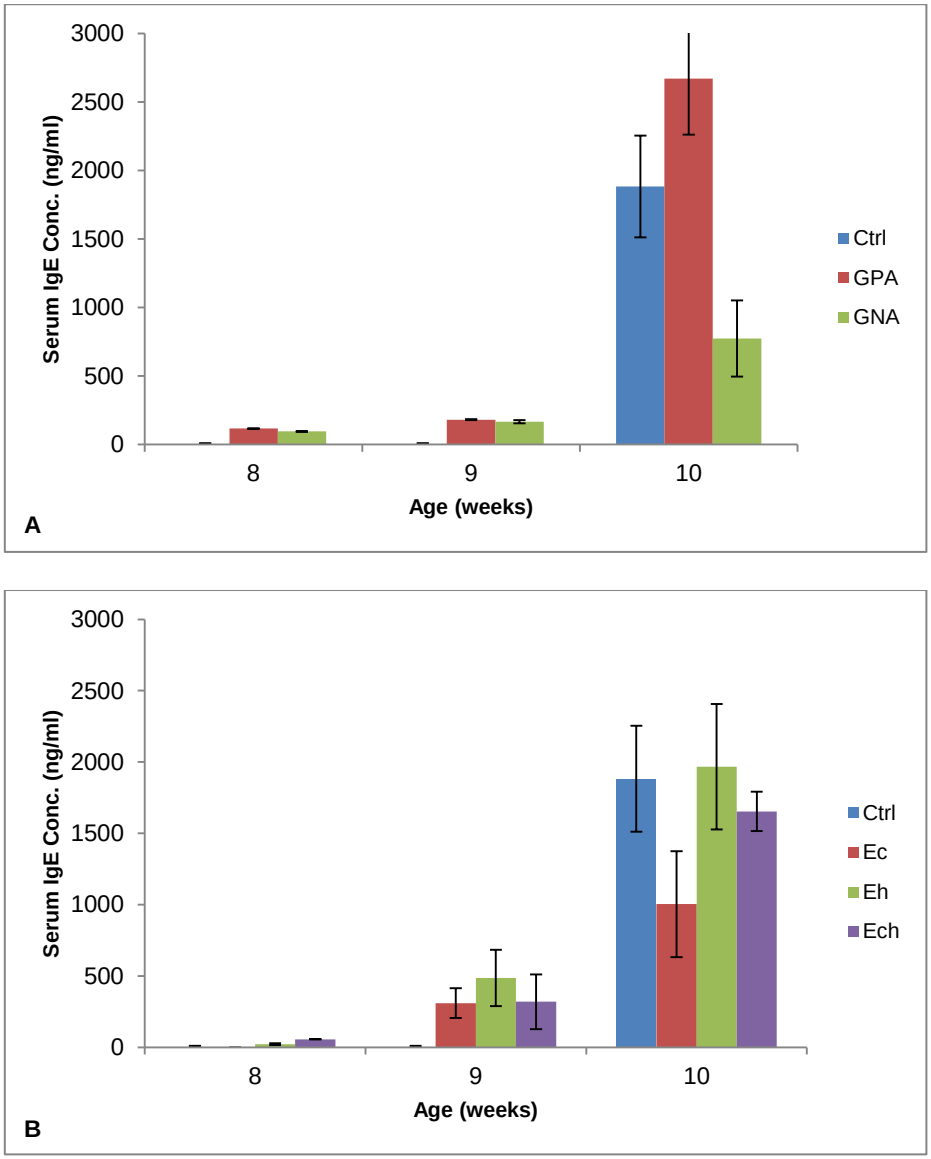


Figure 9. Serum IgE level during antibiotics treatment and bacterial administration.

Blood samples were collected each week and their serum IgE levels were measured using ELISA. All samples were measured in triplicate. **A.** Serum IgE level of NC/Nga mouse administered with antibiotics through oral gavage. **B.** Serum IgE concentration of NC/Nga mouse administered with bacteria through oral gavage (n = 4).

2-2 Confirmation of effect of antibiotics and colonization of orally administered bacteria.

After administrating antibiotics and bacteria, bacterial DNA from stool was extracted from each mouse. Antibiotics treatment significantly decreased total cell number, as total bacterial count decrease form 5×10^8 cells to 2×10^7 cells per mg of stool. GPA also showed effect by decreasing phylum Firmicutes form 2×10^7 cells to 7×10^5 cells per mg of stool. However effect of GNA in stool bacteria was not confirmed as all bacteria including Gram positive bacteria was decreased in the number (Fig. 10). Administration of bacteria showed increase in the specific bacteria as long as the bacteria were administered. This was true for only *Enterobacter hormaechei*, mouse administered with *Enterobacter cowanii* or both bacteria did not show the colonization effect. More than 70% of bacteria detected in stool sample of Eh mouse were identified as *Enterobacter hormaechei* (Fig. 11).

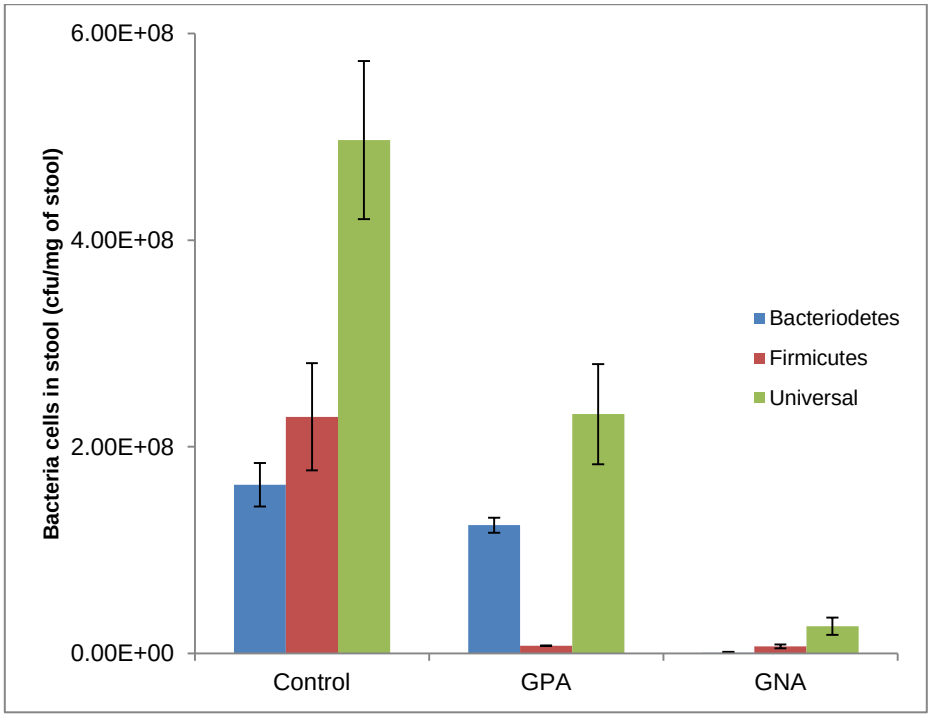


Figure 10. Effect of antibiotics cocktail on gut microbiota at day 3 of the administration

Bacteria from stool samples of NC/Nga mice at day 3 of antibiotic cocktail administration were measured (age 4 weeks + 3 days). Quantitative PCR method using specific primers for 16s rDNA was performed. Universal primer for all bacteria, Firmicutes primer to detect bacteria in phylum Firmicutes, and Bacteroidetes primer to detect bacteria in phylum Bacteroidetes were used. Graph represents average values of triplicated PCR reactions with standard errors (n = 3).

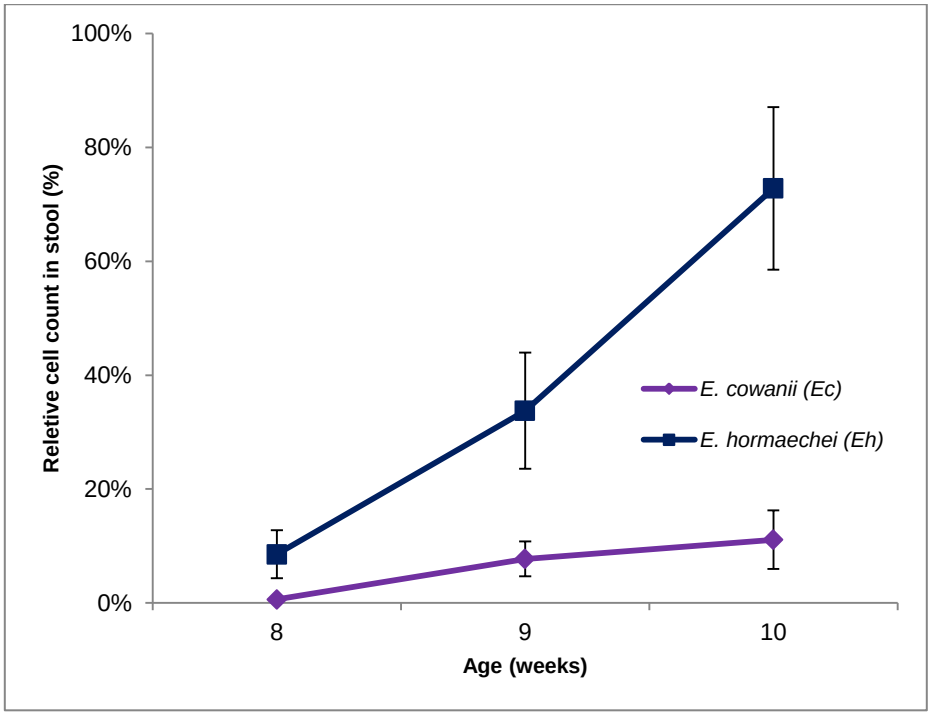


Figure 11. Percent of detected bacteria in stool sample

Stool samples collected every week were analyzed for relative abundance to confirm colonization of administered bacteria. Relative cell number was calculated by quantitative PCR using specific primers for each species and universal bacteria primers to normalize the values. Represented values are average of triplicated PCR with standard error (n = 3).

2-3 Administration of antibiotic cocktail through oral gavage and oral administration of bacteria shows distinct shift in gut microbiota composition.

Gut microbiota of mouse used in the second experiment showed change in their composition. However, it did not showed similar composition change as the first experiment, and individual composition was different so that consensus composition was not observed. The two species observed in the previous experiment was not observed. Gut microbiota composition of the second experiment was different from those of the first. Negative control group had 49% Bacteroidetes, 41% Firmicutes, and 10% Deferribacteres. GNA mouse 58% of Firmicutes and 37% of Bacteroidetes, and 4% of Proteobacteria. Gut microbiota of GPA mouse was composed of 80% Firmicutes, 14% of Deferribacteres, and 6% of Firmicutes (Figure 12).

The administration of bacteria did not showed significant change in their gut microbiota. The colonization effect observed from stool bacteria was not detected in gut microbiota composition analysis data. The percent composition of Proteobacteria was from 0 to 17 percent. Gut microbiota of Ec mouse was 80% Bacteriodetes, 15% Firmicutes, and 4% Proteobacteria. For Eh mouse, gut microbiota was 10% Bacteroidetes and 90% Firmicutes. Ech mouse had gut microbiota with 61% Bacteroidetes, 25% Firmicutes and 13% Proteobacteria (Figure 13).

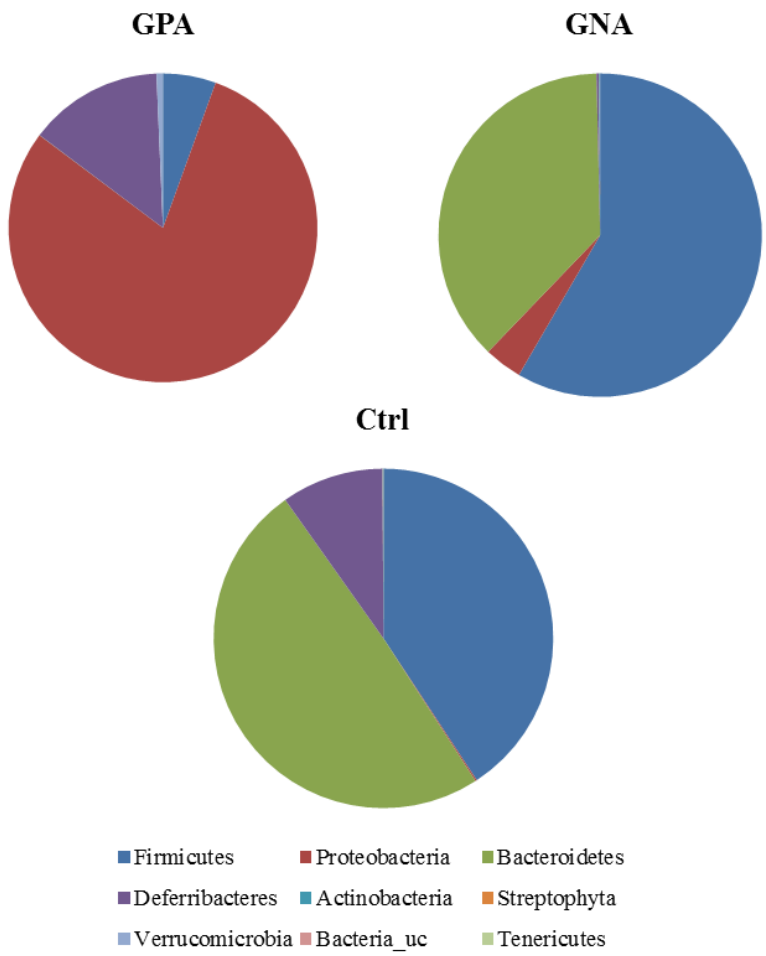


Figure 12. Relative abundances of cecal microbiome of NC/Nga mice after antibiotics treatment

Genomic DNA was extracted from cecal content of each mouse. Each DNA samples were analyzed for the bacterial composition by pyrosequencing of the bacterial 16S rDNA (n = 4)..

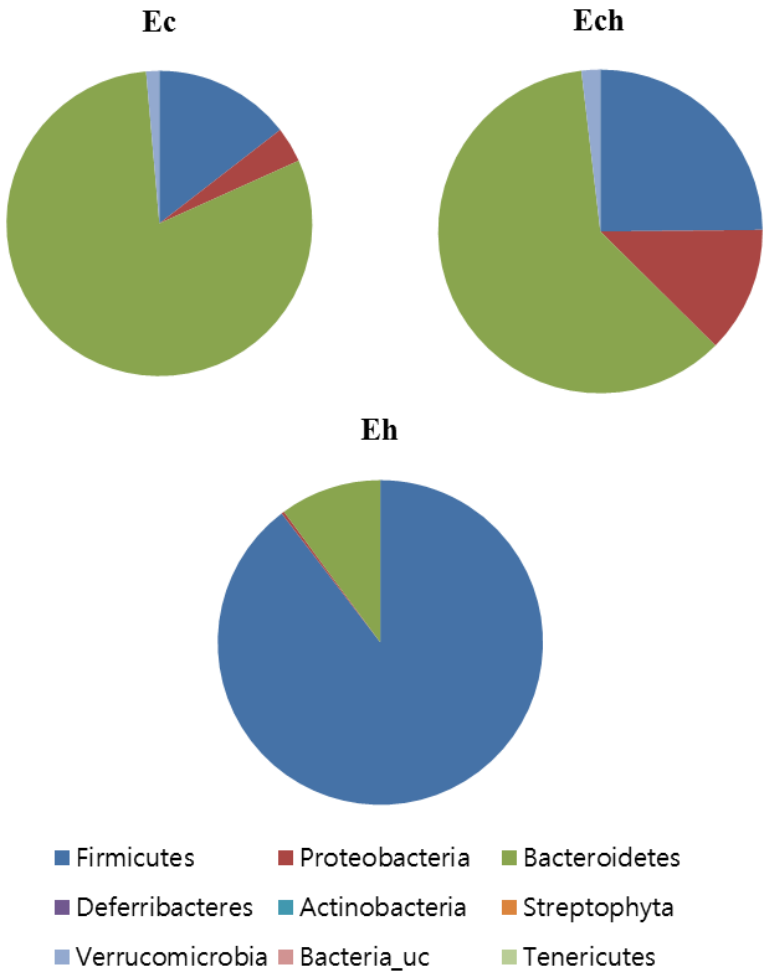


Figure 13. Relative abundances of cecal microbiome of NC/Nga mice after antibiotics treatment

Gut microbiota composition of NC/Nga mouse treated with bacteria through oral gavage.1 × 10^8 bacteria cells in 100μl of PBS was orally administered. Genomic DNA was extracted from cecal content of each mouse. Samples were analyzed for the bacterial composition by pyrosequencing of the bacterial 16S rDNA (n = 4).

IV. Discussion

Gut microbiota is composed of several different phyla of bacteria, and plays significant role in host physiology. Atopic dermatitis is one of the diseases known to be associated with gut microbiota but unclear on its pathophysiology. This research revealed that the administration of antibiotics targeting Gram positive bacteria, making Gamma Proteobacteria the major bacteria in the intestine, can cause intensified symptoms of atopic dermatitis. With increased skin damage and elevated serum IgE level, mouse administered with antibiotics against Gram positive bacteria (GPA) showed more severe signs of atopic dermatitis. Gut microbiota of antibiotics administered mice showed significant shift in their compositions, and two bacterial species where identified to be associated with the increased symptoms of atopic dermatitis. However, these species were not observed in the second set of experiment (EXP 2), where the only variable changed was antibiotics delivery method, from drinking water to oral gavage.

Reikvam et al wrote antibiotics administration by drinking water may expose the mouse to severe health risk, such as dehydration. This phenomenon however was not observed in this study, as water consumption and weight change in both sets of experiment did not differ significantly (data not shown). Even though health risk caused by antibiotics in drinking water may not be present in all experiment, it would be ideal to administer antibiotics through oral gavage because it would eliminate the unknown

variable of difference in exact amount of antibiotics administered per mouse when antibiotics are provided in drinking water *ad libitum*

The only differences between the sets in this study were serum IgE concentration and gut microbiota composition. The first set, where mice were treated with antibiotic cocktails by drinking water showed more biased shift in gut microbiota composition than the second set where mice were treated with the same antibiotics cocktails but through oral gavage. The serum IgE concentration of the second set, however, was far more elevated than the IgE concentration of the first set. The difference in IgE level could be explained by different degree of atopic dermatitis, meaning mice from second set of experiment showed more severe signs of atopic dermatitis than those of the first set

The difference in Gut microbiota composition, however, cannot be explained by experimental variables. Gut microbiota of mouse in set 1 (antibiotics administered with drinking water) showed extremely biased composition, where GPA mouse showed 96% of Proteobacteria, and 63% of total gut microbiota was comprised of the two Gamma Proteobacteria species. Microbial composition at phylum level was similar in both experiment set 1 and 2, but in set 2, the two Enterobacters were not the major species identified. Bacteria called *Klebseiella michiganensis* were major species in phylum Proteobacteria but it was not significantly increased as *Enterobacter cowanii* and *hormaechei* in the first set. Much more studies should be done to understand the gut microbiota shift and possible connection with human physiology.

The two Enterobacters identified from the GPA group of the first experiment, E*nterobacter cowanii* and *hormaechei*, are relatively newly discovered strains of bacteria. Thus not much of research has been done on either of the two. Initial hypothesis for the bacteria administration experiment was that since the rise of the two bacteria in total gut microbiota composition and increase in severity of atopic dermatitis seems to be connected, administration of the two bacteria could induce increased severity of atopic dermatitis. From the skin score data, this hypothesis seemed to be correct, but more subjective biomarker, serum IgE level, did not confirm such. Mouse administered with each bacterium or mixture of the two did not show increased amount of IgE level compared to the control group.

When host gene expression level in intestinal tissue was analyzed, CXCL9 was under expressed in GPA mouse compared to the control mouse, whereas gene expression in GNA mouse was within the normal range. This is opposite of what was previously reported, as CXCL9 of atopic dermatitis patient was reported to be elevated, not decreased (17). Also TLR2 and 4 in both GNA and GPA mouse were significantly overexpressed relative to the control mouse. As TLR2 is reported to augment IgE dependent allergic reaction, over expression of TLR2 in GPA mouse is reasonable, but it is not clear why both GPA and GNA mouse have elevated TLR2 gene expression (18). The unexpected host gene expression level changes in CXCL9 and TLR2, 4 suggest close correlation between atopic dermatitis and immune response in previously undocumented way.

This study concluded that administration of antibiotics could change

murine gut microbiota composition, and altered gut microbiota may led increase or decrease in symptoms of atopic dermatitis. Gram negative bacteria, more specifically Gamma Proteobacteria seemed to worsen the symptoms of atopic dermatitis, as indicated by increase skin damage score and elevated serum IgE level. However, what species of bacteria triggers or worsens the atopic dermatitis symptom is sill unknown. Much more animal studies with large number of subject must be conducted to narrow down and find out key player of atopic dermatitis.

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국문 초록

근래 들어 미생물의 연구를 통한 인간의 질병을 이해하려는 시도가 늘어나고 있다. 그러한 질병들 중 하나는 아토피성 피부염으로서 발생과 진행이 잘 알려지지 있지 않은, 하지만 장내미생물과 관련이 있을 것으로 추정되는 질병 중 하나이다. 아토피성 피부염과 관련이 있는 장내미생물을 찾기 위하여 16s rRNA 의 유전자 태그를 이용한 Pyrosequencing 방법을 통해 장내 미생물의 군집을 알아보려 하였다. 아토피성 피부염의 모델 동물인 NC/Nga 를 통해 각각 그람 양성균을 대상으로 하는 항생제 (GPA) 나 그람 음성균을 대상으로 하는 항생제 (GNA) 또는 아무것도 들어있지 않는 물을 먹여서 총 6주간 실험하였다. 실험은 경구투여를 하거나 식용수에 항생제를 첨가하는 식으로 진행되었다.

GPA를 먹은 쥐들에게서 IgE 의 혈중 농도가 높아짐을 발견하였고, 피부의 변성 상태 또한 다른 쥐들에 비해서 심각해짐을 확인할 수 있었다. Pyrosequencing 을 통한 쥐들의 장내 미생물 군집을 관찰한 결과 GPA 를 먹은 쥐들에게서 특별하게 *Enterobacter cowanii* 와 *Enterobacter hormaechei* 라는 두 가지의 박테리아가 나타남을 확인할 수 있었다. 이 두 가지의 박테리아를 경구투여 하는 방식으로 이 박테리아들과 아토피성 피부염의 상관성을 알아보려 하였다.

Enterobacter hormaechei 를 먹은 쥐들에게서만 IgE 의 증가와 피부 변성이 심화됨을 확인할 수 있었다. 이 실험을 통해 *Enterobacter hormaechei* 가 아토피성 피부염의 증상과 관련이 있음을 확인할 수 있었다.

주요어 : 아토피성 피부염, NC/Nga, 파이로시퀀싱, 항생제, 마이크로바이옴

학 번 : 2011-24028