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Dietary soyasaponin attenuates 2,4-dinitrofluorobenzene-induced contact

hypersensitivity via gut microbiota in mice

Short title: SS attenuates CHS via gut microbiota

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Summary

Soyasaponins (SSs) are abundant in soybeans and display inhibitory activity against contact hypersensitivity (CHS), which is often used as a mouse model for allergic contact dermatitis (ACD); however, their therapeutic mechanisms remain unknown. Here, we attempted to clarify the role of gut microbiota in the inhibition of CHS by dietary soyasaponins. For antibiotic treatment, mice were administered a mixture of ciprofloxacin and metronidazole or vancomycin. These antibiotics and SSs were given to mice via drinking water 3 weeks prior to CHS induction with 2,4-dinitrofluorobenzene, and the mice were analysed for ear swelling, tissue oedema, infiltration of Gr-1-positive immune cells, the composition of faecal microbiota, and regulatory T (T_{reg}) cells. The soyasaponin diets attenuated ear swelling and tissue oedema, and reduced the number of Gr-1-positive cells infiltrating ear tissues. CHS caused changes in the structure of the gut microbiota, but dietary SSs blocked the changes in the microbiota composition. Ciprofloxacin and metronidazole treatments significantly enhanced the severity of CHS symptoms, whereas vancomycin treatment blocked the suppressive effect of dietary SSs on CHS. These antibiotic treatments differed in their effects on the gut microbiota composition. T_{reg} cells in auricular lymph node and spleen increased under SS-enriched diets, but this increase was blocked by vancomycin treatment. These results suggest that dietary SSs exert their inhibitory activity on CHS via the gut microbiota in mice, suggesting that dietary supplementation

with SSs may have beneficial effects on ACD patients, but that the gut microbiota is a critical determinant of the therapeutic value of dietary SSs.

Introduction

Soybean-based foods are rich in biologically active phytochemicals such as soy isoflavones (SIs) and soyasaponins (SSs). The SS content of soybeans, and their protein fraction, is 0.47 % and 0.76 %, respectively [1]. SSs are triterpenoid saponins, varying in the type of aglycones (soyasapogenols) and the position of attached sugar chains, which are classified into four groups: A, B, E, and DDMP [2].

Soyasaponins have been reported to provide a range of health benefits, including antioxidant, anti-viral and anti-cancer activity, cardiovascular protection, and inhibition of inflammation [3,4]. Soyasaponins suppress the release of proinflammatory mediators, such as cytokine tumour necrosis factor (TNF)- α and chemokine monocyte chemoattractant protein (MCP)-1, and inhibit the activation of transcription factor NF- κ B in LPS-stimulated peritoneal macrophages [5]. The main components of SSs, A1, A2, and I, have been shown to attenuate inflammation by inhibiting reactive oxygen species (ROS)-mediated activation of the NF- κ B pathway in LPS-stimulated RAW264.7 murine macrophage [6]. In mice, orally administered SSs Ab and I attenuated trinitrobenzene sulfonic acid (TNBS)-induced colitis by ameliorating colon shortening and inhibiting myeloperoxidase activity, cyclooxygenase-2 (COX-2) expression, and NF- κ B activation in the colon [7,8]. Soyasaponins are expected to exert their effects mostly in the gastrointestinal tract, because they are poorly absorbed [4].

Allergic contact dermatitis (ACD) is one of the most common skin diseases caused by delayed-type hypersensitivity (DTH) responses to antigens that come into

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contact with the skin [9]. Contact hypersensitivity (CHS) is an experimental animal model for human ACD [10–12]. In our previous study, we showed that dietary SSs suppressed 2,4-dinitrofluorobenzene (DNFB)-induced CHS: the attenuation of 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 blocked by dietary SSs. These results demonstrated that SSs alleviated CHS symptoms, perhaps through a link with the function of the intestinal microbiota [13]. However, the role of gut microbiota in the inhibitory activity of SSs on CHS remains unknown.

In the present study, we used antibiotics to clarify the role of gut microbiota in the inhibitory activity of dietary SSs on CHS. In addition, regulatory T (T_{reg}) cells in auricular lymph node and spleen were analysed, because they have taken centre stage in our understanding of how to reduce allergic inflammation in conditions ranging from CHS to allergies [14–16].

Materials and methods

Diet and SS

The soybean-free diet F2PLD1 was purchased from Oriental Yeast (Tokyo, Japan), and the SS-enriched formulation, Soyhealth SA (SA), containing 58.4 % SSs, was obtained from Fuji Oil (Izumisano, Japan).

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 ± 2 °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 dietary SSs, mice were divided into non-CHS (NC, n = 5), SS-treated non-CHS (SN, n = 5), CHS control (CC, n = 5), and SS-treated CHS (SC, n = 5) groups. The NC and CC groups were given water, while the SN and SC groups were given 0.004 % SA, respectively. For the antibiotic treatments, mice were given a mixture of ciprofloxacin and metronidazole or vancomycin (Sigma Aldrich, St. Louis, MO, USA). The mice were divided into CHS control (CC, n = 6), SS-treated CHS (SC, n = 6), ciprofloxacin, metronidazole, and SS-treated CHS (CM, n = 6), and vancomycin and SS-treated CHS (VS, n = 6) groups. The CC and SC groups were given water and 0.004 % SA, respectively. The CM group was given 0.02 % ciprofloxacin, 0.1 % metronidazole, and 0.004 % SA, while the VS group was given 0.05 % vancomycin and 0.004 % SA. To investigate effects of vancomycin treatment, the mice were divided into CHS control (CC, n = 6), vancomycin-treated CHS (VC, n = 6), SS-treated CHS (SC, n = 6), and vancomycin and SS-treated CHS (VS, n = 6) groups. Each mice group was divided into two cages (three per cage). The CC and SC groups were given water and 0.004 % SA, respectively. The VC group was given 0.05 % vancomycin, while the VS group was given 0.05 % vancomycin and 0.004 % SA. These antibiotics and SA were administered to mice via a water bottle, starting 3

weeks prior to the first DNFB (Kanto Chemical, Tokyo, Japan) challenge and continuing for 29 days, until the end of testing.

Contact hypersensitivity test

The CHS experiments in mice were performed as described previously [17]. Briefly, the mice were sensitised on days 0 and 1 by applying 50 μ 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 μ L 0.2 % DNFB on the dorsal side of both ears on days 5 and 12. The non-CHS mice were treated with the vehicle alone (without DNFB). The ear thickness of the mice was measured using a soft touch micrometre. Ear swelling was calculated as the difference in thickness before and after challenge.

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 using Ventana Discovery XT (Ventana Medical Systems, Tucson, AZ, USA) as described previously [17]; anti-mouse Ly-6G (Gr-1) and anti-rat IgG biotin (eBioscience, San Diego, CA, USA) were applied as the primary and secondary antibodies, respectively. The number of Gr-1-positive cells was counted in five randomly selected areas ($\sim 210 \mu\text{m}^2$) of tissue section, using a microscope.

Bacterial DNA sequencing and taxonomic analysis

To collect faecal samples, we further divided the three mice in each cage into one mouse per cage after the second DNFB challenge on day 12. Fresh faecal pellets were collected on day 13 and stored at -80°C until use. Bacteria were recovered from faecal samples' lysates using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA). PCR and sequencing were performed using the Illumina Miseq platform (Illumina, San Diego, CA, USA), as described previously [13]. 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) [18].

T_{reg} analysis

The mice were sacrificed under anaesthesia 24 h after the second DNFB challenge on day 13 of the experiment. The spleen and auricular lymph node were immediately excised, and single cells were suspended in CELLBANKER[®] 1plus (Nippon Zenyaku Kogyo, Koriyama, Japan), then stored at -80°C before use. The thymic T_{reg} cells (CD4⁺, CD25⁺ and FoxP3⁺) were fixed and stained using a Mouse Regulatory T Cell Staining Kit #1 (eBioscience) according to the manufacturer's instruction. The stained cells were analysed using a BD FACSCANTO II[™] (BD Bioscience).

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 $P < 0.05$.

Results

Dietary SSs inhibit CHS and influence the gut microbiota composition

The inhibitory effect of SS-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, the SS-treated CHS group showed reduced ear swelling. Histopathological analysis of ear tissues 24 h after the second DNFB challenge revealed tissue oedema and increased infiltration of immune cells in the CHS controls compared to the non-CHS mice, which was alleviated by the SS-enriched diets (Fig. 1b). The infiltration of myeloid cells was also assessed by staining the Gr-1 antigen (Fig. 1c), a marker of granulocytes and monocytes typically recruited in large numbers to the site of hapten challenge during the effector phase. Compared to the CHS controls, fewer Gr-1-positive cells infiltrated the ear tissue in the SS-treated CHS group, as shown in Fig. 1d.

The intestinal microbiota composition was analysed by sequencing bacterial 16S rDNA. In principal component analysis (PCA) at the family level (Fig. 2a), the microbiota composition of non-CHS and CHS mice were separated on the PC1 axis (43.5 % of explained variance). The PCA plot also showed that the microbiota composition of the SS-treated CHS and CHS control mice could be separated on the PC2 axis (20.2 % of explained variance). Lactobacillaceae, Lachnospiraceae, and

Porphyromonadaceae were major gut bacteria in a BALB/c mouse (Fig. 2b). There were significant differences in the relative abundance of Lachnospiraceae (Fig. 2c), Porphyromonadaceae (Fig. 2d), and Desulfovibrionaceae (Fig. 2e). Lachnospiraceae and Desulfovibrionaceae were significantly higher in relative abundance among the CHS controls compared to non-CHS, SS-treated non-CHS, or SS-treated CHS mice. There was no significant difference in the relative abundance of Porphyromonadaceae between the SS-treated CHS and CHS control mice, although they were more prevalent in the SS-treated CHS mice compared to that in the non-CHS mice.

Antibiotic treatments decrease the CHS-inhibitory activity of dietary SSs and alter the gut microbiota composition

The effects of antibiotic treatments on the inhibitory activity of dietary SSs toward CHS were evaluated by measuring ear swelling 24 h after the second DNFB challenge (Fig. 3a). Compared to the CHS controls, the ear swelling significantly increased in mice treated with ciprofloxacin, metronidazole and SSs, whereas no significant difference in ear swelling was observed in mice treated with vancomycin and SSs. Histopathological analysis of inflamed ear tissues 24 h after the second DNFB challenge revealed that the tissue oedema and increased infiltration of immune cells in the CHS mice had been alleviated by the SS-enriched diets. These alleviations were blocked by treatment with ciprofloxacin and metronidazole or vancomycin (Fig. 3b). The infiltration of myeloid immune cells was also assessed by staining for the Gr-1 antigen (Fig. 3c), allowing the number of the Gr-1-positive cells to be counted (Fig. 3d). Compared to the CHS controls, the number of Gr-1-positive cells significantly increased in the mice treated

with ciprofloxacin, metronidazole and SSs, whereas no significant difference was observed in the vancomycin and SS-treated mice.

The intestinal microbiota composition was analysed by sequencing bacterial 16S rDNA. In the phylum-level PCA (Fig. 4a), microbiota composition is well separated between antibiotic treated and untreated mice on the PC1 axis (68.8 % of explained variance). The antibiotic-treated mice showed an increase in Proteobacteria and a reduction in Bacteroidetes and Actinobacteria compared to the antibiotic-untreated mice (Fig. 4b). At family-level, the PCA (Fig. 4c) did not separate the microbiota compositions of SS-treated and CHS control mice. However, the microbiota composition of the mice treated with ciprofloxacin, metronidazole and SSs was different from that of the vancomycin and SS-treated mice on the PC2 axis (26.8 % of explained variance). Compared to the CHS controls, Streptococcaceae and Paenibacillaceae 1 increased in the mice treated with ciprofloxacin, metronidazole and SSs, whereas Lactobacillaceae and Enterobacteriaceae increased in the vancomycin and SS-treated mice (Fig. 4d).

Vancomycin treatment decreases T_{reg} cell percentage in the SS-treated mice

To investigate why vancomycin treatment blocked the inhibitory activity of SSs on CHS, ear swelling was evaluated 24 h after the second DNFB challenge (Fig. 5a). There were no significant differences in ear swelling between the vancomycin-treated and CHS control mice. The ear swelling was reduced in the SS-treated mice compared to the CHS controls. In contrast, no significant difference in ear swelling was observed between the vancomycin and SS-treated and CHS control mice. Histopathological analysis of inflamed ear tissues 24 h after the second DNFB challenge showed that the

alleviations of tissue oedema in the SS-treated mice were blocked by vancomycin treatment (Fig. 5b). The infiltration of myeloid cells into the ear tissues was also assessed by staining for the Gr-1 antigen (Fig. 5c) and counting the number of Gr-1-positive cells (Fig. 5d). There were no significant differences in the number of Gr-1-positive cells between the vancomycin-treated and CHS control mice. The number of Gr-1-positive cells decreased in the SS-treated mice compared to that in the CHS controls. In contrast, no significant difference in the number of Gr-1-positive cells was observed between the vancomycin and SS-treated and CHS control mice.

The intestinal microbiota composition was analysed by sequencing bacterial 16S rDNA. In the PCA at the family level (Fig. 6a), the gut microbiota composition can be separated well between the vancomycin treated and untreated mice on the PC1 axis (73.0 % of explained variance). The microbiota composition in the vancomycin-treated mice was similar as that in the vancomycin and SS-treated mice (Fig 6b).

T_{reg} cells from auricular lymph node (Fig 6c) and spleen (Fig 6d) were analysed. There were no significant differences in the percentage of T_{reg} cells in the auricular lymph node and spleen of the vancomycin-treated CHS mice compared to the CHS controls. However, significant increases in the percentage of T_{reg} cells in the auricular lymph node and spleen were observed in the SS-treated mice compared to the CHS control and vancomycin-treated mice. In contrast, no significant differences in the percentage of T_{reg} cells were observed in the auricular lymph node and spleen of the vancomycin and SS-treated mice compared to the CHS controls.

Discussion

Our results demonstrated that dietary SSs exert their inhibitory activity on CHS via gut microbiota in mice. Specifically, we observed that the inhibitory activity of dietary SSs on CHS was blocked by vancomycin treatment, as evidenced by ear swelling and the infiltration of Gr-1-positive immune cells into the ear tissues. T_{reg} cells in auricular lymph node and spleen of the SS-treated mice were also reduced by vancomycin treatment. Moreover, the gut microbiota compositions of the SS-treated mice were completely altered by vancomycin treatment. In a previous study, Russell et al. indicated that neonatal, but not adult, vancomycin-treated mice showed increased severity of experimental allergy asthma. Both neonatal and adult antibiotic treatments altered the gut microbiota composition; however, the neonatal vancomycin-treated mice possessed microbiota compositions that were completely different from mice treated with vancomycin only as adults. In addition, they found colonic T_{reg} cells to be significantly reduced in the neonatal mice with vancomycin treatment compared to the controls [19]. In another study, Strzępa et al. showed that oral treatment with a broad-spectrum antibiotic enrofloxacin increased the production of IgE and Th2 cytokines, such as IL-4 and IL-13, which is indicative of allergy in young mice, but not adult mice [20]. Furthermore, Atarashi et al. also observed that vancomycin treatment reduced colonic T_{reg} cells [21]. These results showed that the inhibitory activity of dietary SSs on CHS was blocked with vancomycin treatment because the altered gut microbiota composition resulted in a reduction of T_{reg} cells.

The microbiota plays a fundamental role on the induction, training, and function of the immune system. Disruption of the microbial functions is associated with diseases and development of immune disorders [14,22,23]. In our previous study, taxonomic

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analysis revealed that the non-CHS and CHS mice belonged to two separate clusters, indicating that CHS caused changes in the gut microbiota structure. In contrast, the gut microbiota composition of the SS-treated CHS mice clustered with that of non-CHS mice, suggesting that the inhibitory activity of SSs on CHS blocked the changes in the microbiota composition. However, in that experiment, faecal samples were obtained from each cage (3 mice/cage), but not each mouse [13]; therefore, in the present study, faecal samples were collected from each mouse to obtain a deeper understanding. The PCA plot showed the shift in the microbiota composition that resulted in CHS, supporting our previous study, showing that CHS caused changes in the gut microbiota structure. The PCA plot also indicated that the difference in the PC1 axis was smaller between the non-CHS controls and SS-treated CHS mice compared to that between the non-CHS and CHS controls. In addition, the relative abundances of Lachnospiraceae and Desulfovibrionaceae were higher in the CHS controls compared to the non-CHS and SS-treated CHS mice, suggesting that these bacteria are correlated with CHS. In another study, dietary fructo-oligosaccharide reduced CHS symptoms and was found to correlate negatively with the number of bifidobacteria [24]. These results also support our previous study, which found that the inhibitory activity of SSs on CHS blocked the changes in the microbiota composition and suggest that the severity of CHS symptoms may be linked to the gut microbiota composition.

The antibiotic treatments were used to gain insights into the role of intestinal microbiota on CHS. Oral administration of enrofloxacin induced suppression of CHS and altered the gut microbiota composition in mice. The antibiotic treatment resulted in increased levels of *Bacteroidetes*, *Bifidobacterium*, and *Clostridium*, while levels of segmented filamentous bacteria were decreased [25]. In our previous study,

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ciprofloxacin and metronidazole treatments blocked the suppressive activity of dietary SIs on CHS, whereas vancomycin treatment had little effect on their inhibitory activity against CHS [26]. Ciprofloxacin and metronidazole are Gram-negative-targeting broad-spectrum antibiotics whereas vancomycin is a Gram-positive-targeting antibiotic [27]. In addition, oral vancomycin is poorly absorbed from the intestine [28]. These antibiotic treatments had different effects on the gut microbiota composition [26]. In this study, ciprofloxacin and metronidazole treatment significantly enhanced the severity of CHS symptoms, whereas vancomycin treatment blocked the suppressive activity of dietary SSs on CHS. These differential effects of these antibiotic treatments on the gut microbiota composition were, however, similar to those observed in our previous study [26]. These different results on the inhibitory activity of CHS by the antibiotic treatments suggest that there are differences in the therapeutic mechanisms between SIs and SSs. Soy isoflavones are absorbed in intestines, and the injection of genistein, a major component of SI aglycone, has been shown to suppress DTH, whereas SSs are poorly absorbed. These different properties of SIs and SSs could explain why the antibiotic treatments worked differently on the inhibitory activities of CHS by dietary SIs and SSs.

T_{reg} cells play a suppressive role at inflammation sites and reduce CHS responses [11,16]. Ring et al. demonstrated that the administration of T_{reg} significantly suppressed the ear swelling response and inflammatory cell infiltration into the skin. These suppressive effects were mediated by Interleukin (IL)-10 [29]. T_{reg} from IL-10-deficient mice failed to suppress the CHS reactions by inhibiting leukocyte infiltration into the inflamed skin [30]. In addition, oral administration of heat-killed *Lactobacillus acidophilus* strain L-92 upregulated the number of T_{reg} cells in the spleen

and cervical lymph nodes, thereby suppressing the progression of CHS in mice [31]. In this study, T_{reg} cells significantly increased in the auricular lymph node and spleen of SS-treated mice compared to the CHS controls, which were reduced by vancomycin treatment. There were no significant differences in the percentage of T_{reg} cells in auricular lymph node and spleen of the mice treated with SSs and vancomycin, compared to the CHS controls. These results demonstrated that T_{reg} cells increased by SS-enriched diets, and this increase was blocked by vancomycin treatment; however, the mechanism remain incompletely understood. Further investigations are necessary to clarify the mechanism underlying the inhibitory activity of SS on CHS via intestinal microbiota.

In conclusion, our data provide evidence that dietary SSs exert their inhibitory activity on CHS via the gut microbiota in mice. The inhibitory activity of dietary SSs on CHS was blocked because the gut microbiota composition was altered, resulting in a reduction in the number of T_{reg} cells. These results suggest that the gut microbiota is an important determinant of the therapeutic value of dietary SSs for ACD patients.

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Conflict of interest

M. K. and K. T. are employees of Fuji Oil. T. N. declares no conflict of interest.

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Figure legends

Fig. 1. Dietary soyasaponins (SSs) reduce 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 dinitrofluorobenzene (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 μ m. (c) Immunohistochemistry images stained with an anti-Gr-1 antibody. Dark-brown, Gr-1-positive cells; blue, nuclei stained with haematoxylin. Scale bars, 100 μ m. (d) The number of Gr-1-positive cells infiltrating ear tissues. NC, non-CHS group (n=6); SN, SS-treated non-CHS group (n=6); CC, CHS control group (n=6); SC, SS-treated CHS group (n=6). The data are presented as the means $\pm$ SEM; ** P < 0.01 vs. CC.

Fig. 2. Dietary soyasaponins (SSs) and contact hypersensitivity (CHS) influence the gut microbiota composition in mice. The gut microbiota composition was analysed by sequencing the bacterial 16S ribosomal RNA gene, followed by principal components

analysis (a) and taxonomic distribution (b) at the family level, and the relative abundance of Lachnospiraceae (c), Porphyromonadaceae (d), and Desulfovibrionaceae (e). NC, non-CHS group (n=5); SN, SS-treated non-CHS group (n=5); CC, CHS control group (n=5); SC, SS-treated CHS group (n=5). The data are presented as the means $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$ vs. CC.

Fig. 3. Antibiotic treatments affect 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 dinitrofluorobenzene (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 μ m. (c) Immunohistochemistry images stained with an anti-Gr-1 antibody. Dark-brown, Gr-1-positive cells; blue, nuclei stained with haematoxylin. Scale bars, 100 μ m. (d) The number of Gr-1-positive cells infiltrating ear tissues. CC, CHS control group (n=6); SC, soyasaponin (SS)-treated CHS group (n=6); CM, ciprofloxacin, metronidazole and SS-treated CHS group (n=6); VS, vancomycin and SS-treated group (n=6). The data are presented as the means $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$ vs. CC.

Fig. 4. Antibiotic treatment alters the gut microbiota composition in mice with contact hypersensitivity (CHS). The composition of gut microbiota was analysed by sequencing the bacterial 16S ribosomal RNA gene, followed by principal component analysis (PCA) (a) and taxonomic distribution (b) at the phylum level, and PCA (c) and taxonomic distribution (d) at the family level. CC, CHS control group (n=6); SC,

soyasaponin (SS)-treated CHS group (n=6); CM, ciprofloxacin, metronidazole and SS-treated CHS group (n=5); VS, vancomycin and SS-treated CHS group (n=6).

Fig 5. Vancomycin treatment blocks the inhibitory activity of dietary soyasaponins (SSs) on contact hypersensitivity (CHS). (a) Ear thickness was measured before and 24 h after the second dinitrofluorobenzene (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 μ m. (c) Immunohistochemistry images stained with an anti-Gr-1 antibody. Dark-brown, Gr-1-positive cells; blue, nuclei stained with haematoxylin. Scale bars, 100 μ m. (d) The number of Gr-1-positive cells infiltrating ear tissues. CC, CHS control group (n=6); VC, vancomycin-treated CHS group (n=6); SC, SS-treated CHS group (n=6); VS, vancomycin and SS-treated CHS group (n=6). The data are presented as the means $\pm$ SEM; ** $P < 0.01$ vs. CC.

Fig. 6. Vancomycin treatment alters the gut microbiota composition and reduces regulatory T (T_{reg}) cells in soyasaponin (SS)-treated mice with contact hypersensitivity (CHS). The gut microbiota composition was analysed by sequencing the bacterial 16S ribosomal RNA gene, followed by principal component analysis (PCA) (a) and taxonomic distribution (b) at the family level. T_{reg} cells in auricular lymph node (c) and spleen (d). CC, CHS control group (n=6); VC, vancomycin-treated CHS group (n=5); SC, SS-treated CHS group (n=5); VS, vancomycin and SS-treated group (n=6). The data are presented as the means $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$.

Figure 1

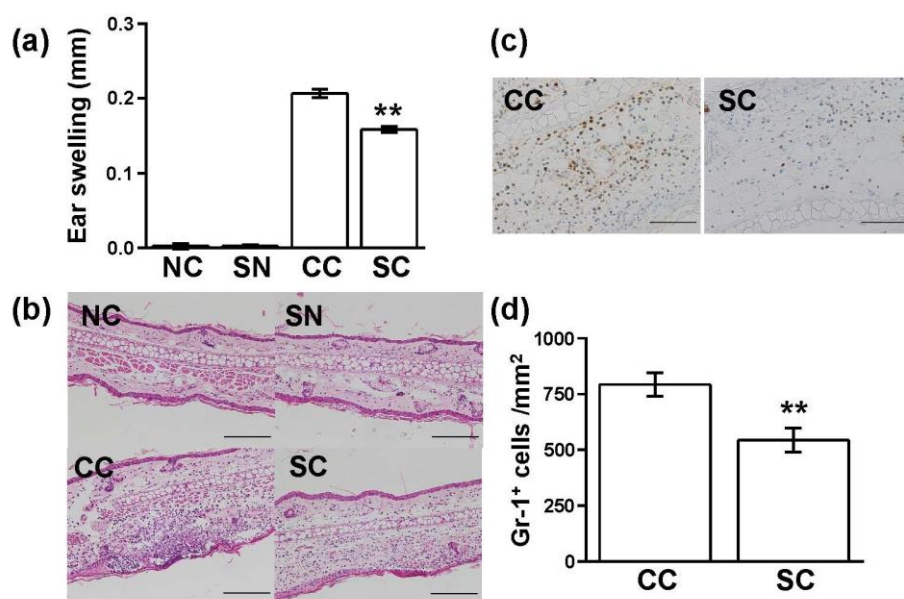


Figure 2

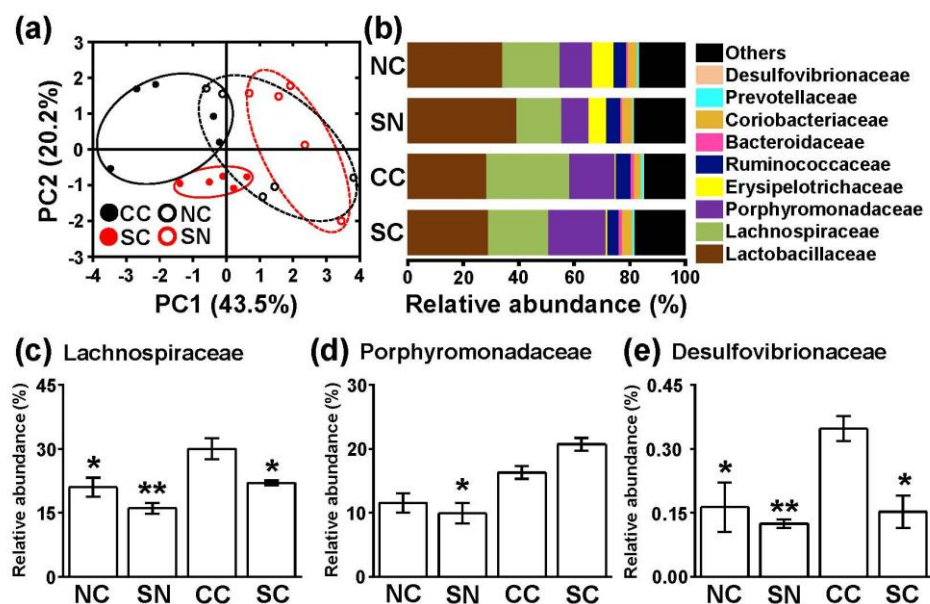


Figure 3

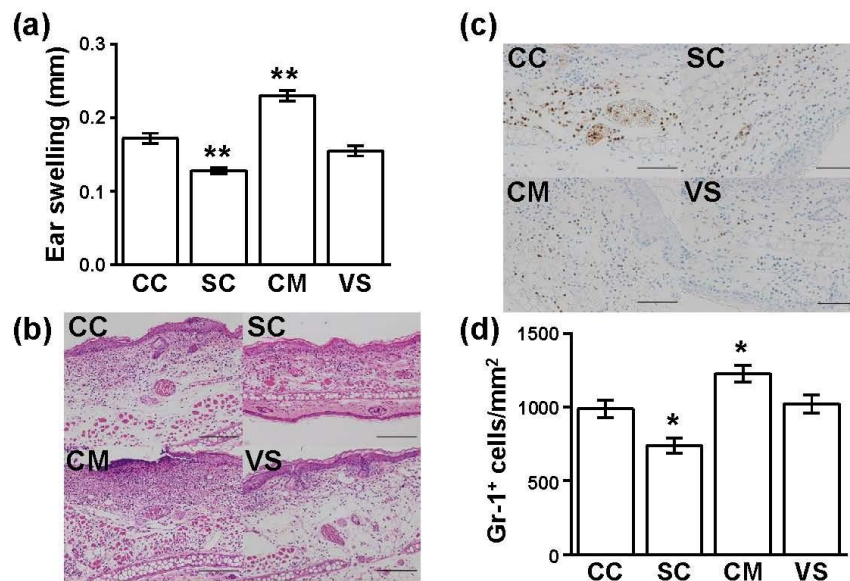


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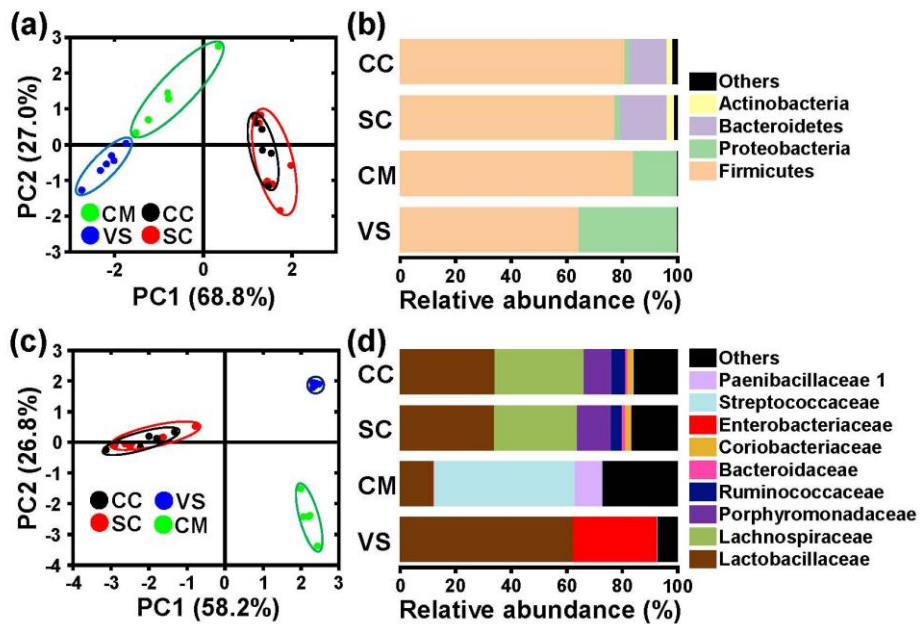


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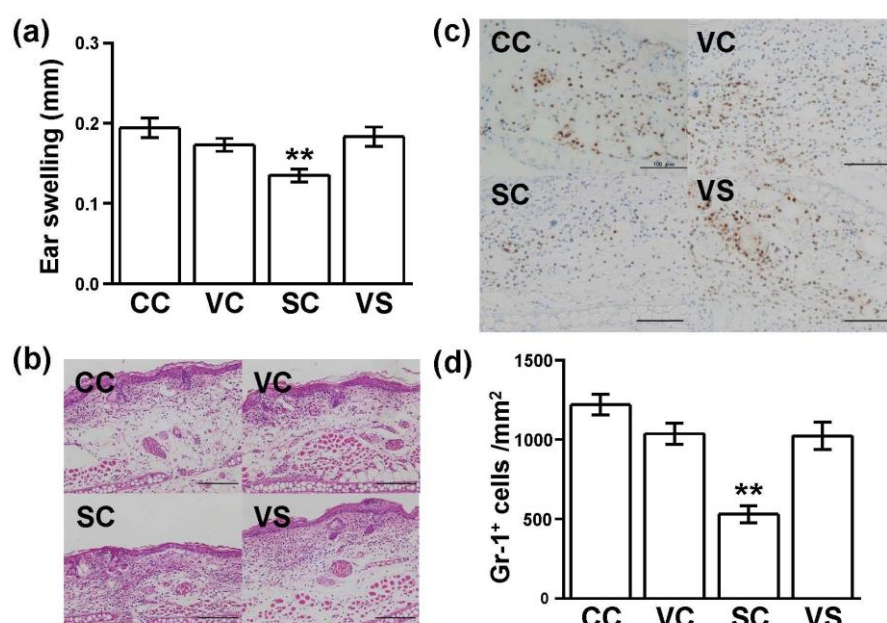
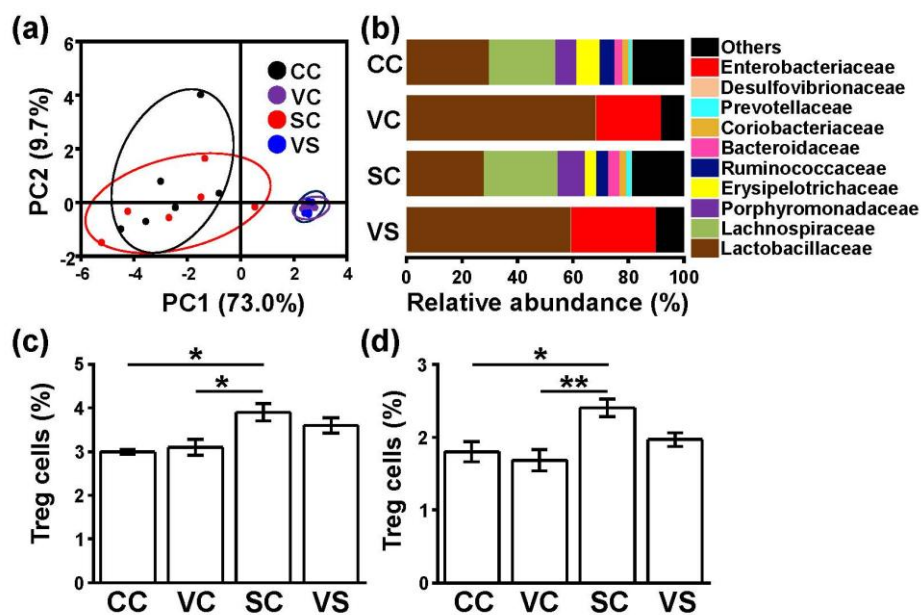
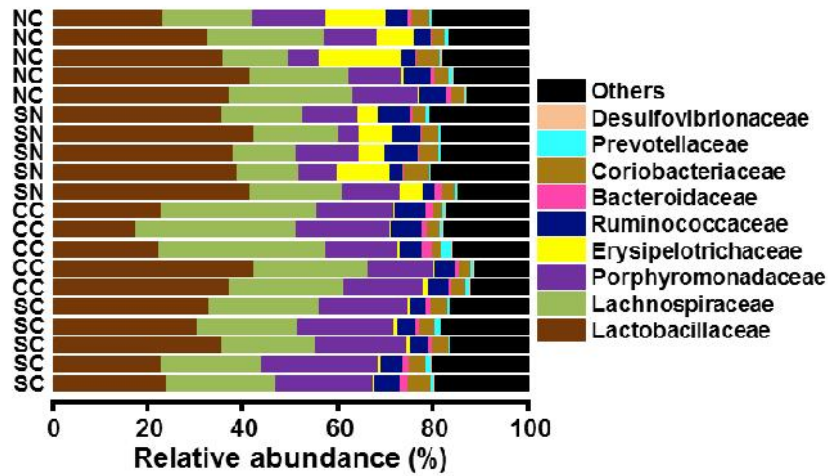


Figure 6

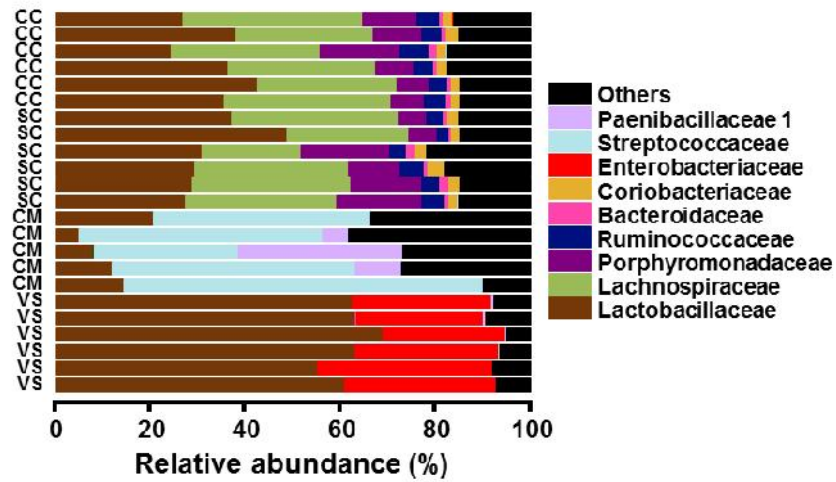


Supplementary figure 1



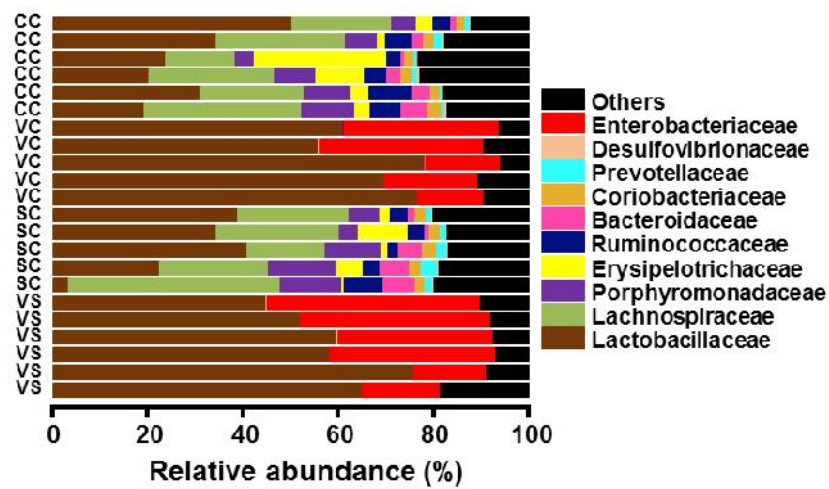
Taxonomic distribution at the family level. The gut microbiota composition was analysed by sequencing the bacterial 16S ribosomal RNA gene. NC, non-contact hypersensitivity (CHS) group (n=5); SN, soyasaponin (SS)-treated non-CHS group (n=5); CC, CHS control group (n=5); SC, SS-treated CHS group (n=5).

Supplementary figure 2



Taxonomic distribution at the family level. The gut microbiota composition was analysed by sequencing the bacterial 16S ribosomal RNA gene. CC, contact hypersensitivity (CHS) control group (n=6); SC, soyasaponin (SS)-treated CHS group (n=6); CM, ciprofloxacin, metronidazole and SS-treated CHS group (n=5); VS, vancomycin and SS-treated CHS group (n=6).

Supplementary figure 3



Taxonomic distribution at the family level. The gut microbiota composition was analysed by sequencing the bacterial 16S ribosomal RNA gene. CC, contact hypersensitivity (CHS) control group (n=6); VC, vancomycin-treated CHS group (n=5); SC, soyasaponin (SS)-treated CHS group (n=5); VS, vancomycin and SS-treated CHS group (n=6).

Supplementary Table 1. **Overview of the groups for contact hypersensitivity experiments.**

1) Experiment 1 (Figs. 1 and 2)

Abbreviation	Groups
NC	non-contact hypersensitivity (CHS) group
SN	soyasaponin (SS)-treated non-CHS group
CC	CHS control group
SC	SS-treated CHS group

2) Experiment 2 (Figs. 3 and 4)

Abbreviation	Groups
CC	CHS control group
SC	SS-treated CHS group
CM	ciprofloxacin, metronidazole and SS-treated CHS group
VS	vancomycin and SS-treated CHS group

3) Experiment 3 (Figs. 5 and 6)

Abbreviation	Groups
CC	CHS control group
VC	vancomycin-treated CHS group
SC	SS-treated CHS group
VS	vancomycin and SS-treated CHS group