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IN VITRO AND ANIMAL STUDIES



Impact of soymilk consumption on 2,4-dinitrofluorobenzene-induced contact hypersensitivity and gut microbiota in mice

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ABSTRACT

Soymilk is rich in phytochemicals such as soy isoflavones (SIs) and soyasaponins (SSs). Dietary SIs and SSs display inhibitory effects on contact hypersensitivity (CHS), which was reported in a mouse model for allergic contact dermatitis (ACD); however, the beneficial effects of soymilk consumption on CHS remain unknown. Here, we studied the effects of drinking soymilk on CHS and gut microbiota. Soymilk consumption attenuated ear oedema and swelling, decreased the infiltration of Gr-1-positive cells into ear tissues, and reduced the production of chemokine (C-X-C motif) ligand 2 and triggering receptor expressed on myeloid cells-1 in ear tissues. The analysis of bacterial 16S ribosomal RNA gene sequences indicated that CHS caused changes in the gut microbiota structure and that consuming soymilk reduced these changes. These results suggest that soymilk consumption may be of therapeutic value for patients with ACD and may help control the balance of intestinal microbiota.

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Introduction

Soymilk, a water extract of soybeans, is widely consumed in Asian countries as a traditional protein beverage (Chen and Ono 2014; Peng et al. 2016). In addition, soymilk and its products are recommended as healthy foods because they are cholesterol and lactose free and rich in phytochemicals such as soy isoflavones (SIs) and soyasaponins (SSs) (Guang et al. 2014; Messina 2014). SIs and SSs have beneficial effects on health, such as the inhibition of inflammation, anti-cancer activity, lowering of the blood cholesterol level, reducing the risk of cardiovascular disease, and antioxidant activity (Kang et al. 2010; Li et al. 2010; Nagata et al. 2014).

Contact hypersensitivity (CHS) is often used as an animal model for human allergic contact dermatitis (ACD), which is a common skin disease caused by delayed-type hypersensitivity responses to low molecular weight chemicals that come in contact with the skin (Inagaki and Nagai 2009; Martin et al. 2011; Kaplan et al. 2012; Honda et al. 2013). In our previous study, we studied the CHS-inhibitory effects of SI-enriched diets and found that SI diets reduced CHS symptoms at physiologically relevant doses in mice (Nagano et al. 2019). We also previously investigated

the effects of dietary SSs on CHS and gut microbiota in mice. The results indicated that SS diets attenuated CHS symptoms at physiologically relevant doses. In addition, dietary SSs blocked changes in gut microbiota associated with CHS (Nagano et al. 2017, 2018). Therefore, our findings suggest that physiologically relevant doses of dietary SIs or SSs may yield beneficial effects in individuals with ACD and control the balance of intestinal microbiota. However, the CHS-suppressive effects mediated by soymilk consumption, as well as possible effects on the intestinal microbiota, are not known.

In the present study, we investigated the inhibitory effects of soymilk consumption on CHS in mice. Furthermore, the intestinal microbiota composition was analysed by sequencing bacterial 16S ribosomal RNA genes (16S rDNA) to elucidate the effect of consuming soymilk on the intestinal microbiota in mice with CHS.

Materials and methods

Diet and soymilk

The soybean-free diet F2PLD1 was purchased from Oriental Yeast (Tokyo, Japan). The plain soymilk

containing SI (20 mg/100 mL soymilk) and SS (40 mg/100 mL soymilk) was obtained from Fuji Oil (Izumisano, Japan).

Mice

Six-week-old female BALB/c mice (15–20 g body weight) were obtained from Japan SLC (Hamamatsu, Japan). The mice were fed F2PLD1 and kept in a temperature-controlled room ($25 \pm 2^\circ\text{C}$) under a 12-h light/dark cycle. The mice were divided into non-CHS (NC, $n=6$), soymilk-treated non-CHS (SN, $n=6$), CHS control (CC, $n=6$), and soymilk-treated CHS (SC, $n=6$) groups. The NC and CC groups were provided water, while the SN and SC groups were provided with diluted soymilk. Soymilk was diluted with water to 10% and was provided to mice via a water bottle 3 weeks before the first 2,4-dinitrofluorobenzene (DNFB, Kanto Chemical, Tokyo, Japan) challenge and continuing for 29 days, until the end of testing. 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).

CHS experiment

The CHS experiment was performed as described previously (Nagano et al. 2016). Briefly, the mice were sensitised on days 0 and 1 by applying 0.5% DNFB on the shaved abdominal skin. The animals were then challenged by the application of 20 μL 0.2% DNFB on the dorsal side of both ears on days 5 and 12. The non-CHS mice were sensitised and challenged without DNFB. The ear thickness of the mice was measured using a soft touch micrometer. Ear swelling was calculated as the difference in thickness before the challenge and 24 h after the second challenge.

Histological analysis

Ear tissue was collected 24 h after the second challenge and fixed with IHC Zinc Fixative (BD Biosciences, Bedford, MA) for 24 h. Ear tissues were stained with haematoxylin and eosin. Immunohistochemistry was conducted using a Ventana DISCOVERY XT (Ventana Medical Systems, Tucson, AZ) as described previously (Nagano et al. 2016) using anti-mouse Ly-6G (Gr-1) and mouse anti-rat IgG biotin (eBioscience, San Diego, CA) as 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 sections using a microscope.

Cytokine antibody array and enzyme-linked immunosorbent assay (ELISA)

Ear tissues collected 24 h after the second challenge were stored at -80°C until use. The ears were cut, placed into Lysing Matrix D tubes (MP Biomedicals, Santa Ana, CA), and lysed in PIPA lysis buffer containing protease inhibitors (ATTO, Tokyo, Japan) by homogenisation in a FastPrep-24 instrument (MP Biomedicals). Cytokine expression was determined using the Proteome ProfilerTM mouse cytokine array kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. Chemiluminescence was detected with an ImageQuant LAS 4000 Mini instrument (GE Healthcare Biosciences, Piscataway, NJ), and spot intensity was quantified using Image Studio Lite software (LI-COR Biotechnology, Lincoln, NE). The production of chemokine (C-X-C motif) ligand 2 (CXCL2) and triggering receptor expressed on myeloid cells-1 (TREM-1) was determined using a mouse DuoSet ELISA development system (R&D Systems) as per the manufacturer's instructions. Protein concentration was quantified by the PierceTM BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). The assays of ELISA and protein concentration were performed in duplicate.

Bacterial DNA sequencing and taxonomic analysis

Fresh faecal pellets were collected on day 13 and stored at -80°C until use. Bacteria were recovered from the lysates of faecal samples using the FastDNA Spin Kit for Soil (MP Biomedicals). PCR and sequencing were performed using the Illumina MiSeq platform (Illumina, San Diego, CA). Taxonomic analysis of the 16S rDNA sequences was conducted as previously described (Nagano et al. 2017).

Statistical analysis

The statistical significance of differences between two groups was analysed by a student's *t*-test, and the statistical significance of differences among more than three groups was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests, and the principal component analysis (PCA) was conducted using Origin 2018 software (OriginLab, Northampton, MA). The data were considered significant at $p < 0.05$.

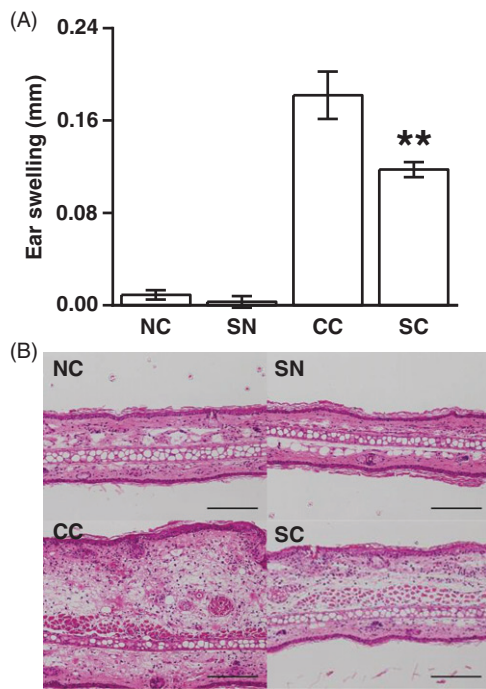


Figure 1. Soymilk consumption reduces ear swelling and tissue oedema in mice with contact hypersensitivity (CHS). (A) Ear thickness was measured before and 24 h after the second dinitrofluorobenzene (DNFB) challenge, and ear swelling was calculated as the difference in ear thickness between these two time points. (B) Images of ear tissues stained with haematoxylin and eosin. Scale bars, 200 μ m. NC: non-CHS group; SN: soymilk-treated non-CHS group; CC: CHS control group; SC: soymilk-treated CHS group. The data are presented as the means $\pm$ SEM; ** p < 0.01 vs. CC.

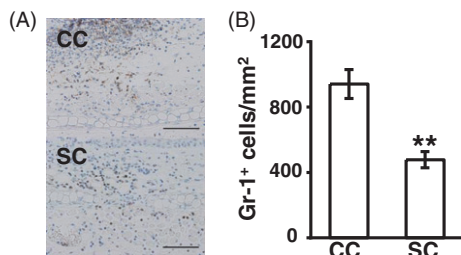


Figure 2. Soymilk consumption decreases the infiltration of myeloid cells into ear tissues in mice with contact hypersensitivity (CHS). (A) Immunohistochemistry images stained with an anti-Gr-1 antibody. Dark brown, Gr-1-positive cells; blue, nuclei stained with haematoxylin. Scale bars, 100 μ m. (B) The number of Gr-1-positive cells infiltrating ear tissues. CC: CHS control group; SC: soymilk-treated CHS group. The data are presented as the means $\pm$ SEM; ** p < 0.01 vs. CC.

Results

Soymilk consumption reduces CHS

The CHS-inhibitory effect of soymilk was examined by measuring ear swelling (Figure 1(A)). Compared to the CHS controls (0.182 ± 0.021 mm), mice in the soymilk-treated CHS group (0.115 ± 0.006 mm) showed

Table 1. Proinflammatory cytokines downregulated by soymilk consumption in mice with contact hypersensitivity.

Cytokines	Fluorescence intensity		Ratio
	Soymilk	CHS control	
CXCL12	3400	11,000	0.31
TNF- α	3400	10,500	0.32
C5/C5a	157,00	34,900	0.45
IL-16	15,400	32,200	0.48
M-CSF	12,700	25,900	0.49
TIMP-1	32,400	61,800	0.52
CXCL2	6400	12,000	0.53
TREM-1	11,800	20,400	0.58

CHS: contact hypersensitivity.

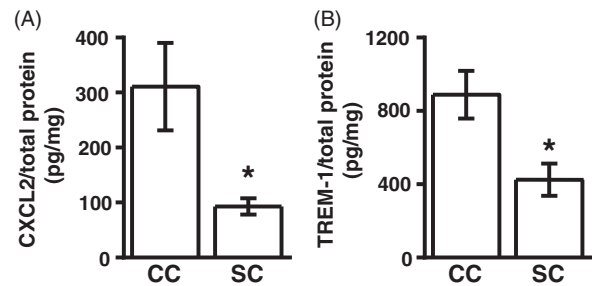


Figure 3. Soymilk consumption suppresses CXCL2 and TREM-1 production in ear tissues of mice with contact hypersensitivity (CHS). CXCL2 (A) and TREM-1 (B) levels were measured by ELISA. CC: CHS control group; SC: soymilk-treated CHS group. The data are presented as the means $\pm$ SEM; * p < 0.05 vs. CC.

decreased ear swelling (37%, p < 0.01). Microscopic observation of ear tissues 24 h after the second DNFB challenge revealed prominent ear tissue oedema and swelling in the CHS control mice compared to the non-CHS mice, which was alleviated by soymilk consumption (Figure 1(B)). The infiltration of myeloid cells was also evaluated by staining for Gr-1, which is a marker of granulocytes and monocytes (Figure 2(A)). The soymilk-treated CHS group (470 ± 49 cells/mm²) showed decreased infiltration of Gr-1-positive cells into the inflamed ear tissues (50%, p < 0.01) compared to the CHS controls (941 ± 88 cells/mm²) as shown in Figure 2(B).

Soymilk consumption attenuates the production of cytokines in mice with CHS

Cytokine antibody array analyses were used to determine the cytokine production in the ear tissues of the soymilk-treated and CHS control mice. The results of the antibody array showed that the specific cytokines with the greatest decrease in the soymilk-treated mice were CXCL12, tumour necrosis factor- α (TNF- α), complement component 5a (C5/C5a), interleukin-16 (IL-16), macrophage colony-stimulating factor (M-CSF), tissue inhibitor of metalloproteinase-1 (TIMP-1), CXCL2, and TREM-1 (Table 1). The production of

CXCL2 and TREM-1 in the ear tissues was also examined via ELISA. Levels of these cytokines in the ear tissues were reduced in the soymilk-treated mice compared with the CHS controls (Figure 3(A,B)).

Soymilk consumption influences the gut microbiota composition in mice

The gut microbiota composition was assessed by sequencing the bacterial 16S rDNA. In the PCA, at the family level (Figure 4(A)), the microbiota composition of soymilk-treated non-CHS was separated from that of the CHS control mice, but not from that of non-CHS or soymilk-treated CHS mice on the PC1 axis (46.1% of explained variance). The PCA plot also

showed that the microbiota composition of the soymilk-treated non-CHS and non-CHS mice could be separated on the PC2 axis (15.3% of explained variance).

Lactobacillaceae, Lachnospiraceae and Turicibacteraceae were major gut bacteria in a BALB/c mouse (Figure 4(B)). The relative abundance of Lactobacillaceae was significantly lower in the CHS controls compared with the soymilk-treated non-CHS ($p < 0.05$, Figure 5(A)). In contrast, the relative abundance of Lachnospiraceae ($p < 0.05$, Figure 5(B)), Ruminococcaceae ($p < 0.01$, Figure 5(C)), Desulfovibrionaceae ($p < 0.05$, Figure 5(D)) and Rhodothermaceae ($p < 0.01$, Figure 5(E)) was significantly increased in the CHS controls compared with the soymilk-treated non-CHS mice. The relative abundance of Ruminococcaceae in the CHS

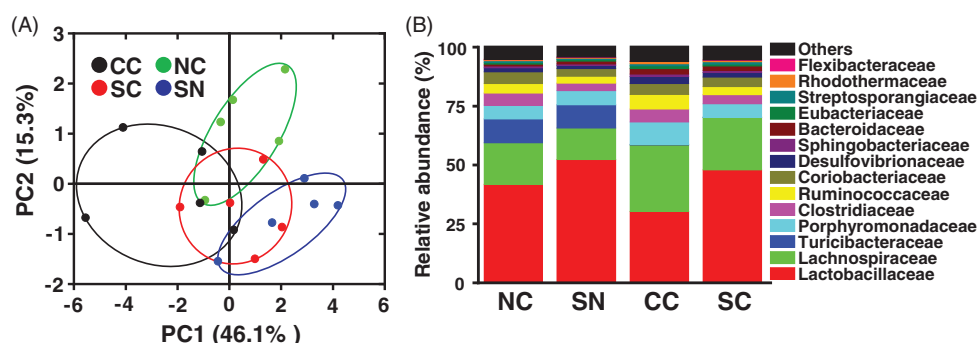


Figure 4. Soymilk consumption affects the gut microbiota composition in mice with contact hypersensitivity (CHS). The gut microbiota composition was analysed by sequencing the bacterial 16S ribosomal RNA gene, followed by principal component analysis (A) and determination of the taxonomic distribution (B) at the family level. NC: non-CHS group; SN: soymilk-treated non-CHS group; CC: CHS control group; SC: soymilk-treated CHS group.

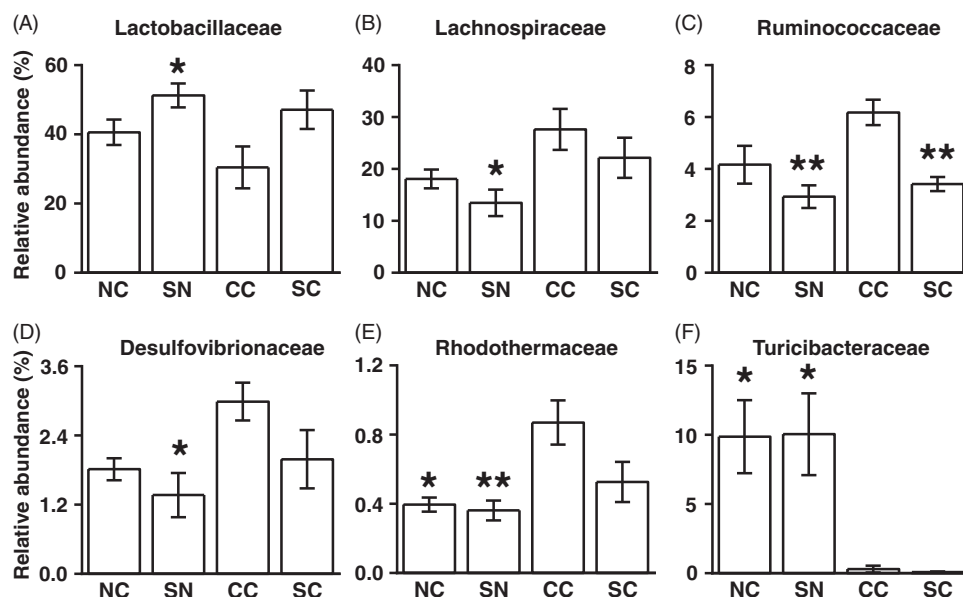


Figure 5. Soymilk consumption influences the relative abundance of intestinal bacteria in mice with contact hypersensitivity (CHS). (A) Lactobacillaceae, (B) Lachnospiraceae, (C) Ruminococcaceae, (D) Desulfovibrionaceae, (E) Rhodothermaceae, (F) Turicibacteraceae. NC: non-CHS control group; SN: soymilk-treated non-CHS group; CC: CHS control group; SC: soymilk-treated CHS group. The data are presented as the means $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$ vs. CC.

controls was significantly higher than in the soymilk-treated CHS mice ($p < 0.01$). The relative abundance of Rhodothermaceae in the CHS controls was significantly higher than that in the non-CHS mice ($p < 0.05$). Furthermore, the relative abundance of Turicibacteraceae in non-CHS and soymilk-treated non-CHS mice was significantly higher than that in the CHS control and soymilk-treated CHS mice ($p < 0.05$, Figure 5(F)).

Discussion

Our results demonstrated that CHS symptoms were attenuated by soymilk consumption in mice. Specifically, we observed alleviated ear tissue oedema in the CHS mice, as evidenced by reduced ear swelling, decreased Gr-1-positive cell infiltration, and reduced levels of pro-inflammatory cytokines such as CXCL2 and TREM-1. Our previous studies demonstrated that CHS symptoms were reduced by the consumption of physiologically relevant doses of SIs or SSs (Nagano et al. 2018, 2019). In this study, we used a plain soymilk which contained 20 mg SI and 40 mg SS per 100 mL soymilk. We diluted the soymilk to 10% with water and administered this to the mice; this intake corresponds to approximately 75 mg SI or 380 mL soymilk for a human per day. SI intake ranges from 25 to 50 mg per day in Asian countries, although this is variable across populations (Messina et al. 2006). Per Japanese nutritional guidelines, the maximum safe level for dietary SI intake is 70–75 mg per day as aglycone. These results suggest that drinking soymilk may have beneficial effects on ACD patients.

In this study, cytokine antibody arrays and ELISA analyses showed that drinking soymilk decreased the production of CXCL2 and TREM-1 in ear tissues of CHS mice. CXCL1 and CXCL2 are neutrophil-recruiting chemokines, and the infiltration of inflammatory cells into ear tissues during CHS arises from the sequential expression of chemokine genes (Niwano et al. 2008). At the early time, there was increased gene expression of CXCL1 and CXCL2 at the elicitation site on CHS mice (Mitsui et al. 2004). These results support our present study, which demonstrated that the infiltration of Gr-1-positive cells into the ear tissues was reduced in soymilk-treated mice compared with the CHS controls.

TREM-1 is a receptor of the immunoglobulin superfamily expressed on neutrophils and monocytes/macrophages (Bouchon et al. 2001). Levels of soluble TREM-1 increase in the body fluids of animals and human patients exhibiting strong inflammatory reactions because of various diseases such as inflammatory

bowel disease and sepsis, and its levels often correlate with disease severity (Ford and McVicar 2009). These results suggest that the level of TREM-1 may be related with the severity of CHS symptoms.

The gut microbiota plays a fundamental role in the induction and function of the immune system. Thus, disruption of microbial functions is associated with diseases and the development of immune disorders (Guarner and Malagelada 2003; Russell et al. 2013; Belkaid and Hand 2014; Hirata and Kunisawa 2017). In our previous study, the effects of CHS and dietary SS on the gut microbiota composition in mice were studied. The PCA plot showed a shift in the gut microbiota composition in response to CHS. 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 between the non-CHS and CHS controls. Furthermore, the relative abundances of Lachnospiraceae and Desulfovibrionaceae were higher in the CHS controls than in the non-CHS and SS-treated CHS mice. These results suggest that dietary SSs reduce the changes in the microbiota composition of the mice with CHS (Nagano et al. 2018). In the present study, the PCA plot showed that the microbiota composition of soymilk-treated non-CHS is different from that of the CHS controls. The difference in the PC1 axis was smaller between the soymilk-treated non-CHS mice and non-CHS or soymilk-treated CHS mice compared to between the CHS controls and non-CHS mice. In addition, compared to the soymilk-treated non-CHS mice, the relative abundance of Lactobacillaceae decreased, and the relative abundances of Lachnospiraceae, Ruminococcaceae, Desulfovibrionaceae and Rhodothermaceae increased in the CHS controls. These changes in the microbiota composition of CHS mice were absent in that of soymilk-treated CHS mice, suggesting that soymilk consumption has beneficial effects in controlling the balance of intestinal microbiota in mice with CHS. In contrast, the relative abundance of Turicibacteraceae in the non-CHS and soymilk-treated non-CHS mice is significantly higher than in the CHS control and soymilk-treated CHS mice, indicating that Turicibacteraceae levels are not affected by soymilk consumption. Further study is necessary to elucidate the relationship between the CHS-inhibitory effects of soymilk and its effects on the gut microbiota.

Conclusions

Our data provide evidence that soymilk consumption attenuates CHS by inhibiting CXCL2 and TREM-1

expression in CHS mice. In addition, our analysis of the gut microbiota indicates that soymilk consumption reduces the changes in gut microbiota composition induced by CHS in mice. These results suggest that the consumption of soymilk has beneficial effects in controlling the balance of intestinal microbiota and that it may be of therapeutic value for patients with ACD.

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Disclosure statement

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

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