Effects of Astaxanthin on Intestinal Microflora in Mice Fed a High-fat Diet

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Abstract

Objective: The human enteric flora changes due to aging and various life environmental factors, and is involved in our health status and diseases, including cancer. To retain a normal enteric flora is important to the maintenance of a young, healthy body. This study was conducted to assess the effects of astaxanthin on the enteric gut flora.

Methods: We examined the changes in the enteric flora in mice fed a high-fat diet and the effects of astaxanthin on said mice using real-time PCR. The mice were divided into 4 groups: those receiving an ordinary diet (fat 3.9%) alone, those receiving an ordinary diet plus astaxanthin (0.02%), those receiving a high-fat diet (fat 35%) alone, and those receiving a high-fat diet plus astaxanthin (n=5 for each group). Feces were collected at 14 and 28 days. Bacterial DNA was extracted to be used for template DNA. Each extract was analyzed using 16S rRNA-targeted group-specific PCR primers as follows: all bacteria, Bacteroides, Bifidobacterium, Prevotella, Lactobacillus, Streptococcus, Clostridium coccoides, and Clostridium leptum. The relative ratio of each test bacteria to all bacteria was measured.

Results: An analysis of the enteric flora revealed that ingestion of the high-fat diet led to increased prevalences of the genus Bacteroides, the Clostridium coccoides group, and the Clostridium leptum group, and a decreased prevalence of the Streptococcus group (lactobacilli). These changes were suppressed in the mice receiving astaxanthin in their diet.

Conclusion: Administration of astaxanthin acts to suppress enteric flora derangement induced by administering a high-fat diet to mice.

KEY WORDS: astaxanthin, high-fat diet, enteric flora, Lactobacillus, real-time PCR

Materials and Methods

Preparation of ordinary and high-fat diets containing the test material

Labo MR Stock (Nosan Corporation, Nishi-ku, Yokohama, Kanagawa, Japan) was used for the ordinary diet, and HFD-60 (Oriental Yeast Co., Ltd., Itabashi-ku, Tokyo, Japan) for the high-fat diet. The ordinary and high-fat diets were prepared to obtain a test material (astaxanthin) concentration of 0.02%. Astaxanthin (AstaREAL® Oil 50F) was kindly supplied by Fuji Chemical Industry, Co., Ltd. (Kamiichimachi, Nakaniikawa-gun, Toyama, Japan). Nutritional ingredients were as follows: fat 3.9%, protein 18.8%, carbohydrates 54.7% and dietary fiber 6.6% in the ordinary diet, and fat 35%, protein 23%, carbohydrates 25.3%, and dietary fiber 6.6% in the high-fat diet.

Animal husbandry and assay procedures

Twenty ICR mice at 4 weeks of age were individually reared for acclimation in separate cages for 1 week. They were then individually weighed and divided into 4 groups of 5 animals: the ordinary diet group (Group C), the ordinary diet...
plus astaxanthin group (Group CA), the high-fat diet group (Group H), and the high-fat diet plus astaxanthin group (Group HA). On the final day of acclimation (Day 0), 24-hour pooled feces were recovered. Starting on Day 1, the specified diets were fed to the respective groups, and each animal was weighed once weekly. They were fed once daily for the ordinary diet group, or every 2 days for the high-fat diet group (the feed was used after being returned to room temperature on the day before feeding). Feces were recovered over the 13th and 14th days of rearing (Day 14) and the 27th and 28th days of rearing (rearing ended on Day 28). The recovered feces were stored at −20°C.

**Extraction of DNA**

After being thawed and weighed, the feces recovered on Days 0, 14, and 28 were dried at room temperature overnight. The following day, the dry feces were weighed, and then totally powdered. About 100 mg of each powdered fecal sample was transferred to a micro-test tube and subjected to DNA extraction using the QIAamp® DNA Stool Mini Kit (Qiagen, Venlo, Netherlands). The concentrations of the DNA extract solutions were determined using NanoDrop.

**Real-time PCR**

DNA solutions were diluted 100 fold for all test bacteria together, and for *Lactobacillus*, *Streptococcus*, *Clostridium cocoides*, and *Clostridium leptum*, and adjusted to a final concentration of 20 μg/μL for the other targets. Primer sequences were determined according to the protocol for LightCycler® 480 SYBR Green I Master (Roche Diagnostics K.K., Minato-ku, Tokyo, Japan) by real-time PCR analysis using DNA extraction using the QIAamp® DNA Stool Mini Kit (Qiagen, Venlo, Netherlands). The concentrations of the DNA extract solutions were determined using NanoDrop.

**Statistical analyses**

All measurements obtained in this study are expressed herein as mean ± standard deviation. Mean data for each group were statistically analyzed using Tukey’s multiple comparison test. To compare the ordinary and high-fat diets, bacterial count change rates during ordinary diet ingestion (Group C and Group CA) and high-fat diet ingestion (Group H and Group HA) on Days 0, 14, and 28 were statistically analyzed using Mann-Whitney’s U test (two-tailed test). To compare the addition and non-addition of astaxanthin, bacterial count change rates were statistically analyzed using Mann-Whitney’s U test (one-tailed test). A p-value of <0.05 was considered to indicate a significant difference. All statistical analyses were performed using SPSS for Windows Ver. 15.0 (SPSS Inc. Chicago, IL, USA).

**Results**

**Changes in body weight over time and nature of feces**

Changes in body weight over time are shown in Fig. 1. Group H experienced remarkable weight gain compared with Group C (p<0.01, p<0.05). Group HA experienced remarkable weight gain compared with Group CA (p<0.05). No significant difference was found between the astaxanthin addition groups (Group CA, Group HA) and the non-addition groups (Group C, Group H).

Fecal color was dark green for Group C, red-brown for Group CA, light greenish gray for Group H, and red to orange for Group HA (Fig. 2). On Day 14 and Day 28, fecal weight was significantly lighter for Group H than for Group C, and for Group HA than for Group CA (Fig. 3, p<0.001).

**Comparison of bacterial quantities by real-time PCR**

**DNA content**

The fecal DNA content (mean for Days 0, 14, and 28) was determined by the NanoDrop method to be 89.8±21.5 ng/μL for Group C, 102.7±28.5 ng/μL for Group CA, 70.2±21.2 ng/μL for Group H, and 66.9±28.4 ng/μL for Group HA (Fig. 4). The DNA content tended to decrease in the high-fat diet groups (Group H + Group HA) compared with the ordinary diet groups (Group C + Group CA) (p=0.001).

**Genus Bacteroides**

The bacterial count of the genus *Bacteroides* was low, accounting for less than 0.003% of the total bacterial count of all test bacteria together (Fig. 5). With high-fat diet ingestion, the bacterial count increased significantly (p=0.006). In Group H, a significantly increased bacterial count was found on Day 14 (p<0.01 versus Day 0, p<0.05 versus Group C); however, this increase was transient, and on Day 28, the bacterial count returned to the Day 0 level. No such transient increase was found in Group HA. When comparing the different diets, the bacterial count increase rate was higher in the high-fat diet group than in the ordinary diet group (p=0.002). Regarding the effect of addition of astaxanthin, no significant difference was found between the addition groups (Group CA, Group HA) and the non-addition groups (Group C, Group H).

**Bifidobacteria**

The bacterial count of bifidobacteria was extremely low, accounting for less than 0.000006% of the total bacterial count of all test bacteria together (Fig. 6). In all groups, no significant difference was found to result from the addition of astaxanthin, nor was there any significant difference among the groups. Since the standard deviation was great among the individual animals, the findings were handled for reference purposes only.

**Genus Prevotella**

The bacterial count of the genus *Prevotella* was low, accounting for less than 0.06% of the total bacterial count of all test bacteria together (Fig. 7). With the high-fat diet, the bacterial count decreased remarkably (p<0.001). When comparing the different diets, the bacterial count increase rate
was higher in the ordinary diet group than in the high-fat diet group \((p<0.001)\). No significant difference was found to result from addition of astaxanthin.

**Streptococcus group (lactic acid bacteria)**

The bacterial count of the *Streptococcus* group accounted for less than 16% of the total bacterial count of all test bacteria together (Fig. 8). Although the bacterial count increased remarkably with ingestion of an ordinary diet, the bacterial count change caused by the high-fat diet was minor \((p=0.028)\). The bacterial count increase rate was remarkably lower in the high-fat diet group than in the ordinary diet group \((p=0.008)\). In Group HA (fed astaxanthin), the rate returned to the level for the ordinary diet groups (Group C and Group CA) on Day 28. During high-fat diet ingestion, a significant difference was found between the groups with and without addition of astaxanthin \((p=0.044)\).

![Fig. 1. Comparison of changes in body weight over time](image)

*C*: ordinary diet group, CA: ordinary diet plus astaxanthin group, *H*: high-fat diet group, HA: high-fat diet plus astaxanthin group (\(n=5\) for each group). Mean± standard deviation. **\(p<0.01\), *\(p<0.05\) vs. Group C, #\(p<0.05\) vs. Group CA, Tukey’s multiple comparison test.

![Fig. 2. Colors of fecal samples](image)

Alterations of Mouse Intestinal Microflora by a High-fat Diet and Astaxanthin

**Fig. 3.** Comparison of fecal weight
C: ordinary diet group, CA: ordinary diet plus astaxanthin group, H: high-fat diet group, HA: high-fat diet plus astaxanthin group (n=5 for each group). Mean±standard deviation. ***p<0.001, Tukey’s multiple comparison test.

**Fig. 4.** DNA content determined using the NanoDrop method
C: ordinary diet group, CA: ordinary diet plus astaxanthin group, H: high-fat diet group, HA: high-fat diet plus astaxanthin group (n=5 for each group). Mean±standard deviation. Unit of measurement: ng/mL. The DNA copy number change rates were compared by diet using Mann-Whitney’s U test.
**Fig. 5.** Putative copy number (%) of bacteria of the genus *Bacteroides* relative to total primer measurement for all test bacteria
C: ordinary diet group, CA: ordinary diet plus astaxanthin group, H: high-fat diet group, HA: high-fat diet plus astaxanthin group (n=5 for each group). Mean± standard deviation. The groups were compared using Tukey’s multiple comparison test, and the bacterial count change rates (dotted lines) were compared by diet using Mann-Whitney’s U test.

**Fig. 6.** Putative copy number (%) of bifidobacteria relative to total primer measurement for all test bacteria
C: ordinary diet group, CA: ordinary diet plus astaxanthin group, H: high-fat diet group, HA: high-fat diet plus astaxanthin group (n=5 for each group).
**Fig. 7.** Putative copy number (%) of bacteria of the genus *Prevotella* relative to total primer measurement for all test bacteria

C: ordinary diet group, CA: ordinary diet plus astaxanthin group, H: high-fat diet group, HA: high-fat diet plus astaxanthin group (n=5 for each group). Mean±standard deviation. The groups were compared using Tukey’s multiple comparison test, and the bacterial count change rates (dotted lines) were compared by diet using Mann-Whitney’s U test.

**Fig. 8.** Putative copy number (%) of bacteria of the *Streptococcus* group (lactic acid bacteria) relative to total primer measurement for all test bacteria

C: ordinary diet group, CA: ordinary diet plus astaxanthin group, H: high-fat diet group, HA: high-fat diet plus astaxanthin group (n=5 for each group). The addition of astaxanthin during high-fat diet ingestion raised the bacterial count change rate (dotted lines) (p<0.05). Mean±standard deviation. The groups were compared using Tukey’s multiple comparison test, and the bacterial count change rates (arrows) were compared using Mann-Whitney’s U test.
Lactobacillus group (lactic acid bacteria)

The bacterial count of the Lactobacillus group accounted for less than 3% of the total bacterial count of all test bacteria together (Fig. 9). With the administration of the high-fat diet, the bacterial count increased significantly (p=0.004). In the ordinary diet groups (Group C and Group CA), the bacterial count tended to increase over time through Day 14 and Day 28. No significant difference was found in the bacterial count increase rate between the ordinary diet and the high-fat diet (p=0.069). The bacterial count increase rate increased with the addition of astaxanthin (p=0.031).

Clostridium coccoides group

The bacterial count of the Clostridium coccoides group accounted for less than 1.4% of the total bacterial count of all test bacteria together (Fig. 10). With the administration of the high-fat diet, the bacterial count increased significantly (p=0.016). The bacterial count increase rate was higher in the high-fat diet group than in the ordinary diet group (p=0.012). The bacterial count increase rate decreased with the addition of astaxanthin during high-fat diet ingestion (p=0.029).

Clostridium leptum group

The bacterial count of bacteria of the Clostridium leptum group accounted for less than 1.6% of the total bacterial count of all test bacteria together (Fig. 11). With the high-fat diet, the bacterial count increased significantly (p=0.017). The bacterial count increase rate was higher in the high-fat diet group than in the ordinary diet group (p=0.002). In Group HA, the bacterial count increased transiently on Day 14 (p<0.001, versus Group H); however, on Day 28, no difference was found between Group H and Group HA. No significant difference was found to result from the addition of astaxanthin.

Phylum level analysis

The ratios of bacterial counts of the phylum Firmicutes, the phylum Bacteroidetes, and other phyla, determined by the phylum level analysis, are shown in Fig. 12.

The bacterial count of the phylum Firmicutes accounted for about 20% of the total bacterial count of all test bacteria together on Day 0, but increased to 40% in the high-fat diet group on both Day 14 and Day 28 (p<0.001, Fig. 13). The bacterial count change rate was significantly higher in the high-fat diet group than in the ordinary diet group (p<0.001). No significant difference was found to result from the addition of astaxanthin.

The bacterial count of the phylum Bacteroidetes accounted for about 45-50% of the total bacterial count of all test bacteria together on Day 0, but decreased to about 20% in the high-fat diet group on both Day 14 and Day 28 (p<0.001, Fig. 14). The bacterial count change rate was significantly lower in the high-fat diet group than in the ordinary diet group (p<0.01). No significant difference was found to result from addition of astaxanthin.
**Fig. 10.** Putative copy number (%) of bacteria of the Clostridium coccoides group relative to total primer measurement for all test bacteria
C: ordinary diet group, CA: ordinary diet plus astaxanthin group, H: high-fat diet group, HA: high-fat diet plus astaxanthin group (n=5 for each group). Ingestion of high-fat diet raised the bacterial count change rates (dotted lines), and addition of astaxanthin lowered the bacterial count change rate (arrows) \((p<0.05)\). Mean±standard deviation. The groups were compared using Tukey’s multiple comparison test, and the bacterial count change rates were compared by diet using Mann-Whitney’s U test.

**Fig. 11.** Putative copy number (%) of bacteria of the Clostridium leptum group relative to total primer measurement for all test bacteria
C: ordinary diet group, CA: ordinary diet plus astaxanthin group, H: high-fat diet group, HA: high-fat diet plus astaxanthin group (n=5 for each group). Mean±standard deviation. The groups were compared using Tukey’s multiple comparison test, and the bacterial count change rates (dotted lines) were compared by diet using Mann-Whitney’s U test.
**Fig. 12.** Phylum level analysis
Shown are the ratios of the bacterial quantities of the phylum *Firmicutes*, the phylum *Bacteroidetes*, and other phyla relative to the total primer measurement for all test bacteria. C: ordinary diet group, CA: ordinary diet plus astaxanthin group, H: high-fat diet group, HA: high-fat diet plus astaxanthin group (n=5 for each group).

**Fig. 13.** Putative copy number (%) of bacteria of the phylum *Firmicutes* relative to total primer measurement for all test bacteria
C: ordinary diet group, CA: ordinary diet plus astaxanthin group, H: high-fat diet group, HA: high-fat diet plus astaxanthin group (n=5 for each group). Mean±standard deviation. The groups were compared using Tukey’s multiple comparison test (***, vs. Day 0), and the bacterial count change rates (#, dotted lines) were compared by diet using Mann-Whitney’s U test.
Discussion

The human intestine is inhabited by a wide variety of bacteria with a total cell count of 600 trillion, about 10 times the number of cells constituting our body, forming the diverse enteric flora. The enteric flora changes due not only to aging, but also to daily life environmental factors, such as diet, exercise, drinking, and smoking, and is involved in various aspects of health such as obesity, rough skin, atopy, allergies, and cancer development. To retain a normal enteric flora is important to the maintenance of a young, healthy body. In particular, increased fat ingestion in recent years has been reported to alter the enteric flora 5-8).

Under the recently developing concepts of prebiotics and probiotics, ingestion of oligosaccharides, lactic acid bacteria, bifidobacteria, and other bacteria has so far been recommended as an approach to improving our enteric flora. We previously investigated the effects of astaxanthin as a food-derived antioxidant, and reported that administration of astaxanthin enhanced antioxidant capacity, lowered blood pressure and ameliorated climacteric complaints in women with high oxidative stress 1). Finding a gut absorption rate of less than 5%, the same study suggested that astaxanthin could potentially have an effect on the enteric flora as well.

Conventionally, culture methods have been commonly used to analyze the enteric flora; in recent years, however, molecular biological approaches using the 16S rRNA gene domain, known as real-time PCR and terminal restriction fragment length polymorphism (T-RFLP), have been developed, enabling us to achieve highly reliable analyses with simple procedures 2-4, 9-11). Against this background, the present study was conducted for the purpose of examining the changes in the enteric flora in mice fed a high-fat diet, and to assess the effects of astaxanthin on said mice using real-time PCR.

Description of real-time PCR

Numerous unidentified enteric bacteria inhabit the intestinal tract in a symbiotic fashion, forming a complex bacterial community 12,13). Recent progress in the development of DNA sequencers has dramatically advanced research into the complex, highly diverse enteric flora. Ribosomal genes are a group of genes that have been preserved over the boundaries of bacterial species. It has become possible to make an efficient approach to elucidating the reality of the complex, highly diverse enteric flora by comprehensively and quantitatively analyzing the 16S ribosomal RNA gene of the ribosomal small subunit of enterobacterial origin 14).

The measuring principle of this technique is as follows: PCR-based detection with specific primers involves directly extracting DNA of bacterial origin from a fecal sample, selectively amplifying the target gene using bacterial group/ species-specific primers, and detecting the desired bacterium.

Genus Bacteroides

The bacteria belonging to the genus Bacteroides, including B. fragilis, are Gram-negative obligatory anaerobic non-sporeulating bacilli 9). They occur in large numbers in the intestinal tracts of humans and other animals, fermenting sugars to lactic acid, acetic acid, and other products. In human feces, these bacteria occur at 10 billion to 100 billion cells per gram. Although these bacteria are normally non-pathogenic,
they can cause opportunistic infections. Since these bacteria are resistant to many clinically used penicillins and cephalosporins, their use in combination with β-lactamase inhibitors is recommended.

**Genus Lactobacillus (lactic acid bacteria)**

The bacteria belonging to the genus *Lactobacillus* are Gram-positive bacilli. Some species of this genus produce lactic acid only (lactic acid homo-fermentation), and others produce both lactic acid and other products (lactic acid hetero-fermentation). These bacteria are abundant in the digestive tracts of humans and other animals, from the feces of which they are isolated. The genus *Lactobacillus* constitutes a group of bacteria inhabiting a broad range of environments, from fermented foods to animal digestive tracts. With their highly useful functions, these bacteria occupy an extremely important position in research on the effects of lactic acid bacteria on fermented foods and host health. Currently, this genus consists of more than 100 species with diverse profiles.

**Genus Streptococcus (lactic acid bacteria)**

The bacteria belonging to the genus *Streptococcus* are also known as streptococci, referring generically to eubacteria that are Gram-positive cocci. Each measuring about 1 μm across, individual streptococcal cells assume a regular linear arrangement. Biochemically, these bacteria differ from other Gram-positive cocci insofar as they lack catalase. Generally, streptococci do not produce energy by respiration, but gain energy mainly by lactic acid fermentation. Many members of this genus act as lactic acid bacteria in the intestinal tract.

Enterococci were separated from the group of bacteria that had previously been classified as the genus *Streptococcus* and reclassified as an independent family (*Enterococcaceae*). The new family consists of *E. faecalis*, *E. faecium*, and other members, inhabiting the ileum, cecum, and large intestine. Streptococcus mutans is detected in the oral cavity, and, along with *Lactobacillus plantarum* and *Micrococcus micros*, mediates the onset of pulpitis. Pulpitis is induced as the dental pulp is invaded by caries-related bacteria with the progression of dentin caries. Diplococci that do not assume a regular linear arrangement exhibit a preventive effect on influenza virus infection and ameliorating action against allergic symptoms such as in pollinosis. Although these bacteria are abundant in the feces of breast-fed babies, their ratio in the enteric flora decreases gradually due to aging and dietary conditions.

**Genus Prevotella**

The bacteria belonging to the genus *Prevotella* are Gram-negative anaerobic bacilli found in the oral cavity and the intestinal tract. Most oral infections are caused by normal bacteria or other weakly pathogenic bacteria; however, infections with such low-virulence bacteria are characterized by occasional induction of opportunistic infections. Bacteria of the genus *Prevotella* are among the many causative organisms for oral infections such as bacteremia following tooth extraction and aspiration pneumonia in the elderly. Most cases of human anaerobic bacterial infections occur as mixed infections with aerobic and anaerobic bacteria, and bacteria of the genus *Prevotella* can cause these type of infections. They are also detected in tongue fur; the isovaleric acid produced thereby volatilizes and produces an unpleasant odor, causing halitosis.

**Genus Clostridium**

**Clostridium coccoides group**

*Clostridium leptum group*  
*Clostridium* is a genus of eubacteria, consisting of obligatory anaerobic sporulating Gram-positive bacilli. As obligatory anaerobes inhabiting environments with low oxygen concentrations, such as soil and the intestinal tract, these bacteria are unable to proliferate in the presence of oxygen. Generally, obligatory anaerobes lack the antioxidant enzymes superoxide dismutase and catalase, and are hence inactive in the presence of oxygen; however, the bacteria of the genus *Clostridium* produce highly durable spores and have a long dormancy in the presence of oxygen. With this property, they can survive conditions under which other obligatory anaerobes cannot.

*Clostridium tetani*, *C. botulinum*, *C. perfringens*, gas gangrene bacilli (*C. novyi*, *C. septicum*, etc.), and *C. difficile* are well-known members of this genus. Of the *Clostridium* bacteria inhabiting the intestinal tract, *C. coccoides* and *C. leptum* are predominant; however, *C. perfringens* and *C. difficile* are also found. Although *C. perfringens* is a normal bacterium inhabiting the intestines of humans and other animals, some strains produce toxins that can cause food poisoning. *C. difficile* is also found in the intestinal tracts of humans and other animals, and is resistant to antibiotics; during high-dose administration of antibiotics, it can be involved in superinfection, causing pseudomembranous colitis. As the gastric juice secretion decreases during oral treatment with proton pump inhibitor (PPI) and in other situations, the *C. coccoides* concentration increases. One study suggested that some species of the genus *Clostridium* could potentially induce IL-10-producing Treg cells in the large intestine, thus contributing to the control of intestinal inflammation.

**Changes in the enteric flora due to high-fat diet ingestion**

Ingestion of a high-fat diet increases bile acid excretion in the intestinal tract, causing a broad range of changes in the enteric flora, which are more pronounced in the large intestine than the small intestine. A high-fat diet reduces intestinal immunity, impairs mucosal barrier function, and causes abnormal gut fermentation, thus raising the risk of carcinogenesis.
Regarding the changes in the enteric flora induced by high-fat diets, results of animal experiments and human studies have been reported. In the cecum of the KK-Ay mouse, an animal model of type 2 diabetes, the bacterial count of the genus Bacteroides decreased, whereas that of the Clostridium coccoides group tended to increase 7). In a mouse model of high-fat diet-induced obesity, decreased cecal and fecal bacterial counts of bifidobacteria were found 6). In rats, the bacterial counts of the genera Bacteroides and Prevotella increased 65). In humans, a decreased bacterial count of the genus Bacteroides was documented as a change in the enteric flora of obese subjects who were likely to have a high-fat diet 65). Phylum level analyses revealed an increased bacterial count of the phylum Firmicutes and a decreased bacterial count of the phylum Bacteroidetes 66-68).

The phylum level analysis in the present study yielded results similar to those reported from previous studies 66-68). Feeding a high-fat diet (Group H) increased the bacterial counts of the genus Bacteroides, the Clostridium coccoides group, and the Clostridium leptum group, and decreased the bacterial count of the Streptococcus group (lactic acid bacteria). The observed difference in the bacterial count of the genus Bacteroides between our study and the aforementioned studies may be due to the difference in animal species (rats versus mice) or the difference in testing method; however, the reason could not be clarified in the present study. Taking into account the fact that the bacteria of the genus Bacteroides are “bad bacteria”, however, the results of the present study appear to provide more reliable evidence.

The mice used in this study were 5 to 9 weeks old, which in human years would be roughly 20 to 30 years, since the average life span of mice is approximately 100 weeks old. We examined the composition change of intestinal microflora in young adult mice and elucidated the preventive effect of astaxanthin, which was the main purpose of this study.

**Effects of astaxanthin on the enteric flora**

The effects of astaxanthin observed in the present study are summarized as follows:

First, astaxanthin is expected to suppress short-term increases in the bacterial count of the genus Bacteroides (Gram-negative bacteria) resulting from ingestion of a high-fat diet. With regard to lactobacilli (lactic acid bacteria), an interesting finding was obtained: the addition of astaxanthin increased the bacterial count of the genus Lactobacillus. Likewise, the addition of astaxanthin during high-fat diet ingestion increased the bacterial count of streptococci (lactic acid bacteria). These findings suggest that the administration of astaxanthin could potentially improve the enteric flora (by suppressing the rapid growth of Gram-negative bacteria and increasing the bacterial count of the lactobacillus group). In the future, a comprehensive analysis will be needed for other bacterial species through “metagenomics analysis using next-generation sequencers” for 16S rRNA genes.

In this study, the test product was administered to mice by feeding 0.02% astaxanthin-containing food, not by tube feeding. A specific amount of astaxanthin can be ascertained by tube feeding, however this method is invasive and stressful for mice; it often causes upper gastrointestinal injury, and it may result in microfloral composition change. Also the individual differences can be large. In contrast, when mice are fed astaxanthin-containing food, it is not stressful for mice, however the amount depends on food intake and it can vary.

The astaxanthin intake amount (average weight at Day 20; Group C = 38–41g, Group H = 44–48g) was estimated from the daily food intake: Group C = 5g, Group H = 3g, daily fat; Group C = 0.2g, Group H = 1.1g, daily protein amount; Group C = 0.9g, Group H = 0.7g, astaxanthin; Group CA = 1.0mg/day, Group HA = 0.6mg/day. These doses of astaxanthin in human terms (60 kg body weight) may be equivalent to 1500mg/a day (Group CA) and 800mg/day (Group HA).

There were differences in astaxanthin intake amount between Groups CA and HA; the amount was smaller in Group HA. The fact that the mice were fed by astaxanthin-containing food is a limitation of the present method, however the study purpose was the comparison between groups with or without astaxanthin administration, rather than that between Groups CA and HA.

The astaxanthin amounts used in this study are much larger than doses used humans; 6 to 12 mg/day. Thus, it still remains unclear whether or not the experimental results as such could be applied to human cases.

In fact, 95% of orally taken astaxanthin remains in the intestinal tract without being absorbed. Less than 5% of the compounds are absorbed from the small intestine and enter the systemic circulation. Only a small part, if any, of astaxanthin can act on the large intestine. To date, there is no report indicating its direct effect on the intestinal mucosal cells or mucosal immune system. The effect on the microflora seems mainly due to the direct action by the astaxanthin which remains in the intestinal lumen without being absorbed.

The mechanism still remains unclear in the present study how astaxanthin acts in the intestinal tract on preventing the microfloral composition change induced by a high-fat diet. Some possible mechanisms are as follows:

Firstly, astaxanthin may improve the enteric flora by its anti-oxidative activity. Alterations of the enteric flora, particularly increases in the bacterial count of Gram-negative bacilli, induced by high-fat diet ingestion, can increase oxidative stress. It should be elucidated whether or not improvement in the enteric flora resulting from astaxanthin ingestion lessens oxidative stress.

Secondly, astaxanthin, degenerated by enteric bacteria, provides substrates for the production of small molecules that influence the improvement of the pathological condition especially in high-fat fed mice. The metabolic pathways of astaxanthin in the intestinal tract need to be clarified.

Thirdly, astaxanthin or its metabolites may affect the intestinal immune system. The index for the enteric natural immune system (i.e., immunoglobulin A, defensin) should be examined.

Further demonstrative studies on the action mechanisms of astaxanthin in the intestinal tract are awaited to clarify the precise mechanism.

**Conclusion**

The human intestinal tract is inhabited by many normal bacteria constituting the enteric flora characteristic of each individual. Normally, an equilibrium exists between the enteric flora and the host, and among the bacterial species constituting the flora. If this equilibrium is broken by a certain
cause, however, superinfection and/or opportunistic infection occur, resulting in major impacts on host senescence, nutrition, pharmacological effects, physiological function, immune mechanisms, and cancer development. The present study showed that administration of astaxanthin had suppressive effects on enteric flora derangement induced by imposing a high-fat diet on mice, suggesting that astaxanthin could potentially remain in the intestinal tract without being absorbed and could continue to have a mitigating effect on enteric flora derangement resulting from high-fat diets in humans. We would like to proceed by investigating the effects of astaxanthin on the human enteric flora.

A presentation of this study was made at the 13th Meeting of the Japanese Society of Anti-Aging Medicine (held in Yokohama in June 2013).

Conflicts of Interest

The authors declare that they have no conflict of interest associated with this study.

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