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Effect of *Aster scaber* extract on the Growth of Bifidobacteria and *Clostridium perfringens*

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Growth responses of some intestinal bacteria such as bifidobacteria and *Clostridium perfringens* to the extracts of certain foodstuffs were investigated *in vitro*. Among edible mountain herbs, the extracts of several chui-na-muls (*Aster tataricus*, *Ligularia fischeri* and *Aster scaber*) had an inhibitory activity against *C. perfringens* on the agar plate and the water extract of *Aster scaber* worked selectively on it among intestinal bacteria. The water extract showed growth-promoting effect toward bifidobacteria such as *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. infantis* and *B. thermophilum* in the broth culture. When the faecal inoculum was incubated in the culture with the extract, the population of *C. perfringens* decreased, whereas that of bifidobacteria increased by 10^3 scale. β-glucuronidase activity in the culture with the water extract of *Aster scaber* digested with pepsin and pancreatin was lower than that in the control culture.

Recently, improvements in anaerobic culture techniques and development of gnotobiotic technology have revealed complexity of the normal microflora in the gastrointestinal tract and their important influences toward the animal host.

Population of *Bifidobacterium* constitutes 5 to 10% of the total flora in the faeces of children and adults (14). Fujikawa *et al.* (5) reported that more than 25% of the bifidobacteria in the human intestine inhibits the formation of putrefaction product. The main physiological effects of bifidobacteria in the intestine are proposed to be as follows: (a) suppress the putrefactive bacteria as well as intestinal putrefaction, so as to prevent constipation and geriatric diseases, including cancer; (b) prevent and treat antibiotic-associated diarrhea; and (c) stimulate immunological response, thus contributing to a greater resistance to infection (10, 14).

Clostridium perfringens, belonging to the smallest group among intestinal microflora, is commonly found in the gastrointestinal tract of both human and other animals, as well as in soil and sewage. It has been shown to be a cause of human diseases, food poisoning, necrotizing enterocolitis of infants, and enteritis necroticans etc. (7, 13). The organism also seemed to be related to the aging process because of its marked increase in the intestine of the aged (14). These diseases are mediated *via* the production of extracellular enzymes or toxins such as phospholipase C, a thiol-activated hemolysin, collagenase, hyaluronidase, DNase, neuraminidase, ξ-toxin and β-toxin etc.

In the intestine of elderly persons, bifidobacteria decrease or disappear, whereas clostridia including *C. perfringens* significantly increase, and lactobacilli, streptococci and enterobacteriaceae also increase (14). The abnormal flora are generally characterized by a remarkable increase in bacterial counts in the small intestine, by an increase of aerobes, mostly enterobacteriaceae and streptococci, by reduction or disappearance of bifidobacteria, and often by the incidence of *C. perfringens*.

Therefore, it is recommended for health and longevity to maintain the ideal microflora balance (15), which is the population of beneficial bacteria kept high, and that of harmful bacteria in the intestine maintained low.

The size and activities of the microbial populations and communities comprising the normal microflora are regulated by allogenic and autogenic factors (21). Allogenic factors originate from the body's ecosystems (the host's environment and diet) whereas autogenic factors

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are generated within the ecosystems, largely by the microbial populations themselves.

Summing up, these balances of normal microflora are maintained mainly by the competition between microbial species for space and nutrients, and partly by the inhibitors of pH, redox potential, bacteriocin, fatty acids, bile salts and hydrogen sulfide (4) under the strict anaerobic condition, bowel movement and continual secretion. Although little is known of these autogenic factors, some of the health-promoting influences of the normal microflora by diet are currently being studied (3, 12), and are worth studying.

Some unabsorbable oligosaccharides (11, 17) for selective growth-promoting on bifidobacteria are on a market and screening substances (20) of growth-inhibiting on *C. perfringens* is attempted.

To improve the intestinal environment, we screened the substances from foodstuffs, which promote the growth of bifidobacteria and/or inhibit the growth of *C. perfringens* among the human intestinal bacteria.

Chui-na-mul, one of the favored edible mountain herbs in Korea, showed such an effect that we report the results in this paper. Also the harmful enzymes and the metabolite in the intestine such as β-glucuronidase, β-glucosidase and indole (12) were analyzed.

MATERIALS AND METHODS

Strains

Two main intestinal genera were used here; *Clostridium perfringens* ATCC 13124 as the harmful bacteria and *Bifidobacterium* such as *B. adolescentis* ATCC 15073, *B. animalis* ATCC 25527, *B. bifidum* ATCC 29521, *B. breve* ATCC 15700, *B. infantis* ATCC 15697, *B. longum* ATCC 15707 and *B. thermophilum* ATCC 25525, as the beneficial bacteria. Others are *Bacteroides fragilis* ATCC 25285, *E. coli* ATCC 11775, *Clostridium butyricum* ATCC 19398, *C. paraputrificum* ATCC 25 780, *Eubacterium limosum* ATCC 8486, *Lactobacillus acidophilus* KCTC 3145, *L. acidophilus* KCTC 3151, *L. acidophilus* KCTC 3168, *L. plantarum* ATCC 14917, *Staphylococcus aureus* ATCC 12600, *Streptococcus faecalis* ATCC 19433.

Media and Reagents

The strains were subcultured every month and were activated just before use on reinforced clostridial media (Difco). With the EG agar (Eiken, Tokyo), agar diffusion method was used for the detection of growth. In observing the growth in the liquid media, PYF (17) and György (8) (Table 1) media were used to determine carbon-dependent and non carbon-dependent growth promoting factors, respectively.

Table 1. Composition of György medium for observing the growth effect of bifidobacteria by non-carbon growth factor

Composition	Amount
K_2HPO_4	2.5 g
Lactose	35.0 g
$CH_3COONa \cdot 3H_2O$	25.0 g
Bacto casamino acid (vitamin free, Difco)	5.0 g
Alanine, L-cysteine, tryptophan	each 200 mg
Asparagine	100 mg
Adenine, guanine, uracil, xanthine	each 10 mg
Thiamin HCl	200 μg
Riboflavin	200 μg
Pyridoxin-HCl	1200 μg
Nicotinic acid	600 μg
p-Amino benzoic acid, folic acid, biotin	each 12.5 μg
$MgSO_4 \cdot 7H_2O$	200 mg
$FeSO_4 \cdot 7H_2O$	10 mg
NaCl	10 mg
$MnSO_4$	6.74 mg
D.W.	1 L
pH 6.8	

Table 2. Composition of Neomycin-Nagler (NN) agar medium for the selective viable count of *C. perfringens*

Composition	Amount
Peptone	40.0 g
Na_2HPO_4	5.0 g
KH_2PO_4	1.0 g
NaCl	2.0 g
$MgSO_4$	0.1 g
Glucose	2.0 g
Bacto-agar	25.0 g
D.W.	1000 ml
pH 7.6	
Egg yolk solution	100 ml
Neomycin sulfate (2% solution)	10 ml

EGF (16) was used as the medium for the culture of the human faeces, while BS bifidobacteria selective agar (16), *C. perfringens* selective agar (Table 2) and BL agar (16) for total anaerobes were used for viable counts in the culture broth. The reagents of the media came mainly from Difco but the chemicals of phenolphthalein-β-D-glucuronic acid, nitrophenyl-β-D-glucoside, p-dimethyl-aminobenzaldehyde came from Sigma Chemicals Co. Other chemicals used were guaranteed grade. The horse blood for the media was taken by courtesy of Korean Horse Affairs Association.

Chui-na-mul, one of edible mountain herbs, was collected in a dried state after blanching from Hongchon, Chulwon and Chongsong in Korea.

Sample Preperation from Chui-na-mul

Typical chui-na-muls of Gai-mi-chui (*Aster tataricus*), Gom-chui (*Ligularia fischeri*) and three Charm-chuis (*Aster scaber*) were pulverized by conventional mixer, and extracted with the solvents of water, acetone, ethyl acetate and butanol by the same weight/volume basis. Centrifugation (5,000 rpm, 10 minutes) was followed after standing overnight at 4℃ so that the supernatents were taken for the application into the holes of EG agar lawned with target intestinal bacteria. On the other hand, the solvent of 80% methanol with water was also used to extract from chui-na-mul. Further studies, the water extract was lyophilized (GT2, Leybold-Heraeus, Germany) and other solvent extracts were prepared by vacuum rotary evaporator (RE121, Büchi, Switzerland). To use the lyophilized water extract of *Aster scaber*, it was dissolved in distilled water to the appropriate concentration and ultrafiltrated with 0.45 μm filter (Corning Lab. Sci. Co., U.S.A.).

The herbs was digested with pepsin and pancreatin sequentially according to the method of Phillips *et al.* (18) and lyophilized.

Cultivation Methods

Holes of 7 mm diameter were made on the EG agar lawned with 5% of the target bacteria, and 70 μl of the extracts were applied. The agar plates were incubated in an anaerobic glove box (Coy Laboratory Products Inc.) of 37℃ under the mixed gas of H_2 (5%), CO_2 (15%) and N_2 (80%) for two days. An inhibitory zone was detected around each hole.

For the liquid cultivation, the water extract put into the PYF and György broth of the pressure tube to be 0.5 percentage (weight/volume basis). They were cultivated at 37℃ for two days after inoculating the washed inoculum (1) of bifidobacteria. The culture broth was monitored for any decrease in the pH, which would indicate that the growth of bifidobacteria had been affected. For cultivation of the human faeces, the fresh faeces were diluted by 100 times, inoculated to the pressure tube by 2.5% inoculum (v/v) and analyzed after two days.

Anaerobic medium-preparation and other anaerobic procedures were according to the VPI manual (9).

Enzyme Activities and Indole Determination

The activities of β-glucuronidase and β-glucosidase in the culture broth were measured by the method of Goldin *et al.* (6) and the amount of indole in the broth was determined by Sigma's protocol. 2 ml of the culture broth and 2 ml of toluene were mixed vigorously. To the 0.2 ml of the upper toluene layer, 1 ml of 5% p-dimethylaminobenzaldehyde solution (w/v) and 8.8 ml of acid-alcohol reagent (12N HCl 8 ml plus 95% ethyl alcohol 92 ml) was added. By letting it stand for 10 minutes after inverting, the absorbance at 540 nm was determined and the amount of indole in the broth was calculated by the indole standard curve.

RESULTS

Effect of Chui-na-mul Extracts on the Growth of *C. perfringens* and *B . bifidum*

The extracts were applied into the EG agar lawned with *C. perfringens* and *B. bifidum*, and cultivated.

All of the water extracts and the acetone extracts showed growth-inhibition against *C. perfringens* except the acetone extract of *Ligularia fischeri*. It seems that the inhibitory substance(s) toward *C. perfringens* is in the extract of solvent of higher polarity. On the other hand, those extracts did not inhibit the growth of *B. bifidum* except the *Aster tataricus* water extract from Table 3. The butanol itself inhibited the growth of all two genera and the butanol extract did not seem to contain the inhibiting substances.

Apart from the effects against *C. perfringens*, the three *Aster scaber*'s from the different regions indicated no inhibitory effect on *B. bifidum*, so that one of *Aster scaber*'s, collected from Hongchun, was selected further study.

Effect of the Water Extract of *Aster scaber* on the Growth of Some Intestinal Bacteria

In order to find out how the water extract of *Aster scaber* affects the growth of some intestinal bacteria in human, the extracts were applied into the EG agar lawned with some intestinal bacteria.

Only the growth of *C. perfringens* was inhibited by it. Even at the amount of 1 mg of the extract per hole, an inhibitory halo zone was observed around *C. perfringens*, whereas *B. bifidum* was inhibited only a little bit at the amount of 10 mg. Even at the amount of 10 mg of the extract, such an inhibitory effect was not detected against other bacteria of *B. adolescentis, B. animalis, B. breve, B. infantis, B. longum, B. thermophilum, Bacteroides fragilis, C. butyricum, C. ramosum, C. paraputrificum, Escherichia coli, Eubacterium limosum, Lactobacillus acidophilus, L. plantarum, Staphylococcus aureus* and *Streptococcus faecalis* (Table 4).

Growth Reponse of Bifidobacteria to the Water Extract

The György medium for screening non-carbon growth factor and the PYF medium for carbon growth factor were used and pH drop of the culture broth with the control culture was measured to decide whether bifidobacteria grew well or not.

Table 3. Growth inhibition of *C. perfringens* and *B. bifidum* by each solvent extract of *Aster scaber* with agar diffusion method

Samples	*C. perfringens*				*B. bifidum*			
	water	acetone	ethylacetate	butanol	water	acetone	ethylacetate	butanol
Aster scaber from								
Hongchun	−	−	n	−	n	n	n	−
Chulwon	−	− −	n	−	n	n	n	−
Chongsong	− −	−	n	−	n	n	n	−
Aster tataricus	− −	−	n	−	− −	n	n	−
Ligularia fischeri	−	n	n	−	n	n	n	−

*Symbols: Inhibitory halo diameter including 7 mm hole size; 7 mm< − ≤9 mm, 9 mm< − − ≤14 mm, n=no inhibitory effect

**70 μl solvent extracts (solvent:dried sample=1 : 1, W/V) were applied into the hole of EG agar plate.

Table 4. Growth effect on the principal intestinal bacteria by the water extract of *Aster scaber* with agar diffusion method

	Water extract of *Aster scaber* (mg/hole)	
	1	10
Bifidobacterium adolescentis	n	n
B. animalis	n	n
B. bifidum	n	−
B. breve	n	n
B. infantis	n	n
B. longum	n	n
B. thermophilum	n	n
Clostridium butyricum	n	n
C. paraputrificum	n	n
C. perfringens	−	−
Bacteroides fragilis	n	n
Escherichia coli	n	n
Eubacterium limosum	n	n
Lactobacillus acidophilus KCTC3145	n	n
L. acidophilus KCTC3151	n	n
L. acidophilus KCTC3168	n	n
L. plantarum	n	n
Staphylococcus aureus	n	n
Streptococcus faecalis	n	n

*Symbols: Inhibitory halo diameter including 7 mm hole size; 7 mm < − ≤ 9 mm, 9 mm < − − ≤ 14 mm, n=no inhibitory effect

The extract promoted the growth of *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. infantis* and *B. thermophilum* at the György medium after cultivating for two days , which indicates the presence of non-carbon growth factor in the water extract (Table 5). The growth-promoting effect for *B. animalis* of animal origin was so much

Table 5. Growth promotion of bifidobacteria by the water extract of *Aster scaber* in the liquid broths

Strains	György medium	PYF medium
Bifidobacterium adolescentis	+	n
B. animalis	++++	n
B. bifidum	+	n
B. breve	n	n
B. infantis	+	n
B. longum	n	n
B. thermophilum	+	n

*Symbols: The degree of medium pH drop after two days of cultivation was shown as number of plus (+); 2.0≥+++, 1.0>+≥0.4, 0.4>n.

**The water extract was added to be 0.5%(w/v) of each broth.

that the pH drop amounted to 2.31.

In the PYF where carbon source was substituted with the extract, any bifidobacteria did not grow. On the other hand, a methanol extract of *Aster scaber* also affected the growth of *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. infantis* and *B. thermophilum* in the György medium (data not shown). Therefore, the water extract of *Aster scaber* seems to contain the non-carbon factor which promotes the growth of bifidobacteria.

Behaviour of the Faecal Inoculum by Incubation with the Water Extract

It is needed to incubate the human faeces as an inoculum with the extract of *Aster scaber* digested for estimating its effect more closer to *in vivo* situation.

Fresh faeces were incubated with the extract in the EGF medium and the culture was analyzed in terms of viable counts of total anaerobes, bifidobacteria and *C. perfringens*. The enzyme activities of β-glucuronidase and β-glucosidase, and the indole concentration were also measured.

Table 6. Viable count in the culture of human-originated faeces with the water exract of *Aster scaber* by Log_{10} viable cells per milliliter

Microorganism	Cultivation hour	*Aster scaber*[1]		*Aster scaber* (digested)[2]		Control	
		0	48	0	48	0	48
Total anaerobes		5.04	8.34	4.95	8.79	4.95	8.43
C. perfringens		1.00	<1.0*	<1.0	<1.0	1.95	4.32
Bifidobacteria		4.85	7.30	4.60	7.60	4.85	<4.0

[1]*Aster scaber*: lyophilized water extract was added finally to be 1%(w/v) in the culture broth of EGF media. [2]*Aster scaber* (digested): *Aster scaber* digested by swine pepsin and pancreatin was extracted with water and lyophilized, and the extract added to be 1%(w/v). *An inequlity sigh(〈) means smaller value than that number.

Table 7. Analysis of the culture of human-originated faeces with the water exract of *Aster scaber* by enzyme activities and indole amount

	Aster scaber	*Aster scaber* (digested)	Control
β-glucuronidase (unit)	14.96	2.33	4.97
β-glucosidase (unit)	5.89	3.10	0.84
Indole (μg/ml)	17.01	14.36	11.63

*One unit of β-glucuronidase and β-glucosidase represent phenolphthalein 1 μg/ml liberated from phenolphthalein-β-D-glucuronic acid during 40 minutes and nitrophenol 1 μg/ml from nitrophenyl-β-D-glucopymoside during 20 minutes, respectively.

By 10^3 scale, the number of *C. perfringens* increased in the control culture, while bifidobacteria did not almost grow after two days of cultivation (Table 6). The growth of bifidobacteria would rather be inhibited. However, in the culture broths with the extracts of *Aster scaber* and *Aster scaber* (digested), the growth of *C. perfringens* was inhibited remarkably and the number of bifidobacteria increased by 10^3 scale. Thus, a compound which inhibits the growth of *C. perfringens* must be in the extract and the extract should be helpful to the growth of bifidobacteria.

Even though the activities of β-glucuronidase and β-glucosidase found here were higher than those in the control culture, β-glucuronidase activity of the extract of *Aster scaber* digested by pepsin and pancreatin was lower. A little larger amounts of indole were found in the culture of the extract (Table 7).

DISCUSSIONS

In the human intestine, more than 100 species of microorganisms were reported (15) to exist on the arrived nutrients and the released host secretion under the conditions of symbiosis and antagonism. The microbial population amount to the number of 10^{11} per gram of wet faeces, which corresponds to one third of the contents in the bowels by volume basis. The microflora related closely to the maintenance of health, aging and diseases including cancer are influenced by the host, environmental and dietary factors. So, we started to explore the relationship between the intestinal microflora and diet. First of all, we screened the foodstuffs that could control the ratio of beneficial and harmful bacteria.

Bifidobacteria and *C. perfringens* were selected as the target microorganisms for screening on the basis of Mitsuoka's suggestion for the longevity that bifidobacteria should be maintained at a high population, and *vice versa* for *C. perfringens* which increases as a person grows older (15).

About 20 dried edible mountain herbs were collected and extracted with four solvents of different polarities. The extracts of chui-na-mul among them showed almost the similar inhibition effects against the growth of *C. perfringens*, but no inhibitory effect on *B. bifidum* by agar diffusion method. It is interesting that the water extract of *Aster scaber* up to 10 mg per hole on agar gave almost no inhibitory effect toward other intestinal bacteria except *C. perfringens*, which seems to be useful as a selective growth inhibitor of *C. perfringens* in the intestine.

Unlike detecting the inhibitory zone on the agar, determinating whether the extract gave the growth-promoting effect toward bifidobacteria was not so easy that pH decrease in the broth after cultivation was checked. To observe the growth effect toward bifidobacteria by the extract, György medium and PYF medium were used.

As shown in Table 5, human-originated bifidobacteria of *B. adolescentis, B. bifidum* and *B. infantis* grew well in the György medium, which indicates that those bifidobacteria utilized some non-carbon compound(s) from the water extract. Therefore, from Table 4 and Table 5, the water extract of *Aster scaber* would be contributive to the growth promotion of bifidobacteria and the inhibition of *C. perfringens* in the mixed culture of those bacteria.

However, it is not easy to expect one compound to have two effects for each bacteria. Although at the extract of 10 mg, the growth of *B. bifidum* was a little inhibited, it was promoted in the culture broth with the extract of 0.5% (w/v), which suggests the presence of several effective compounds in the extract.

A cultivation of human faeces containing bifidobacteria and *C. perfringens* with many kinds of microorganisms would be one of the methods for *in vitro* estimation for the effect of the water extract in the intestine. Also the water extract of *Aster scaber* digested with pepsin and pancreatin was used to estimate the effect of the foodstuffs which arrived to the large intestine through the digestive organs.

In the control culture at Table 6, *C. perfringens* grew well unlike the growth of bifidobacteria after cultivating for two days. However, *C. perfringens* was inhibited remarkably, while bifidobacteria increased in the culture of the *Aster scaber* extracts. In comparison with the control and the cultures of the extracts, activities of β-glucuronidase were three and 0.5 times higher in the cultures of the extracts of *Aster scaber* and *Aster scaber* digested, repectively. Such differences of the two extracts of *Aster scaber* are not known yet. β-glucosidase activity was the highest in the culture of the extract of *Aster scaber* undigested because such high activity probably needed to hydrolyze the numerous plant glycosides encounted in the extract (12).

There have not been many reports (2, 19) regarding plant compounds which reveal some sort of relationship with the intestinal anaerobic bacteria except some oligosaccharides and dietary fibers. This *Aster scaber* water extract, including polar and non carbon-related compound, would be one of ideal food materials as they showed the ideal growth effect toward bifidobacteria and *C. perfringens*. Daily intake of cooked *Aster scaber* would be expected to alter the growth and composition of the microbial community within the intestinal tract, thus protecting from a variety of diseases and helping maintain the optimal human health.

Further work to identify the biologically active materials of *Aster scaber* is in process and a direct administration to human will be attempted.

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