

# Inhibitory effects of dietary soy isoflavone and gut microbiota on contact hypersensitivity in mice

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## ABSTRACT

Soy isoflavones (SIs) are abundant in soybeans and have inhibitory effects on contact hypersensitivity (CHS), which is often used as a mouse model for allergic contact dermatitis (ACD); however, their therapeutic mechanisms remain unknown. We studied the suppressive activity of dietary SI and gut microbiota on dinitrofluorobenzene (DNFB)-induced CHS. Low-dose SI diets alleviated DNFB-induced ear swelling and oedema and decreased infiltration of Gr-1-positive cells into ear tissue. In addition, dietary SIs also decreased interleukin-1 $\beta$  and chemokine (C-X-C motif) ligand 1 production in ear tissue compared to controls. Furthermore, ciprofloxacin and metronidazole treatments blocked the suppressive activity of dietary SIs on CHS, whereas vancomycin treatment had a marginal effect. These antibiotic treatments differed in their effects on the gut microbiota composition. These results demonstrated that consumption of physiologically relevant doses of SIs reduced CHS symptoms, and suggested that the gut microbiota influenced their suppressive activities on CHS.

## 1. Introduction

Soybean-based foods are rich in biologically active phytochemicals, such as soybean isoflavone (SI) and soyasaponin (SS), and have been consumed in Asian countries for thousands of years. The consumption of SI has been attributed to the reduced prevalence of several chronic diseases (Messina, 2014; Setchell & Cassidy, 1999). Recent studies have shown that SI has significant health-enhancing effects on certain types of cancers (Nagata et al., 2014), cardiovascular diseases (Li et al., 2010), osteoporosis (Alekel et al., 2010), and menopausal symptoms (Taku, Melby, Kronenberg, Kurzer, & Messina, 2012).

Allergic contact dermatitis (ACD) is one of the most common skin diseases caused by delayed-type hypersensitivity (DTH) responses to antigens that come into contact with the skin (Kaplan, Igyártó, & Gaspari, 2012). Contact hypersensitivity (CHS) is an experimental animal model for human (Honda, Egawa, Grabbe, & Kabashima, 2013; Inagaki & Nagai, 2009). In our previous study, we have demonstrated

that oral feeding of a SI-enriched diet produced beneficial effects in mice with 2,4-dinitrofluorobenzene (DNFB)-induced CHS, such as the alleviation of ear tissue oedema and suppression of Gr-1-positive cell infiltration. However, the amount of SIs consumed by SI-treated mice was not physiologically relevant to humans (Nagano et al., 2016). In our other study, we demonstrated that dietary SSs suppressed CHS; the attenuation of the oedema corresponded to the inhibition of inflammation in the ear as evidenced by the reduced infiltration of Gr-1-positive immune cells, and the downregulation of proinflammatory mediators such as chemokine (C-X-C motif) ligand 2 (CXCL2) and triggering receptor expressed on myeloid cells (TREM)-1. In addition, taxonomic analysis based on 16S ribosomal RNA gene (16S rDNA) sequences revealed significant alterations in faecal microbiota caused by CHS, which were reversed by dietary SSs. These results demonstrated that SSs alleviated CHS symptoms by attenuating inflammation and improving the composition of intestinal microbiota (Nagano, Katase, Tsumura, Saito, & Matsuda, 2017). However, the role of gut microbiota

**Abbreviations:** ACD, allergic contact dermatitis; CHS, contact hypersensitivity; CXCL1, chemokine (C-X-C motif) ligand 1; DTH, delayed-type hypersensitivity; DNFB, 2,4-dinitrofluorobenzene; ELISA, enzyme-linked immunosorbent assay; IL-1 $\beta$ , interleukin-1 $\beta$ ; SI, soy isoflavone; SS, soyasaponin; PCA, principal component analysis; 16S rDNA, 16S ribosomal RNA gene

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for the inhibitory activity of SIs on CHS remains unknown.

In the present study, we analysed CHS symptoms by feeding DNFB-induced CHS mice with a lower SI-enriched diet. Furthermore, we used antibiotics to clarify the role of gut microbiota for its inhibitory activity on CHS.

## 2. Materials and methods

### 2.1. Diet and SI

The soybean-free diet F2PLD1 was purchased from Oriental Yeast (Tokyo, Japan), and the SI-enriched formulation Soyaflavone HG (HG) containing 58.4% SIs was obtained from Fuji Oil (Izumisano, Japan).

### 2.2. Mice

Six-week-old female BALB/c mice (15–20 g body weight) were purchased from Japan SLC (Hamamatsu, Japan). The animals were fed F2PLD1 and housed in a temperature-controlled room ( $25 \pm 2^\circ\text{C}$ ) under a 12-h light/dark cycle. The experiments were designed in accordance with the guidelines for animal experimentation and were approved by the Animal Experimental Committee of Kawasaki University of Medical Welfare (authorization number 15-009) and Kawasaki Medical School (authorization number 15-118). To investigate effects of SI doses on CHS, mice were divided into non-CHS control (NC,  $n = 3$ ), CHS control (CC,  $n = 6$ ), low-dose SI-treated (LD,  $n = 6$ ), and high-dose SI-treated (HD,  $n = 6$ ) groups. The NC and CC groups were given water, while the LD and HD groups were given 0.004% and 0.1% HG, respectively, by a water bottle 3 weeks before the first DNFB (Kanto Chemical, Tokyo, Japan) challenge. The antibiotics we tested were diluted in drinking water. Mice were given a mixture of ciprofloxacin and metronidazole or vancomycin (Sigma Aldrich, St. Louis, MO, USA) according to a previously reported method (Suez, Korem, Zeevi, Zilberman-Schapira, Thaiss, Maza, et al., 2014). Mice were divided into CHS control (CC,  $n = 6$ ), SI-treated (SI,  $n = 6$ ), ciprofloxacin, metronidazole and SI-treated (CM,  $n = 6$ ), and vancomycin and SI-treated (VS,  $n = 6$ ) groups. The CC and SI groups were given water and 0.004% HG, respectively. The CM group was given 0.02% ciprofloxacin, 0.1% metronidazole, and 0.004% HG, while the VS group was given 0.05% vancomycin and 0.004% HG. These antibiotics and SI were given to mice by a water bottle 3 weeks before the first DNFB challenge.

### 2.3. Contact hypersensitivity test

The CHS experiments in mice were performed as described previously (Nagano, et al., 2017). Briefly, the mice were sensitized on days 0 and 1 by applying 50  $\mu\text{L}$  0.5% DNFB diluted in acetone/olive oil (4:1) on the shaved abdominal skin. The animals were then challenged by the application of 20  $\mu\text{L}$  0.2% DNFB on the dorsal side of both ears on days 5 and 12. The non-CHS control mice were sensitized and challenged with vehicle (without DNFB). The ear thickness of the mice was measured using a soft touch micrometer (CLM1-15QM, Mitutoyo, Tokyo, Japan). Ear swelling was calculated as the difference in thickness before and after the challenge. All animal experimental procedures were performed under anaesthesia with sevoflurane (Maruishi Pharmaceutical, Osaka, Japan).

### 2.4. Histological analysis

Ear tissue was harvested 24 h after the second DNFB challenge and fixed with IHC Zinc fixative (BD Biosciences, Bedford, MA, USA) for 24 h. Paraffin-embedded ear tissues were stained with haematoxylin and eosin, and analysed by immunohistochemistry performed in a Ventana Discovery XT (Ventana Medical Systems, Tucson, AZ, USA) as described previously (Nagano et al., 2016) using purified anti-mouse

Ly-6G (Gr-1) primary antibodies (eBioscience, San Diego, CA, USA), and biotin anti-rat IgG2b (polyclonal or monoclonal antibodies, eBioscience) secondary antibodies. The number of Gr-1-positive cells was counted at five randomly selected areas ( $\sim 210 \mu\text{m}^2$ ) of tissue sections using an Axio Photo microscope (Carl Zeiss AG, Oberkochen, Germany).

### 2.5. Preparation of tissue lysates from mouse ears

Ear tissues collected 24 h after the second DNFB challenge were stored at  $-80^\circ\text{C}$  before use. The ears were cut, placed into Lysing Matrix D tubes, and lysed in PIPA lysis buffer containing protease inhibitors (ATTO, Tokyo, Japan) by homogenization in the FastPrep-24 instrument (MP Biomedicals, Santa Ana, CA, USA). Protein concentration was determined by the Pierce<sup>TM</sup> BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA) performed in duplicate.

### 2.6. Cytokine antibody array

Cytokine expression was analysed using the Proteom Profiler<sup>TM</sup> mouse cytokine array kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction. Chemiluminescence was detected using an ImageQuant LAS 4000 mini instrument (GE Healthcare Biosciences, Piscataway, NJ, USA), and spot intensity was quantified by the Image Studio Lite software (LI-COR Biotechnology, Lincoln, NE, USA).

### 2.7. Enzyme-linked immunosorbent assay (ELISA)

The expressions of CXCL1 and interleukin-1 $\beta$  (IL-1 $\beta$ ) were determined using a mouse DuoSet ELISA development system (R&D Systems) according to the manufacturer's instruction. The assays were performed in duplicate.

### 2.8. Bacterial DNA sequencing and taxonomic analysis

Fresh faecal pellets were collected on day 13 and stored at  $-80^\circ\text{C}$  before use. Bacteria were recovered from the lysates of faecal samples using the FastDNA Spin Kit for Soil (MP Biomedicals) as previously described (Nagano, et al., 2017). PCR and sequencing were performed by the method as described (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013) using the Illumina Miseq platform (Illumina, San Diego, CA, USA). Briefly, the V4 region of the 16S rDNA was amplified with region-specific primers, which included the Illumina flow cell adapter sequences. For sequencing of custom 16S rDNA amplicons, the 300-cycle MiSeq reagent kit v2 and a Miseq reagent cartridge were used. Taxonomic analysis of the 16S rDNA sequences was conducted by the TechnoSuruga laboratory (Shizuoka, Japan) based on the Ribosomal Database Project using the Metagenome@KIN analysis software (World Fusion, Tokyo, Japan).

### 2.9. Statistical analysis

The data were represented as the mean  $\pm$  standard error of the mean (SEM). Statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc tests, using the Origin 2018 software (OriginLab, Northampton, MA, USA). The data were considered significant at  $p < 0.05$ .

## 3. Results

### 3.1. Dietary low-dose and high-dose SIs inhibit CHS

The suppressive effect of SI-enriched diets on CHS was evaluated by measuring ear swelling 24 h after the second DNFB challenge (Fig. 1A). Compared to the CHS control group ( $0.167 \pm 0.007 \text{ mm}$ ), the low-

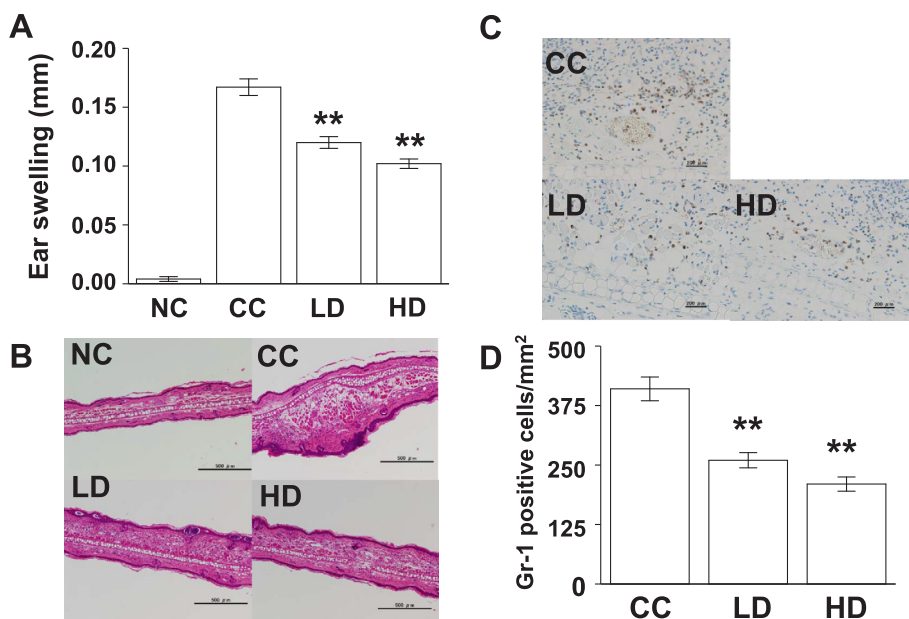


**Fig. 1.** Dietary soy isoflavones (SIs) reduce ear swelling, tissue edema, and the infiltration of myeloid cells into ear tissues in mice with contact hypersensitivity (CHS). (A) Ear thickness was measured before and 24 h after the second DNFB challenge, and ear swelling was calculated as the difference in ear thickness between these two points. (B) Images of ear tissues stained with haematoxylin and eosin. Scale bars, 500 µm. (C) Immunohistochemistry images stained with an anti-Gr-1 antibody. Dark brown, Gr-1-positive cells; blue, nuclei stained with haematoxylin. Scale bars, 200 µm. (D) The number of Gr-1-positive cells infiltrating ear tissues. NC, non-CHS control group; CC, CHS control group; LD, low-dose SI-treated CHS group; HD, high-dose SI-treated CHS group. The data are presented as the means  $\pm$  SEM of two independent experiments; \*\* $P$  < 0.01 vs. CC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

( $0.119 \pm 0.005$  mm) and high-dose SI-treated ( $0.101 \pm 0.004$  mm) groups showed reduced ear swelling (28% and 40%, respectively;  $P$  < 0.01). Histopathological analysis of inflamed ear tissues 24 h after the second DNFB challenge revealed prominent tissue oedema and increased infiltration of immune cells in the CHS mice compared to the non-CHS control mice, which was alleviated by the SI-enriched diets (Fig. 1B). The infiltration of myeloid immune cells was also assessed by staining for the Gr-1 antigen (Fig. 1C), a marker of granulocytes and monocytes typically recruited in large numbers to the site of allergen challenge during the effector phase. Compared to the CHS control group ( $413 \pm 25$  cells/mm<sup>2</sup>), the low-dose SI-treated ( $259 \pm 16$  cells/mm<sup>2</sup>) and high-dose SI-treated ( $210 \pm 15$  cells/mm<sup>2</sup>) groups showed decreases in Gr-1-positive cells that infiltrated into the inflamed ear tissues (39% and 49%, respectively;  $P$  < 0.01) as shown in Fig. 1D.

### 3.2. Attenuating effects of dietary SI on the secretion of cytokines

Cytokine antibody array analyses were used to examine the differences in cytokine expression in the inflamed ear tissues 24 h after the second challenge with DNFB in the high-dose SI-treated and CHS control mice. The comparison between the high-dose SI-treated and CHS control mice showed that the suppressed cytokines were CXCL1, IL-1 $\beta$ , monocyte chemoattractant protein 1 (MCP-1), and chemokine (C-C motif) ligand 3 (CCL3) (Table 1). The productions of IL-1 $\beta$  and CXCL1 in the ear tissues were also examined via ELISA. The productions of IL-1 $\beta$  in the ear tissues were reduced in the low- and high-dose SI-treated groups than in the CHS control group (Fig. 2A). The levels of CXCL1 were significantly suppressed in the high-dose SI-treated group compared to the CHS control group, whereas no significant differences were

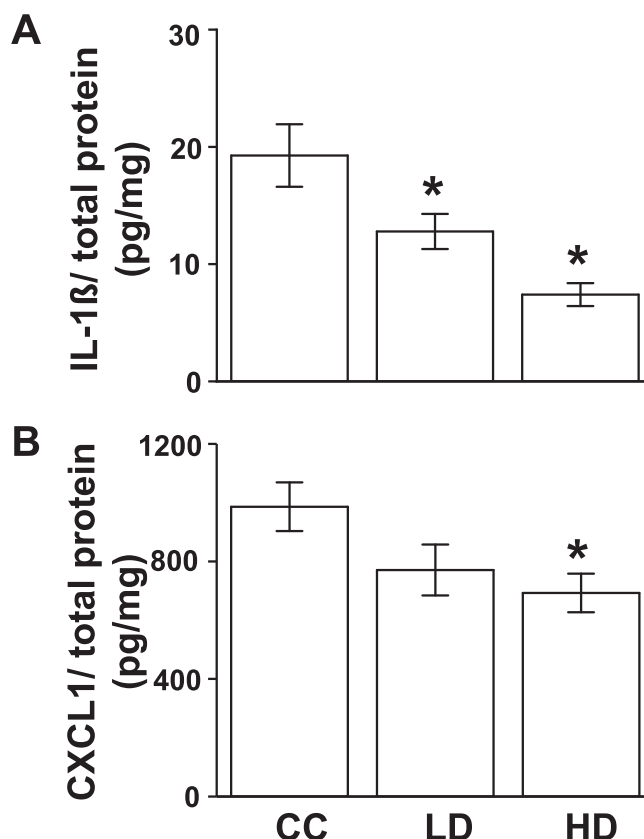


**Fig. 2.** Dietary soy isoflavones (SIs) suppress CXCL1 and IL-1 $\beta$  production in ear tissues of mice with contact hypersensitivity (CHS). IL-1 $\beta$  (A) and CXCL1 (B) levels were measured by ELISA. CC, CHS control group; LD, low-dose SI-treated CHS group; HD, high-dose SI-treated CHS group. The data are presented as the means  $\pm$  SEM of two independent experiments; \* $P$  < 0.05 vs. CC.

**Table 1**

Proinflammatory cytokines downregulated by a high dose of dietary soy isoflavones in mice with contact hypersensitivity.

| Cytokines    | Fluorescence intensity |             |       |
|--------------|------------------------|-------------|-------|
|              | High-dose SIs SSs      | CHS control | Ratio |
| CXCL1        | 5600                   | 2600        | 0.46  |
| IL-1 $\beta$ | 8200                   | 4900        | 0.59  |
| MCP-1        | 14,000                 | 9000        | 0.64  |
| CCL-3        | 8200                   | 5400        | 0.66  |

SSs, soy isoflavones; CHS, contact hypersensitivity.

observed between the low-dose SI-treated and CHS control groups (Fig. 2B).

### 3.3. Antibiotic treatment blocked the inhibitory activity of dietary SI on CHS

To investigate the role of intestinal microbiota for the inhibitory

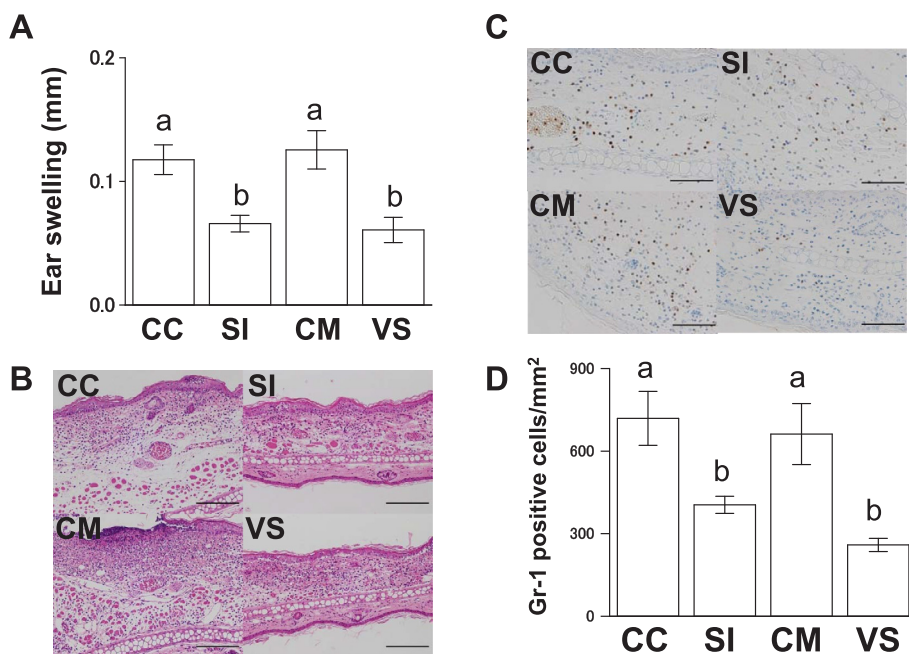


**Fig. 3.** Antibiotic treatment influences ear swelling, tissue oedema, and the infiltration of myeloid cells into ear tissues in mice with contact hypersensitivity (CHS). (A) Ear thickness was measured before and 24 h after the second DNFB challenge, and ear swelling was calculated as the difference in ear thickness between these two points. (B) Images of ear tissues stained with haematoxylin and eosin. Scale bars, 200 µm. (C) Immunohistochemistry images stained with an anti-Gr-1 antibody. Dark brown, Gr-1-positive cells; blue, nuclei stained with haematoxylin. Scale bars, 100 µm. (D) The number of Gr-1-positive cells infiltrating ear tissues. CC, CHS control group; SI, soy isoflavone (SI)-treated group; CM, ciprofloxacin, metronidazole, and SI-treated group; VS, vancomycin and SI-treated group. The data are presented as the means ± SEM; different letters indicate significant differences ( $P < 0.01$ ). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activity of SIs on CHS, we treated the SI-treated mice with antibiotics. The effects of antibiotics on the suppressive activity of CHS by SIs were evaluated by measuring ear swelling 24 h after the second DNFB challenge (Fig. 3A). Compared to the SI-treated mice ( $0.066 \pm 0.007$  mm), the ear swelling significantly increased in the mice treated with ciprofloxacin, metronidazole, and SIs ( $0.126 \pm 0.016$  mm). In addition, compared to the CHS control group ( $0.118 \pm 0.012$  mm), no significant differences were observed in the mice treated with ciprofloxacin, metronidazole, and SIs, indicating that the inhibitory activity of SIs on CHS was blocked by ciprofloxacin and metronidazole treatment. In contrast, there was no significant difference between SI-treated mice and the mice treated with vancomycin and SIs ( $0.061 \pm 0.010$  mm). Histopathological analysis of inflamed ear tissues 24 h after the second DNFB challenge revealed that the prominent tissue oedema and increased infiltration of immune cells in the CHS mice were alleviated by the SI-enriched diets. These alleviations were blocked by ciprofloxacin and metronidazole treatments, but not by vancomycin (Fig. 3B). The infiltration of myeloid immune cells was also accessed by staining for Gr-1 (Fig. 3C), and the number of infiltrating myeloid immune cells into the inflamed ear tissues was counted (Fig. 3D). Compared to the SI-treated mice ( $405 \pm 32$  cells/mm<sup>2</sup>), the number of Gr-1-positive cells that infiltrated to the inflamed ear tissues increased in the mice treated with ciprofloxacin, metronidazole, and SIs ( $662 \pm 111$  cells/mm<sup>2</sup>). In addition, compared to the CHS control group ( $719 \pm 98$  cells/mm<sup>2</sup>), there were no significant differences in the mice treated with ciprofloxacin, metronidazole, and SIs. In contrast, there were no significant differences between the SI-treated mice and mice treated with vancomycin and SIs ( $259 \pm 24$  cells/mm<sup>2</sup>). These results demonstrated that the inhibitory activities of dietary SI on CHS were blocked by ciprofloxacin and metronidazole treatments, but not by vancomycin.

#### 3.4. Antibiotic treatment alters the composition of gut microbiota

The composition of the intestinal bacterial community was analysed by sequencing bacterial 16S rDNA. In the principal component analysis (PCA) at the phylum level (Fig. 4A), the microbiota composition between the antibiotic-treated and untreated groups can be well separated on the PC1 axis (70.6% of explained variance), thus emphasizing the large shift in microbiota composition that resulted after the antibiotic treatment. In the PCA at the family level (Fig. 4B), the compositions of

gut microbiota between the SI-treated and CHS control mice cannot be separated, suggesting that dietary SIs did not alter the composition of gut microbiota in CHS mice. The PCA plot also showed that the microbiota compositions of mice treated with ciprofloxacin and metronidazole were different from those treated with vancomycin as indicated by the PC2 axis (15.8% of explained variance), indicating different anti-bacterial effects of these antibiotics.

At the phylum level, the antibiotic-treated mice demonstrated an increase in Proteobacteria and a reduction in Bacteroidetes and Actinobacteria compared to the untreated mice, which led to an increase in the Firmicutes to Bacteroidetes (F/B) ratio in the gut (Fig. 4C). At the family level, Streptococcaceae increased in mice treated with ciprofloxacin and metronidazole, whereas Lactobacillaceae and Enterobacteriaceae increased in mice treated with vancomycin, compared to the SI-treated and CHS control mice (Fig. 4D).

#### 4. Discussion

Our results demonstrated that low-dose SI diets suppressed CHS in mice. Specifically, we observed attenuated oedema corresponding to the inhibition of inflammation in the ear of CHS mice, as evidenced by the decreased ear swelling, the reduced infiltration of Gr-1-positive immune cells, and the downregulation of pro-inflammatory mediators such as IL-1 $\beta$  and CXCL1. In our previous study, mice were fed only the high-dose of SIs (0.1% HG via drinking), resulting in high levels of SI-intake (Nagano, et al., 2016). HG contained 27.1% SI aglycone, and the measured average daily intake of water was 3–5 g per mouse. As such, each mouse receiving this high dose consumed 0.8–1 mg of SIs per day, or approximately 40 mg SIs/kg bodyweight/day, which corresponds to approximately 200 mg per day for a 50-kg human. For comparison, SI intake, although variable across populations, ranges from 25 to 50 mg/day in Japan and other Asian countries (Messina, Nagata, & Wu, 2006). According to Japanese nutritional guidelines, 70–75 mg/day is the maximum safe level for dietary SI aglycone intake. Thus, the low SI dose in the present study administered to the mice corresponds to approximately 75 mg SI for a human per day. As such, the findings presented here indicate that safely intake levels of dietary SIs may be sufficient to yield beneficial effects in individuals with ACD.

In this study, cytokine antibody array and ELISA analyses showed that dietary SIs reduced the secretion of IL-1 $\beta$  and CXCL1 in contact allergic ear tissues. IL-1 $\beta$  is a pro-inflammatory cytokine that plays

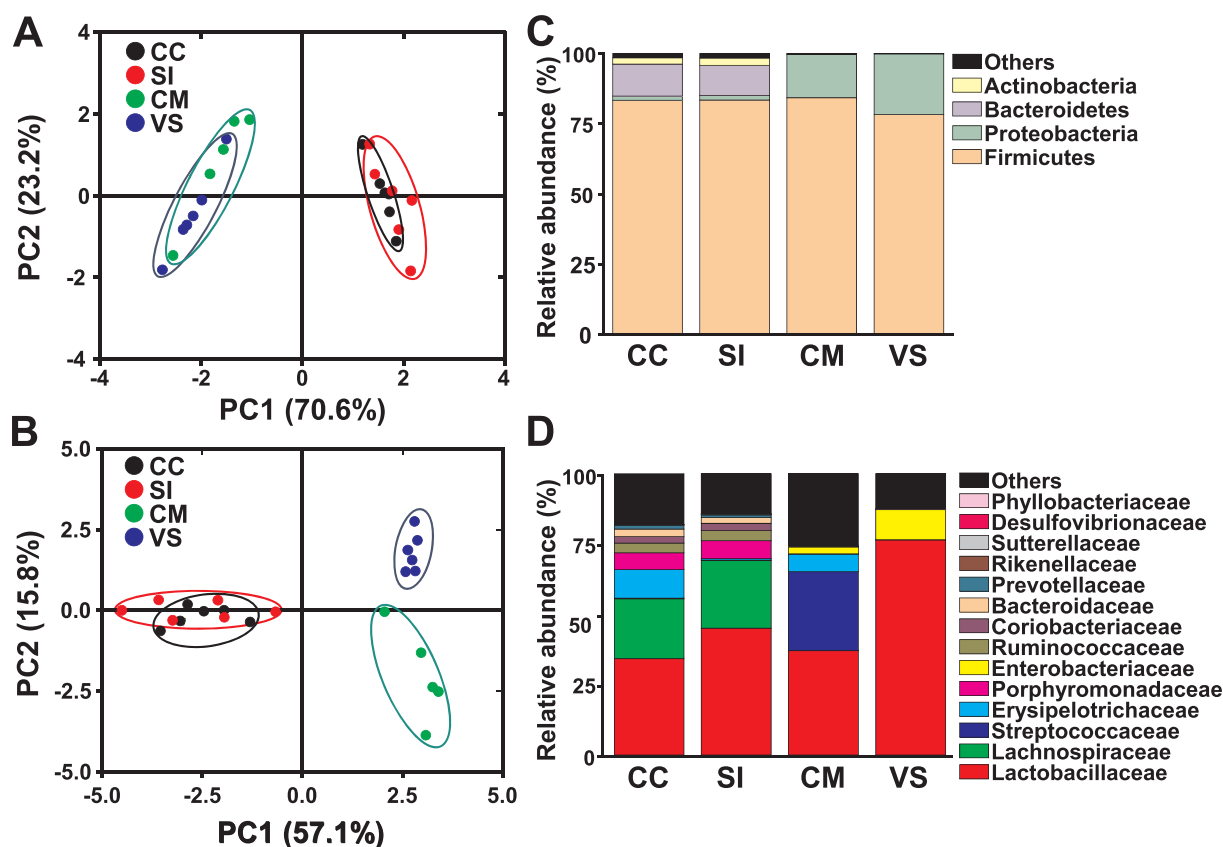


**Fig. 4.** Antibiotic treatment alters the composition of gut microbiota in mice with contact hypersensitivity (CHS). The composition of gut microbiota was analysed by sequencing the bacterial 16S ribosomal RNA gene, indicating principal component analysis (PCA) at the phylum (A) and family levels (B), and taxonomic distribution at the phylum (C) and family levels (D). CC, CHS control group; SI, soy isoflavone (SI)-treated group; CM, ciprofloxacin, metronidazole and SI-treated group; VS, vancomycin and SI-treated group.

critical roles in eliciting CHS (Grabbe & Schwarz, 1998; Honda et al., 2013). In antigen-nonspecific inflammation, haptens stimulate keratinocytes to produce pro-inflammatory cytokines such as IL-1 $\beta$  and tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ). The IL-1 $\beta$ -deficient mice exhibited defective CHS responses to the hapten trinitrochlorobenzene (TNCB) (Shornick et al., 1996). Moreover, CHS responses are dependent on the NLRP3 inflammasome and IL-1 receptor (IL-1R) signalling. The NLRP3 inflammasome activates caspase-1, which cleaves the inactive precursors of IL-1 $\beta$ . Mice deficient in NLRP3 and the adaptor protein ASC showed impaired CHS responses to trinitrophenylchloride (Sutterwala et al., 2006). Similar effects were observed in caspase-1- and IL-1R-deficient mice, and in mice treated with an IL-1R antagonist (Watanabe et al., 2007). In contrast, CXCL1 and CXCL2 are neutrophil-recruiting chemokines. The infiltration of inflammatory cells into ear tissues during CHS arises from the sequential and coordinated expression of chemokine genes (Mitsui et al., 2004; Niwano, Mitsui, & Kohno, 2008). In a previous study, the expression levels of chemokine genes were examined at the elicitation site of TNCB-induced CHS mice. The expression levels of the 11 genes examined were assigned to four groups based on their kinetic patterns. At the early time point, there was increased expression of CXCL1 and CXCL2 (Mitsui et al., 2004). In addition, the blockage of EP3, one of four subtypes of prostaglandin E<sub>2</sub> receptors, by an EP3-selective antagonist (ONO-AE-248) inhibited gene expression of CXCL1 and suppressed CHS during the elicitation phase (Honda et al., 2009). In this study, the ear tissues of SI-fed mice exhibited reduced infiltration of Gr-1-positive myeloid cells. Therefore, the observed suppressive effects of SI diets on the expression of CXCL1 correlates with reduced infiltration of neutrophils and monocytes into the contact allergic ear tissues.

Antibiotic treatment is a useful method to gain insights into the role

of intestinal microbiota in disease animal models (Russell et al., 2012; Suez et al., 2014). In a previous study, Suez et al. (2014) showed that consumption of non-caloric artificial sweeteners (NAS) could induce glucose intolerance in mice. This phenotype was abrogated by antibiotic treatment, as differences in glucose intolerance between NAS-drinking mice and glucose-drinking controls were abolished after a 4-week treatment with ciprofloxacin and metronidazole. Similar effects were observed with vancomycin treatment. These results suggest that the NAS-induced glucose intolerance was mediated through alterations to the commensal microbiota (Suez et al., 2014). In another study, Russell et al. (2012) indicated that neonatal, but not adult, vancomycin-treated mice showed increased severity of experimental allergy asthma. Both neonatal and adult antibiotic treatments altered the compositions of gut microbiota; however, the neonatal vancomycin-treated mice possessed microbiota compositions that were completely different from mice treated with vancomycin only as adults. The different extent of microbiota compositions could explain differences in disease susceptibility (Russell et al., 2012). In this study, ciprofloxacin and metronidazole treatment blocked the suppressive activity of CHS by dietary SIs, as evidenced by the ear swelling and infiltration of Gr-1-positive immune cells into the ear tissues. In contrast, vancomycin treatment had little effect on their inhibitory activity of CHS. These antibiotic treatments changed the composition of gut microbiota differently, as Streptococcaceae increased in ciprofloxacin and metronidazole-treated mice, whereas Lactobacillaceae and Enterobacteriaceae increased in vancomycin-treated mice, compared to the SI-treated and CHS control groups. These results indicated that the gut microbiota influenced the inhibitory activity of SIs on CHS. However, we cannot explain from the present study if the gut microbiota contributed to the inhibitory activity of SIs on CHS.

The SIs were expected to exert an inhibitory activity on CHS without gut microbiota because the injection of genistein, a major component of SI aglycone, has been shown to suppress DTH (Verdrengh, Jonsson, Holmdahl, & Tarkowski, 2003; Yellayi et al., 2003). Verdrengh et al. (2003) demonstrated that DTH response to oxazolone was more significantly suppressed in genistein-treated mice than in the control mice. Yellayi et al. (2003) reported that genistein suppressed the DTH response to 4-hydroxy-3-nitrophenyl acetyl succinimide in ovariectomized mice. There was a 46–67% decrease in the DTH response in the footpads of mice injected with 8–80 mg/kg genistein compared to that in the controls. In this study, ciprofloxacin and metronidazole treatment blocked the inhibitory activity of SIs on CHS. Therefore, the severity of CHS symptoms may be due to the gut microbiota, which were altered by ciprofloxacin and metronidazole treatments. Further investigations are necessary to clarify the role of gut microbiota on the inhibitory activity of SI on CHS.

In conclusion, our data provide evidence demonstrating that low-dose SI diets are capable of attenuating CHS by inhibiting IL-1 $\beta$  and CXCL1 expression at CHS elicitation sites. These results suggest that the consumption of physiologically relevant doses of SI may be of therapeutic value for patients with ACD. In addition, our data support the possibility that the gut microbiota influences the inhibitory activity of SIs on CHS.

### Conflict of interest

MK and KT are employees of Fuji Oil. TN declares no conflict of interest.

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