Bioscience of Microbiota, Food and Health Vol. 36 (1), 33–37, 2017

The effect of soymilk intake on the fecal microbiota, particularly *Bifidobacterium* species, and intestinal environment of healthy adults: a pilot study

Tomohiko FUJISAWA[1*], Yuji OHASHI[1], Ryoichi SHIN[1], Asako NARAI-KANAYAMA[2] and Takenori NAKAGAKI[3]

[1]*Laboratory of Food Hygiene, Faculty of Applied Life Science, Nippon Veterinary and Life Science University, 1-7-1 Kyonan-cho, Musashino-shi, Tokyo 180-8602, Japan*

[2]*Laboratory of Agricultural Food, Faculty of Applied Life Science, Nippon Veterinary and Life Science University, 1-7-1 Kyonan-cho, Musashino-shi, Tokyo 180-8602, Japan*

[3]*Nakagaki Consulting Engineer & Co., Ltd., 10-39 Otorikitamachi, Nishi-ku, Sakai-shi, Osaka 593-8328, Japan*

Received July 15, 2016; Accepted September 29, 2016; Published online in J-STAGE October 15, 2016

The influence of soymilk on the fecal microbiota, particularly *Bifidobacterium* species, and metabolic activities were investigated in eight healthy adult humans. During the soymilk intake period, the number of bifidobacteria in feces was significantly higher ($p<0.05$) on day 14 of the soymilk intake period than before the intake period, whereas that of *Enterobacteriaceae* was significantly lower ($p<0.05$) on days 7 and 14 of the soymilk intake period than before the intake period. In an investigation of *Bifidobacterium* at the species or group level, the numbers of all species and groups studied slightly increased during the soymilk intake period. These results show that the intake of soymilk may contribute to improving the intestinal environment.

Key words: bifidobacteria, soymilk, fecal microbiota, fecal metabolic activity, human

Functional foods have recently been attracting attention because the incidence of lifestyle-related diseases is increasing. Soymilk is derived from soybeans and its constituents include such things as soybean oligosaccharides, isoflavones, proteins, and saponins. Onozawa *et al.* [1] and Zava and Duwe [2] found that the intake of soybean isoflavones reduced the risks of diseases such as breast cancer and prostate cancer. Previous studies also reported that soyasaponin has various beneficial functions such as reducing blood glucose levels [3] in addition to cholesterol-lowering [4], anti-inflammatory [5], and antitumor activities [6]. Soybean oligosaccharides [7–9] and raffinose [10, 11], which is a constituent sugar of soybean oligosaccharide, have exhibited efficacy as prebiotics. The intake of prebiotics has also been shown to selectively increase the number of beneficial bacteria in the gastrointestinal tract. Hayakawa *et al.* [7] previously reported that soybean oligosaccharide intake by healthy adults increased the number of fecal bifidobacteria. This finding clearly indicated that soybean oligosaccharides are *Bifidobacterium* growth promoters. The nutritive value and functionality of soymilk are very high, and soymilk has attracted a large amount of attention as a functional food. The intake of several soybean foods [12–14] and soybean milk-fermented product [15] has been shown to improve the intestinal environment.

A previous study examined soymilk fermented using lactic acid bacteria in order to investigate its effects on the intestinal environment [16] and showed that the numbers of bifidobacteria and lactobacilli in feces increased, while the number of fecal clostridia and the concentration of fecal sulfides were decreased by the intake of fermented soymilk with a sufficient amount of soybean oligosaccharides. Moreover, no significant changes were noted in the fecal microbiota and fecal properties such as pH and the concentrations of organic acids and sulfides during the intake period in the case of non-fermented soymilk [16]. The same study also showed that the number of fecal bifidobacteria slightly increased during intake of non-fermented soymilk [16]. Incidentally, the influence of the intake of soymilk on the

*Corresponding author. Tomohiko Fujisawa, Laboratory of Food Hygiene, Faculty of Applied Life Science, Nippon Veterinary and Life Science University, 1-7-1 Kyonan-cho, Musashino-shi, Tokyo 180-8602, Japan. E-mail: fujisawa@nvlu.ac.jp

intestinal microbiota, particularly the influence on each *Bifidobacterium* species, has not yet been examined. Soymilk contains soybean oligosaccharides that are utilized by bifidobacteria [7, 16, 17]. Therefore, we investigated the influence of non-fermented soymilk on the fecal microbiota, particularly *Bifidobacterium* species, pH, and metabolic activities in humans.

The plain soymilk (non-fermented soymilk) used in the present study was made by a company in Japan and purchased from retail stores in Japan. The main components in 100 ml of plain soymilk were as follows: 5 g protein, 3 g fat and 2 g carbohydrate. Saccharides in soymilk were measured using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a CarboPac^{TM} PA10 column/guard pair (4 × 250/4 × 50 mm) (Dionex, Sunnyvale, CA, USA) and an ED-50 pulsed amperometric detector (Dionex) with a working gold electrode in integrated amperometry mode, as described previously [18]. The subjects comprised four male and four female healthy volunteers (age range 19–23 years). The volunteers consumed a normal free-choice diet for 2 weeks before and after the soymilk intake period. During the intake of soymilk, 100 g of soymilk was consumed once a day in addition to the normal diet for two consecutive weeks. None of the subjects received antibiotic treatments, other therapies, or foods with an abundant viable culture 1 month prior to and during the experiment. This study was carried out in accordance with the Helsinki Declaration as revised in Tokyo in 1975. Informed consent was obtained from all volunteers before the experiment started. Fecal samples collected from each subject were immediately transported under anaerobic conditions at 4°C to the laboratory for analysis. Fecal microbiota and pH were analyzed within 3 hr. The remainder of the samples were frozen at −80°C for later analysis of bacteria metabolites. Fecal pH, fecal concentrations of ammonia and short-chain fatty acids, and the fecal microbiota were measured in these subjects on day 0 before the soymilk intake period, days 7 and 14 during the intake period, and day 14 after the intake of soymilk had ended (day 28). The fecal microbiota was analyzed using the methods of Mitsuoka *et al.* [19, 20] and the heat treatment method of Terada *et al.* [21]. Fecal pH values were measured with a model D-25 pH meter (Horiba Ltd., Kyoto, Japan) [22]. Fecal amounts of short-chain fatty acids (acetic, propionic, and butyric acids) were analyzed using a high-performance liquid chromatography organic acid analysis system (HPLCOA, Shimadzu Corporation, Kyoto, Japan) following the methods of Hara *et al.* [17]. Fecal concentrations of ammonia were measured using the indophenol method. The numbers of fecal *Bifidobacterium* groups or species were also measured by a real-time PCR method [23] using *Bifidobacterium* species-specific primers [24]. Bacterial DNA was extracted from approximately 0.1 g of feces according to Godon *et al.* [25]. The Kruskal-Wallis test and Holm-Bonferroni method were used to statistically analyze changes in the fecal microbiota, bacterial metabolites, and fecal pH values.

The saccharide concentrations of soymilk were as follows: glucose, 1.1 ± 0.0 mM; fructose, 0.8 ± 0.0 mM; sucrose, 44.8 ± 1.9 mM; raffinose, 2.5 ± 0.1 mM; stachyose, 18.4 ± 0.6 mM. Each value is the mean ± SD of three trials.

All subjects remained healthy during the experimental period. The results for the fecal microbiota are shown in Table 1. The number of fecal bifidobacteria significantly increased ($p<0.05$) during the soymilk intake period. The results of measurements taken using the real-time PCR method are shown in Table 2. The copy number of the 16S rRNA gene for each species and group studied slightly increased during the soymilk intake period. The number of *Enterobacteriaceae* was significantly lower ($p<0.05$) on days 7 and 14 of the soymilk intake period than before the intake period.

Changes in fecal short-chain fatty acids, pH values, and ammonia concentrations are shown in Table 3. No significant changes were observed in the concentrations of short-chain fatty acids and ammonia during the soymilk intake period. Fecal pH tended to be lower, but statistical significance was not found.

Bifidobacterium is a predominant member of the intestinal microbiota of humans, and is essential for maintaining the intestinal environment of the host. *Bifidobacterium* exerts various beneficial effects such as those involved in regulation of the intestinal environment, in synthesis of vitamins, in digestion and absorption, in alleviation of constipation, diarrhea, and infection, in enhancements to the immune system, in inhibition of carcinogenesis, in amelioration of lactose intolerance, in therapy and prophylaxis for inflammatory bowel disease, and in reductions in serum cholesterol [26, 27], and it has been used as a probiotic. Soybean oligosaccharides are also regarded as capable prebiotics and *Bifidobacterium* growth promoters [7–9].

We found that the number of fecal bifidobacteria was significantly increased by the intake of soymilk. The copy number of the 16S rRNA gene of each species and group of *Bifidobacterium* also slightly increased. These results suggest that the growths of all species or groups investigated were enhanced by the intake of soymilk. Raffinose and stachyose are utilized by strains of

Table 1. Effect of soymilk intake on the fecal microbiota

Organism	Before intake	During soymilk intake		After intake
	Day 0	Day 7	Day 14	Day 28
Total bacteria	10.7 ± 0.2	10.8 ± 0.2	10.7 ± 0.3	10.7 ± 0.2
Bifidobacterium	9.6 ± 0.5^{b} (100)	10.1 ± 0.6^{ab} (100)	10.1 ± 0.4^{a} (100)	9.9 ± 0.3^{b} (100)
Anaerobic Gram-negative rods	10.3 ± 0.4 (100)	10.4 ± 0.3 (100)	10.4 ± 0.3 (100)	10.4 ± 0.3 (100)
Anaerobic Gram-positive rods	9.9 ± 0.4 (100)	9.8 ± 0.5 (100)	9.6 ± 0.6 (100)	9.9 ± 0.4 (100)
Anaerobic Gram-positive cocci	9.8 ± 0.4 (100)	8.7 ± 1.4 (100)	9.0 ± 1.3 (100)	9.7 ± 0.3 (75)
Veillonella	6.5 ± 1.7 (88)	6.7 ± 1.5 (38)	7.0 ± 0.9 (50)	7.0 ± 0.8 (75)
Clostridium				
Lecithinase-positive	4.8 ± 1.5 (63)	6.1 ± 2.2 (38)	5.3 ± 1.6 (50)	5.3 ± 2.4 (38)
Lecithinase-negative	6.6 ± 1.8 (50)	8.1 ± 1.3 (38)	7.5 ± 1.2 (63)	9.1 ± 0.3 (38)
Lactobacillus	5.7 ± 1.7 (88)	5.6 ± 1.9 (88)	5.3 ± 2.3 (100)	5.3 ± 1.7 (88)
Enterobacteriaceae	9.1 ± 0.4^{a} (100)	8.1 ± 0.6^{b} (100)	8.2 ± 0.8^{b} (100)	8.2 ± 0.9^{ab} (100)
Streptococcus and *Enterococcus*	8.1 ± 0.8 (100)	7.9 ± 0.9 (100)	7.9 ± 1.2 (100)	8.0 ± 0.5 (100)
Staphylococcus	2.8 ± 0.6 (88)	2.7 ± 0.4 (88)	3.2 ± 0.6 (63)	3.0 ± 0.3 (88)
Pseudomonas	3.5 ± 0.7 (38)	3.3 (25)	4.1 ± 0.5 (63)	3.2 ± 1.0 (50)
Yeasts	3.5 ± 0.5 (50)	4.7 ± 1.5 (38)	3.7 ± 0.6 (38)	3.0 ± 0.8 (50)

Data are expressed as the mean ± SD of the $\log_{10}$ number (CFU) per gram wet feces. Figures in parentheses are frequencies of occurrence (%).
^{a,b}Values not sharing a common superscript letter within a row are significantly different ($p<0.05$).

Table 2. Effect of soymilk intake on *Bifidobacterim* groups and species

Organism	Before intake	During soymilk intake		After intake
	Day 0	Day 7	Day 14	Day 28
B. longum	10.6 ± 0.8 (100)	10.9 ± 0.6 (100)	11.0 ± 0.9 (100)	10.4 ± 0.6 (100)
B. adolescentis group	9.3 ± 1.9 (100)	9.2 ± 1.5 (100)	9.5 ± 2.0 (100)	9.0 ± 1.7 (100)
B. catenulatum group	9.9 ± 1.2 (100)	10.1 ± 1.2 (100)	10.2 ± 1.2 (100)	9.9 ± 0.9 (100)
B. bifidum	10.4 ± 3.5 (38)	10.4 ± 2.5 (38)	10.9 ± 2.9 (38)	10.5 ± 2.9 (38)
B. breve	7.7 ± 0.7 (100)	7.9 ± 0.8 (100)	8.1 ± 1.0 (100)	7.5 ± 0.9 (100)

The copy number of the *Bifidobacterium* 16S rRNA gene was measured using a real-time PCR method.
Values are expressed as the mean ± SD of the $\log_{10}$ number (16S rDNA copy number) per gram wet feces.
Figures in parentheses are frequencies of occurrence (%).

Table 3. Effect of soymilk intake on fecal properties

Item	Before intake	During soymilk intake		After intake
	Day 0	Day 7	Day 14	Day 28
pH	6.6 ± 0.4	6.2 ± 0.7	6.5 ± 0.6	6.6 ± 0.4
Ammonia (mM)	50.9 ± 36.3	54.7 ± 27.8	62.2 ± 44.7	47.4 ± 15.7
Short-chain fatty acids (μmol/g)				
Acetic acid	59.4 ± 21.3	61.3 ± 25.9	58.8 ± 23.8	50.2 ± 14.0
Propionic acid	20.1 ± 8.0	19.8 ± 8.8	22.8 ± 12.4	21.0 ± 6.2
Butyric acid	15.8 ± 11.3	17.0 ± 14.3	17.1 ± 17.2	15.3 ± 8.7

Each value is expressed as the mean ± SD.

Bifidobacterium longum [7, 16], *Bifidobacterium breve* [7, 16], *Bifidobacterium catenulatum* [16], *B. catenulatum* group [16], *Bifidobacterium pseudocatenulatum* [16], *Bifidobacterium adolescentis* [7, 16], while *Bifidobacterium bifidum* does not utilize these saccharides [7, 16]. Hara *et al.* demonstrated that *B. bifidum* does not utilize raffinose [17]. In the present study, the number of *B. bifidum* was increased slightly by the intake of soymilk, which was similar to the observations in other *Bifidobacterium* species. The reason for this increase

currently remains unclear. *Bifidobacterium* growth promoters other than soybean oligosaccharides may be present in soymilk. On the other hand, *B. bifidum* has also been reported to utilize raffinose and stachyose [28]. The usage of these sugars may differ depending on the strain and/or culture conditions. The results obtained from the measurement of saccharides indicated the presence of soybean oligosaccharides in soymilk. In a previous study [16], the intake of non-fermented soymilk was shown to slightly increase the number of fecal bifidobacteria. Although soymilk does not contain probiotics, unlike fermented soymilk, its efficacy was still observed. It appears that soybean oligosaccharides are one of the substances that increase the number of bifidobacteria. Most *Bifidobacterium* species were influenced, and the number of *Enterobacteriaceae* was significantly decreased by the intake of soymilk. Wada *et al.* [8] previously reported that the number of fecal *Enterobacteriaceae* significantly decreased during the intake of a soybean oligosaccharides extract (SOE) at 6.2 g/day. The SOE contained 32% soybean oligosaccharides refined (SOR). SOR is a purified stachyose and raffinose fraction that contains 75% stachyose and 25% raffinose [8]. We also observed a decrease in fecal pH during the intake of soymilk, but it was not significant. Although pH was lowered, no significant changes were observed in the fecal properties investigated. Factors other than short-chain fatty acids and ammonia may play a role in reducing fecal pH. Further studies are needed to clarify this cause and effect relationship.

The results obtained in this study suggest the potential of soymilk as a functional food. Fermented soymilk containing probiotics such as *Lactobacillus* and *Bifidobacterium* may also be a candidate synbiotic. Since the number of volunteers was small in the present study, further studies are needed in order to clarify the functions of soymilk.

REFERENCES

1. Onozawa M, Fukuda K, Ohtani M, Akaza H, Sugimura T, Wakabayashi K. 1998. Effects of soybean isoflavones on cell growth and apoptosis of the human prostatic cancer cell line LNCaP. Jpn J Clin Oncol 28: 360–363. [Medline] [CrossRef]
2. Zava DT, Duwe G. 1997. Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells *in vitro*. Nutr Cancer 27: 31–40. [Medline] [CrossRef]
3. Tanaka M, Watanabe T, Uchida T, Kanazawa T, Osanai T, Okumura K. 2006. Hypoglycemic effect of soyasaponin B extracted from hypocotyl on the increasing blood glucose in diabetic mice (KK-Ay/Ta). J Jpn Soc Clin Nutr 27: 358–366 (in Japanese with English abstract).
4. Lee SO, Simons AL, Murphy PA, Hendrich S. 2005. Soyasaponins lowered plasma cholesterol and increased fecal bile acids in female golden Syrian hamsters. Exp Biol Med (Maywood) 230: 472–478. [Medline]
5. Lee IA, Park YJ, Yeo HK, Han MJ, Kim DH. 2010. Soyasaponin I attenuates TNBS-induced colitis in mice by inhibiting NF-κB pathway. J Agric Food Chem 58: 10929–10934. [Medline] [CrossRef]
6. Zhang W, Popovich DG. 2010. Group B oleanane triterpenoid extract containing soyasaponins I and III from soy flour induces apoptosis in Hep-G2 cells. J Agric Food Chem 58: 5315–5319. [Medline] [CrossRef]
7. Hayakawa K, Mizutani J, Wada K, Masai T, Yoshihara I, Mitsuoka T. 1990. Effects of soybean oligosaccharides on human faecal flora. Microb Ecol Health Dis 3: 293–303. [CrossRef]
8. Wada K, Mizutani J, Watabe J, Suzuki H, Shirayanagi S. 1991. Effects of soybean oligosaccharides intake on fecal microflora. Bifidus- Flores. Fructus et Semina 5: 51–54 (in Japanese with English abstract).
9. Wada K, Watabe J, Mizutani J, Tomoda M, Suzuki H, Saitoh Y. 1992. Effects of soybean oligosaccharides in a beverage on human fecal flora and metabolites. Nippon Nōgeikagaku Kaishi 66: 127–135 (in Japanese with English abstract). [CrossRef]
10. Benno Y, Endo K, Shiragami N, Sayama K, Mitsuoka T. 1987. Effects of raffinose intake on human fecal microflora. Bifidobacteria Microflora 6: 59–63. [CrossRef]
11. Nagura T, Muraguchi H, Uchino K, Aritsuka T, Benno Y. 1999. Effects of ingestion of raffinose-rich soup on the fecal flora and daily defecation in humans. J Intestinal Microbiol 13: 1–7 (in Japanese with English abstract).
12. Terada A, Yamamoto M, Yoshimura E. 1999. Effect of the fermented soybean product “Natto” on the composition and metabolic activity of the human fecal flora. Jpn J Food Microbiol 16: 221–230. [CrossRef]
13. Fujisawa T, Shinohara K, Kishimoto Y, Terada A. 2006. Effect of miso soup containing Natto on the composition and metabolic activity of the human faecal flora. Microb Ecol Health Dis 18: 79–84. [CrossRef]
14. Yogo T, Ohashi Y, Terakado K, Harada Y, Nezu Y, Hara Y, Tagawa M, Kageyama H, Fujisawa T. 2011. Influence of dried okara-tempeh on the composition and metabolites of fecal microbiota in dogs. Int J Appl Res Vet Med 9: 176–183.
15. Shin R, Itoh Y, Kataoka M, Hara H, Ohashi Y, Miura S, Miura R, Mizutani T, Fujisawa T. 2014. Effect of soybean milk-fermented product on intestinal microbiota and colon carcinogenesis. J Intestinal Microbiol 28: 15–24 (in Japanese with English

abstract).
16. Inoguchi S, Ohashi Y, Narai-Kanayama A, Aso K, Nakagaki T, Fujisawa T. 2012. Effects of non-fermented and fermented soybean milk intake on faecal microbiota and faecal metabolites in humans. Int J Food Sci Nutr 63: 402–410. [Medline] [CrossRef]
17. Hara H, Li ST, Sasaki M, Maruyama T, Terada A, Ogata Y, Fujita K, Ishigami H, Hara K, Fujimori I, Mitsuoka T. 1994. Effective dose of lactosucrose on fecal flora and fecal metabolites of humans. Bifidobacteria Microflora 13: 51–63. [CrossRef]
18. Narai-Kanayama A, Tokita N, Aso K. 2007. Dependence of fructooligosaccharide content on activity of fructooligosaccharide-metabolizing enzymes in yacon (*Smallanthus sonchifolius*) tuberous roots during storage. J Food Sci 72: S381–S387. [Medline] [CrossRef]
19. Mitsuoka T, Sega T, Yamamoto S. 1965. Eine verbesserte Methodik der qualitativen und quantitativen Analyse der Darmflora von Menschen und Tieren. Zentralbl Bakteiol Parasitenkd Infektionskr Hyg I Orig 195: 455–469.
20. Mitsuoka T, Ohno K, Benno Y, Suzuki K, Namba K. 1976. Die Faekalflora bei Menschen. IV. Mitteilung: Vergleich des neu entwickelten Verfahrens mit dem bisherigen üblichen Verfahren zur Darmfloraanalyse. Zentralbl Bakteriol Hyg I Abt Orig A234: 219–233.
21. Terada A, Hara H, Ikegame K, Sasaki M, Mitsuoka T. 1994. Recommended method for enumeration of lecithinase-positive clostridia in human feces. Bifidobacteria Microflora 13: 27–30. [CrossRef]
22. Shinohara K, Ohashi Y, Kawasumi K, Terada A, Fujisawa T. 2010. Effect of apple intake on fecal microbiota and metabolites in humans. Anaerobe 16: 510–515. [Medline] [CrossRef]
23. Ohashi Y, Sumitani K, Tokunaga M, Ishihara N, Okubo T, Fujisawa T. 2015. Consumption of partially hydrolysed guar gum stimulates Bifidobacteria and butyrate-producing bacteria in the human large intestine. Benef Microbes 6: 451–455. [Medline] [CrossRef]
24. Matsuki T, Watanabe K, Fujimoto J, Kado Y, Takada T, Matsumoto K, Tanaka R. 2004. Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. Appl Environ Microbiol 70: 167–173. [Medline] [CrossRef]
25. Godon JJ, Zumstein E, Dabert P, Habouzit F, Moletta R. 1997. Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. Appl Environ Microbiol 63: 2802–2813. [Medline]
26. Mitsuoka T. 2000. Significance of dietary modulation of intestinal flora and intestinal environment. Biosci Microflora 19: 15–25. [CrossRef]
27. Leahy SC, Higgins DG, Fitzgerald GF, van Sinderen D. 2005. Getting better with bifidobacteria. J Appl Microbiol 98: 1303–1315. [Medline] [CrossRef]
28. Matsuyama J, Hirata H, Yamagishi T, Hayashi K, Hirano Y, Kuwata K, Kiyosawa I, Nagasawa T. 1992. Fermentation profiles and utilization of sugars of bifidobacteria in soymilk. Nippon Shokuhin Kogyo Gakkaishi 39: 887–893 (in Japanese with English abstract). [CrossRef]