Title:

Changes in mouse gastrointestinal microbial ecology with the ingestion of kale

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Kale affects murine intestinal flora

Abstract

Kale, a cultivar of *Brassica oleracea*, has attracted a great deal of attention because of its health-promoting effects, which are thought to be exerted through modulation of the intestinal microbiota. The present study was performed to investigate the effects of kale ingestion on the gastrointestinal (GI) microbial ecology of mice. Twenty-one male C57BL/6J mice were divided into three groups and housed in a specific pathogen-free facility. The animals were fed either a control diet or one of two experimental diets supplemented with different commercial kale products for 12 weeks. Contents of the cecum and colon sampled from the mice were processed for the determination of active bacterial populations by a bacterial rRNA-based quantification method and short-chain fatty acid analysis by HPLC. rRNAs of the *Bacteroides-Prevotella*, the *Clostridium coccoides-Eubacterium rectale* group, and the *Clostridium leptum* subgroup constituted the major fraction of microbiota regardless of the composition of the diet. The ratio of *Firmicutes* to *Bacteroidetes* was higher in the colon samples of one of the kale diet group than in the controls. Colonic butyrate level was also higher with the kale-amended diet. Overall, the ingestion of kale tend to either increase or decrease the activity of specific bacterial groups in mouse GI community, whereas the effect may vary depending on its composition.

1. Introduction

Vegetables from the *Brassica* genus (*Brassicaceae* family) are some of the most widely consumed vegetables in the world. Among *Brassica* vegetables, kale (*Brassica oleracea* L. convar. *acephala*) has been reported to exhibit the highest antioxidant capacity and high concentrations and varieties of vitamins, minerals, dietary fibre, glucoraphanin, carotenoids, and polyphenols (Nilsson *et al.*, 2006; Williams *et al.*, 2013). Glucoraphanin is a kind of glucosinolate, which are metabolized by certain bacteria in the gastrointestinal (GI) tract. This microbial processing may increase the amount of glucosinolates conversion into a bioactive compounds (isothiocyanates), which has been suggested to show health-promoting effects on the host (Mullaney *et al.*, 2013). This includes an antibiotic-like effect, resulting in inhibiting the growth of harmful bacteria

(Aires *et al.*, 2009). In this way, kale ingestion appears to be intimately involved in the change the intestinal flora, while any direct link between them has yet been elucidated. The present study was therefore performed to investigate the effects of kale ingestion upon the GI microbial ecology of mice, which may affect the metabolic potential of the GI tract.

2. Materials and methods

Animals and diets

Mice were cared for according to the Guide for the Care and Use of Experimental Animals of Shinshu University. Twenty-one 5-week-old male C57BL/6 mice were housed in a specific-pathogen-free facility. A control diet (67% carbohydrate, 19% protein, 4% fat, and 4% ash) consisted mainly of casein (190 g/kg diet), corn starch (300 g/kg), sucrose (330 g/kg), cellulose (47 g/kg), soybean oil (22 g/kg) , lard (18 g/kg), vitamins, and minerals. After a one-week acclimatization period on the control diet, the animals were fed either the control diet or one of the two experimental diets supplemented with different commercial kale products (referred to as kale A and B) at 0.1%. To make kale-incorporated feeds, the control diet and a designated amount of either of kale products were mixed, milled and pelletized. Kale A and B had similar nutritional contents (73% carbohydrate, 12% protein, 2% fat) and glucoraphanin was contained only in kale B (3.2 g/kg). Feed and water were supplied for ad libitum intake. All mice were healthy and completed the 12 weeks feeding experiment. Voluntary feed intake did not differ among groups. Intestinal samples were obtained from mice at 18 weeks of age. Mice were sacrificed using CO_2, and their cecal and colonic contents were removed, immediately cooled at 4°C, and then processed for RNA extraction within 1 h after collection.

Sample treatment, RNA extraction, and microbial quantification

Cooled samples (ca. 0.05 g) were suspended in 1 ml of PBS buffer (pH 7.4) and mixed thoroughly to equalize the distribution in the buffer. Total RNAs were extracted from the prokaryotic cells of the suspensions as described previously (Uyeno *et al.*, 2013), followed by purification using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Solutions of the extracted RNA were stored at –80°C until use. An RNA-based, sequence-specific rRNA cleavage method was applied to monitor active bacterial populations of the intestinal samples (Uyeno *et al.*, 2010). Briefly, a mixture comprised of the RNA solution, a hybridization buffer (375 mM Tris-HCl [pH 7.5], 15 mM EDTA, 375 mM NaCl), an oligonucleotide probe solution, a defined amount of formamide and an RNase-free H_2O was subsequently heated at 95°C for 1 min to unfold the RNA molecules. Thereafter, an enzyme solution (25 mM Tris-HCl, 40 mM $MgCl_2$, 25 mM NaCl, 4 mM dithiothreitol, 120 mg bovine serum albumin/ml, 20 U of cloned Ribonuclease H from *Escherichia coli* [TaKaRa, Kyoto, Japan]/ ml) was added to the mixture to initiate the cleavage reaction. After incubation at 50°C for 15 min, a stop solution contained EDTA and sodium acetate was added to the mixture to terminate the reaction. The RNA in the mixture was purified by ethanol precipitation and subjected to electrophoresis by a MultiNA Bioanalyzer (Shimadzu, Kyoto, Japan). The signal intensities of respective peaks in the electropherograms were determined and converted to peak areas to calculate the SSU rRNA population of the target group in total SSU rRNAs. For detection and quantification of respective bacterial groups, the following probes were used: Bac303m (*Bacteroides* and *Prevotella*); Erec482m (*Clostridium coccoides-Eubacterium rectale* group); Rfla1269

(*Ruminococcus flavefaciens*), Rbro730m (*R. bromii*), and Fprau645 (*Faecalibacterium prausnitzii*); Lab158m (*Lactobacillus*); Ralb196, Snm1418, Cvir432, URBI432, and URBII611 (other groups belonging to the phylum *Firmicutes*); and Bif164 (*Bifidobacterium*). These probes were separately applied using the same reaction conditions in previous studies (Uyeno *et al.*, 2013; Uyeno *et al.*, 2010).

Organic acid measurements

Cooled samples (ca. 0.05 g) were weighed and dispersed in 1 ml of sterilized water. As the pH of the suspensions ranged from 6.2 to 6.9, most SCFA and lactic acid were recovered as salts of these organic acids. Suspensions were centrifuged at 1000 ***g*** at 4°C for 5 min. The supernatants were used to analyse the organic acids with an HPLC system equipped with an electroconductivity detector (LC-20 model; Shimadzu Corp., Kyoto, Japan) as described previously (Miyamoto *et al.*, 2005).

Statistical analyses

Measurements were analysed by one-way ANOVA followed by Bonferroni test. All analyses were performed using Stat View 5.0J (SAS Institute, Cary, NC). In all analyses, $P < 0.05$ was taken to indicate statistical significance.

3. Results

Bacterial profiles in cecal and colonic contents of tested mice are shown in Figure 1. Bacterial groups by using six probes (Ralb196, Snm1418, Cvir432, URBI432, URBII611, and Bif164) were found at levels below the detection limit (0.2% of total 16S rRNA) in all the samples (data not shown). rRNAs of *Bacteroides-Prevotella*, *C. coccoides-E. rectale* group, and *C. leptum* subgroup constituted the major fraction of the bacterial community (approximately 60% – 74% of the total 16S rRNA). The majority of change occurred in two major lineages within the phylum *Firmicutes*: *C. coccoides-E. rectale* group and *C. leptum* subgroup, as well as in the phylum *Bacteroidetes*: *Bacteroides* and *Prevotella*. With regard to cecal contents, the populations of *R. flavefaciens*, *R. bromii*, and *F. prausnitzii* (all of which are belonging to *C. leptum* subgroup) in the kale product B ingestion group was higher than those of the control group. Although not significant, the *C. coccoides-E. rectale* group also tended to be more prominent in both the kale product A and the kale product B groups than in the control group ($P < 0.10$). With regard to colonic contents, the populations of *Bacteroides-Prevotella* in the kale product B ingestion group were lower than one sixth that of the control group. Totally, the microbiota in mice that had ingested kale had an elevated proportion of *Firmicutes* and a reduced population of *Bacteroidetes*. As a consequence, the *Bacteroidetes*/*Firmicutes* ratio was lower in the kale product B ingestion group compared to the controls in colon samples. The *Lactobacillus-Enterococcus* group was shown to constitute approximately 1% each of the total rRNA of cecum and colon samples, and there were no differences between treatment groups. Organic acid profiles of the cecal and the colonic contents were also determined, and therefore the butyrate concentrations were higher in the kale product B ingestion group than in the control group (Figure 2). Lactate and valerate were minor constituents (< 0.5 mmol/kg sample) of the total organic acids of the cecal and the colonic contents and were not different among the three groups (data not shown).

4. Discussion

With regard to flora composition, our observations are generally consistent with previous studies using molecular techniques for studying murine GI microbiota, indicating that *Firmicutes* and *Bacteroidetes* are the main bacterial phyla (Abnous *et al.*, 2009). The rest of the bacterial populations in the contents other than the determined groups were not determined, but probably would be mainly comprised of bacteria belonging to the phylum *Actinobacteria* and the phylum *Proteobacteria* as suggested by the results of previous studies (Hildebrandt *et al.*, 2009; Uyeno *et al.*, 2008). Changes of active bacterial populations in the mouse GI tract by kale ingestion observed here may be a reflection of some phenolic compounds that act as antibiotics toward specific microbes (Selma *et al.*, 2009). Quantitative and qualitative differences in such functional compounds may be responsible for the relatively greater degree of change in microbial ecology in the kale product B group, in which glucoraphanin was included.

We observed significant alterations associated with introduction of kale to the diet, including a decrease in *Bacteroidetes* and an increase in *Firmicutes* in GI contents. It has been reported that there is an altered ratio of the abundance of the two dominant bacterial phyla in the GI microbiota, i.e., an increase in the *Bacteroidetes* and a decrease in the *Firmicutes*, in obese humans compared to lean individuals (Flint, 2011; Ji *et al.*, 2012). However, as there have been conflicting reports regarding the abundance of *Bacteroidetes* and *Firmicutes* between lean and obese humans and experimental animals (Jumpertz *et al.*, 2011; Schwiertz *et al.*, 2010), the differences between the gut microbiota in lean and obese individuals remain incompletely understood (Shen *et al.*, 2013). Kale ingestion may induce topological changes in whole genetic functions of the gut microbiome, followed by switching energy harvest, storage, and expenditure mechanisms in the host. In relation to this, *Firmicutes* are known to produce butyrate as one of the fermentation products (Louis and Flint, 2009). Butyrate in the intestine is advantageous for the integrity of the mucosal barrier function of the colon and thereby benefits the host's health. Indeed, the colonic samples from mice that had ingested kale B had an increased butyrate concentration (Figure 2). The predominant butyrate producer in this phylum is belonging to the genus *F. prausnitzii* (Duncan *et al.*, 2002). Also in the present study, it appears there is a positive relevance between the population of *F. prausnitzii* and butyrate production in the GI tract.

The results of this study suggest that alterations of active populations of mouse GI microbiota may be possible by intake of a specific type of food, and improvements in health may be mediated by optimizing the GI microbial community. This represents an expansion of the already known nutritional functions of the microflora, although the detailed effects of dietary factors on gut microbiota and host metabolism, especially in humans, are largely unknown. It will be of interest to determine how microbial communities encode traits that markedly affect host biology, and what is responsible for mediating the linkage between the relative abundance of *Bacteroidetes* to *Firmicutes* and energy deposition and expenditure mechanisms. This study succeeded in quantitatively determining active bacterial groups within mouse GI microbiota affected by the ingestion of a specific food. Basic information provided by the present study benefits for future meta-omics-based studies, which will provide deeper insights into the effects of "health-improving" foods on metabolic functions and interactions between the GI microbiota and host. Further studies to determine the links between gut microbiota and host energy metabolism are warranted, as well as to determine which component of kale is effective to change the community structure.

References

196 Abnous, K., Brooks, S.P.J., Kwan, J., Matias, F., Green-Johnson, J., Selinger, L.B.,
197 Thomas, M. and Kalmokoff, M., 2009. Diets enriched in oat bran or wheat bran
198 temporally and differentially alter the composition of the fecal community of
199 rats. Journal of Nutrition 139: 2024-2031.
200 Aires, A., Mota, V.R., Saavedra, M.J., Rosa, E.A.S. and Bennett, R.N., 2009. The
201 antimicrobial effects of glucosinolates and their respective enzymatic hydrolysis
202 products on bacteria isolated from the human intestinal tract. Journal of Applied
203 Microbiology 106: 2086-2095.
204 Duncan, S.H., Hold, G.L., Barcenilla, A., Stewart, C.S. and Flint, H.J., 2002. *Roseburia*
205 *intestinalis* sp. nov., a novel saccharolytic, butyrate-producing bacterium from
206 human faeces. International Journal of Systematic and Evolutionary
207 Microbiology 52: 1615-1620.
208 Flint, H.J., 2011. Obesity and the gut microbiota. Journal of Clinical Gastroenterology
209 45: S128-S132.
210 Hildebrandt, M.A., Hoffmann, C., Sherrill–Mix, S.A., Keilbaugh, S.A., Hamady, M.,
211 Chen, Y.Y., Knight, R., Ahima, R.S., Bushman, F. and Wu, G.D., 2009.
212 High-fat diet determines the composition of the murine gut microbiome
213 independently of obesity. Gastroenterology 137: 1716-1724.
214 Ji, Y., Kim, H., Park, H., Lee, J., Yeo, S., Yang, J., Park, S., Yoon, H., Cho, G., Franz,
215 C., Bomba, A., Shin, H. and Holzapfel, W., 2012. Modulation of the murine
216 microbiome with a concomitant anti-obesity effect by *Lactobacillus rhamnosus*
217 GG and *Lactobacillus sakei* NR28. Beneficial Microbes 3: 13-22.
218 Jumpertz, R., Le, D.S., Turnbaugh, P.J., Trinidad, C., Bogardus, C., Gordon, J.I. and
219 Krakoff, J., 2011. Energy-balance studies reveal associations between gut
220 microbes, caloric load, and nutrient absorption in humans. American Journal of
221 Clinical Nutrition 94: 58-65.
222 Louis, P. and Flint, H.J., 2009. Diversity, metabolism and microbial ecology of
223 butyrate-producing bacteria from the human large intestine. FEMS
224 Microbiology letters 294: 1-8.
225 Miyamoto, M., Seto, Y., Hai Hao, D., Teshima, T., Bo Sun, Y., Kabuki, T., Bing Yao, L.
226 and Nakajima, H., 2005. *Lactobacillus harbinensis* sp. nov., consisted of strains
227 isolated from traditional fermented vegetables 'Suan cai' in Harbin,
228 Northeastern China and *Lactobacillus perolens* DSM 12745. Systematic and
229 Applied Microbiology 28: 688-694.
230 Mullaney, J.A., Kelly, W.J., McGhie, T.K., Ansell, J. and Heyes, J.A., 2013. Lactic acid
231 bacteria convert glucosinolates to nitriles efficiently yet differently from
232 *Enterobacteriaceae*. Journal of Agricultural and Food Chemistry 61: 3039-3046.
233 Nilsson, J., Olsson, K., Engqvist, G., Ekvall, J., Olsson, M., Nyman, M. and Åkesson,
234 B., 2006. Variation in the content of glucosinolates, hydroxycinnamic acids,
235 carotenoids, total antioxidant capacity and low-molecular-weight carbohydrates
236 in *Brassica* vegetables. Journal of the Science of Food and Agriculture 86:
237 528-538.
238 Schwiertz, A., Taras, D., Schäfer, K., Beijer, S., Bos, N.A., Donus, C. and Hardt, P.D.,
239 2010. Microbiota and SCFA in lean and overweight healthy subjects. Obesity
240 18: 190-195.
241 Selma, M.a.V., Espín, J.C. and Tomás-Barberán, F.A., 2009. Interaction between
242 phenolics and gut microbiota: role in human health. Journal of Agricultural and
243 Food Chemistry 57: 6485-6501.
244 Shen, J., Obin, M.S. and Zhao, L., 2013. The gut microbiota, obesity and insulin

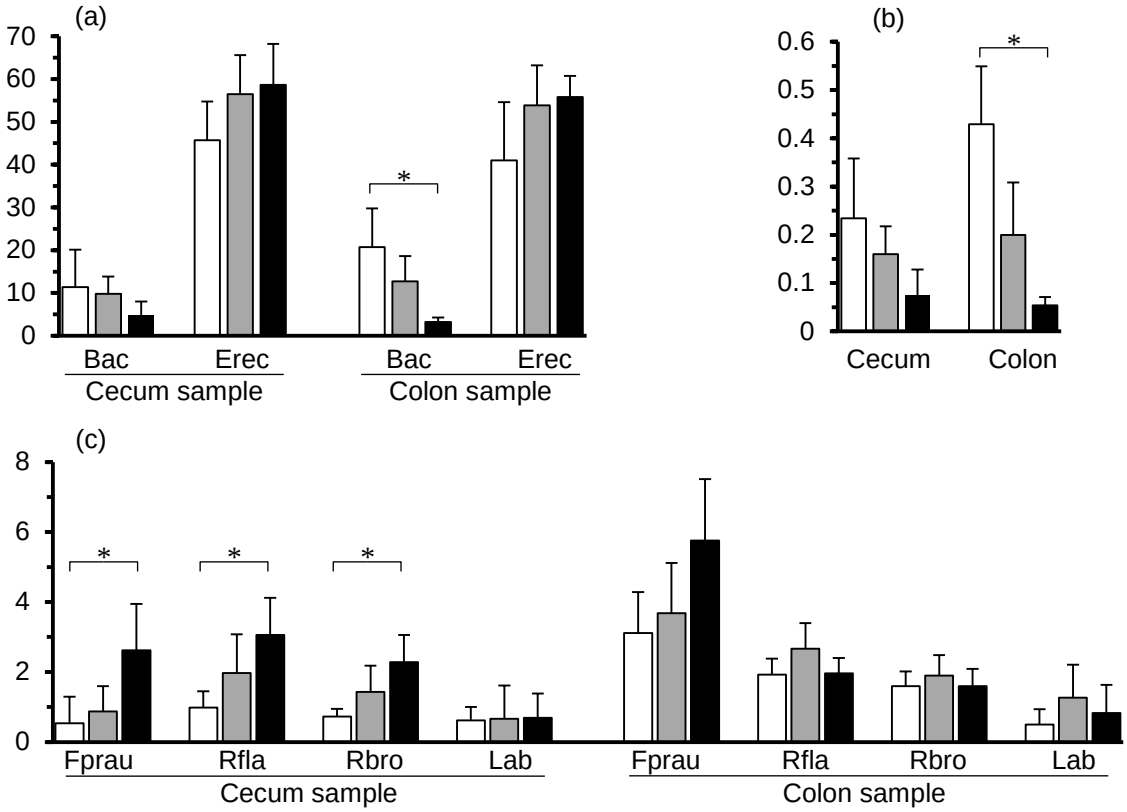
resistance. Molecular Aspects of Medicine 34: 39-58.
Uyeno, Y., Kawashima, K., Hasunuma, T., Wakimoto, W., Noda, M., Nagashima, S., Akiyama, K., Tabata, M. and Kushibiki, S., 2013. Effects of cellooligosaccharide or a combination of cellooligosaccharide and live *Clostridium butyricum* culture on performance and intestinal ecology in Holstein calves fed milk or milk replacer. Livestock Science 153: 88-93.
Uyeno, Y., Sekiguchi, Y. and Kamagata, Y., 2008. Impact of consumption of probiotic lactobacilli-containing yogurt on microbial composition in human feces. International Journal of Food Microbiology 122: 16-22.
Uyeno, Y., Sekiguchi, Y. and Kamagata, Y., 2010. rRNA-based analysis to monitor succession of faecal bacterial communities in Holstein calves. Letters in Applied Microbiology 51: 570-577.
Williams, D.J., Edwards, D., Hamernig, I., Jian, L., James, A.P., Johnson, S.K. and Tapsell, L.C., 2013. Vegetables containing phytochemicals with potential anti-obesity properties: A review. Food Research International 52: 323-333.

Figure legends

Figure 1. Bacterial populations in cecum and colon samples of mice in control (open bar), or fed kale product A (grey bar) or B (closed bar). (a) Bacterial populations detected by probes Bac303m (Bac, *Bacteroides* - *Prevotella*) and Erec482m (Erec, *Cl. coccoides* - *Eu. rectale* group). (b) *Bacteroidetes*/*Firmicutes* ratio. The population of *Bacteroides* - *Prevotella* was applied to *Bacteroidetes*, and the population of *Firmicutes* was calculated by summarizing the values *C. coccoides*-*E. rectale* group, *F. prausnitzii*, *R. flavefaciens*, *R. bromii*, and *Lactobacillus*. (c) Bacterial populations detected by probes Fprau645 (Fprau, *F. prausnitzii*), Rfla1269 (Rfla, *R. flavefaciens*), Rbro730m (Rbro, *R. bromii*), and Lab158m (Lab, *Lactobacillus*). Measurements of bacterial groups are expressed as % of total 16S rRNA in (a) and (c). Statistical significance for the same group is indicated as * ($P < 0.05$).

Figure 2. Organic acid concentrations in cecum and colon samples of mice in control (open bar), or fed kale product A (grey bar) or B (closed bar). Measurements are expressed as mmol/kg sample. Statistical significance for the same group is indicated as * ($P < 0.05$).

（Figure 1）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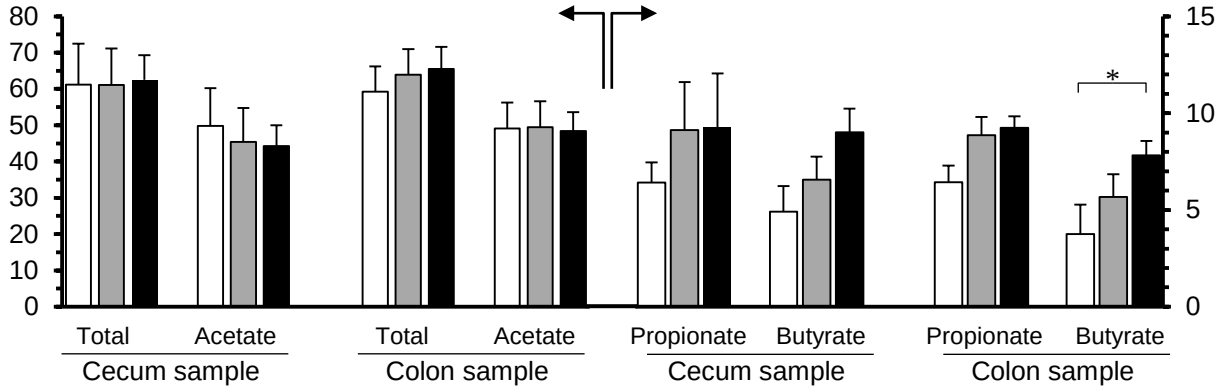
(Figure 2)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