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Note

Growth-promoting Effects of *N*-Acetylneuraminic Acid-containing Substances on Bifidobacteria

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The use of *N*-acetylneuraminic acid, sialyl-lactose, and glycomacropeptide by bifidobacteria and lactobacilli, and their growth-promoting effects on *B. longum*, *B. breve*, *B. bifidum*, and *B. infantis* were investigated. The data presented here suggest that fortification with *N*-acetylneuraminic acid-containing substances of infant formula may provide formula-fed infants with a function that human milk possesses.

Since György *et al.*[1] first suggested that human milk contains factors that stimulate bifidobacterial growth, numerous studies on this subject have been reported. A predominantly bifidobacterial flora in the intestinal tract is considered to inhibit the growth of harmful bacteria such as pathogenic strains of *Escherichia* (*E.*) *coli*, and protect the infants against gastrointestinal diseases.[2,3] Consequently, factors that stimulate the growth of bifidobacteria appear to be promising effective substances for the maintenance of intestinal homeostrains. The fractionation of human milk yielded mixtures of oligosaccharides containing *N*-acetylglucosamine, glucose, galactose, and fucose.[4–6] Of them, *N*-acetylglucosamine appeared to be the potentially active sugar that promote the growth of *Bifidobacterium* (*B.*) *bifidum*.[7] In addition to the oligosaccharide that contains *N*-acetylglucosamine, there are other various indigestible saccharides that increase the number of intestinal bifidobacteria. In this regard, oligosaccharides such as fructo-oligosaccharide,[8] isomalto-oligosaccharide,[9] and galacto-oligosaccharide[10] have been reported as bifidus factors. *N*-Acetylneuraminic acid (NeuAc)-containing oligosaccharides from human milk and sialo glycoproteins such as α1-acid glycoprotein also demonstrated bifidus growth-promoting activity, which was enhanced and/or induced by treatment with neuraminidase.[11,12] In this study, we investigated the effects of NeuAc-containing substances derived from cow's milk, such as sialyl-lactose (SL) and glycomacropeptide (GMP), on the growth of bifidobacteria and lactobacilli.

NeuAc and SL were purchased Sigma Chemical Co. (St. Louis, MO). GMP was isolated from cheese whey by ultrafiltration according to a method described previously.[13] Nine strains of bifidobacteria and lactobacilli, *B. longum* (ATCC15707), *B. longum* (Snow Brand type culture number (SBT) 2928), *B. breve* (ATCC15700), *B. bifidum* (ATCC29521), *B. infantis* (ATCC15697), *B. adolescentis* (ATCC15703), *Lactobacillus* (*L.*) *acidophilus* (SBT2062), *L. casei* (ATCC4646), and *L. salivarius* (ATCC11741), were used in the fermentation tests. Five milliliters of Peptone Yeast Extract Fildes Solution broth[14] containing 0.5% of glucose, NeuAc, SL, or GMP as the carbohydrate source, were inoculated with 0.1 ml (1×10^8 cfu/ml) of the bacteria, which had been cultured in Peptone Yeast Extract Fildes Solution Glucose broth.[14] The inoculated media were anaerobically incubated at 37°C for 96 h by the steel-wool method.[15] Fermentation of the materials by the bacteria was evaluated by measuring the pH of the incubated media. The pH was scored in the following manner; ++: pH <4.9, +: 5.0 <pH <5.9, and −: 6.0 <pH. The growth-promoting effects of NeuAc, SL, and GMP on *B. longum*, *B. breve*, *B. bifidum*, and *B. infantis* were investigated by the method of Yoshioka.[16] Briefly, the bifidobacteria were anaerobically incubated in Tomarelli broth containing 3.5% (wt/vol) lactose[17] at 37°C for 24 h by the steel-wool method.[15] Tomarelli broth was degassed by boiling for 15 min before incubation. After cooling, the broth was inoculated with one ml (1×10^8 cfu/ml) of the bacteria, and then mixed with filter-sterilized materials and one ml of filter-sterilized 1% ascorbic acid solution (pH 6.5) to obtain a final volume of 10 ml. The final concentrations of NeuAc, SL, and GMP in the broth were 0.01, 0.1, and 1.0 mg/ml. The bacterial growth was evaluated by measuring pH, optical density at 660 nm, and titration with a standard solution of 0.01 N sodium hydroxide. A sialic acid determination kit (Kyokuto Seiyaku, Tokyo) was employed to determine the NeuAc concentration in the broth.

The results of fermentation of glucose, NeuAc, SL, and GMP are shown in Table I. *B. longum* (ATCC15707), *L. casei*, and *L. salivarius* effectively fermented NeuAc and glucose. NeuAc was slightly fermented by *B. longum* (SBT2928), *B. breve*, *B. bifidum*, *B. infantis*, and *B. adolescentis*. SL was fermented only by *B. infantis*, which regularly occur in feces of breast-fed infants, suggesting the significance of SL as a growth-promoting factor for *B. infantis*. There was only a little use of GMP by almost all of the strains tested. Table II summarizes the pH, OD, acidity, and NeuAc concentration of the broths in which *B. longum*, *B. breve*, *B. bifidum*, and *B. infantis* were cultivated. The growth promotion rate was calculated by dividing the acidity of material-containing broth by the average acidity of control broth. Absence of NeuAc in the broth after cultivation indicated that NeuAc was consumed by the bacteria. *B. bifidum* var. *pennsylvanicus*, which is a laboratory strain, is apparently able to

Table I. Use of Glucose, NeuAc, SL, and GMP by Bifidobacteria and Lactobacilli

Bacterial species	Glucose	NeuAc	SL	GMP
B. longum (ATCC15707)	++	++	−	+
B. longum (SBT2928)	++	+	−	+
B. breve (ATCC15700)	++	+	−	+
B. bifidum (ATCC29521)	++	+	−	+
B. infantis (ATCC15697)	++	+	++	+
B. adolescentis (ATCC15703)	++	+	−	+
L. acidophilus (SBT2062)	++	−	−	−
L. casei (ATCC4646)	++	++	−	+
L. salivarius (ATCC11741)	++	++	−	+

Bacterial fermentation was evaluated by the pH of the broth after cultivation: ++, pH<4.9; +, 5.0<pH<5.9; −, 6.0<pH.

Table II. Growth of Bifidobacteria in Broth Containing NeuAc, SL, or GMP

Bacterial species	Sample[a]	pH[b]	OD[b]	Acidity[b]	Growth promotion rate[c]	NeuAc concentration in broth (mg/ml)[d]
B. longum (ATCC15707)	Control	4.21	2.60	1.50	1.00±0.07	0 (0)
	NeuAc 0.01	4.18	2.50	1.45	0.96±0.06	0 (0.014)
	NeuAc 0.1	4.18	2.53	1.47	0.98±0.05	0 (0.145)
	NeuAc 1	4.17	2.54	1.48	0.98±0.13	0 (0.948)
	SL 0.01	4.15	2.60	1.53	1.02±0.10	0 (0)
	SL 0.1	4.17	2.57	1.49	0.99±0.03	0 (0.068)
	SL 1	4.22	2.50	1.45	0.96±0.05	0 (0.412)
	GMP 0.01	4.17	2.70	1.55	1.03±0.14	0 (0)
	GMP 0.1	4.20	2.59	1.60	1.06±0.05	0 (0)
	GMP 1	4.17	2.57	1.51	1.01±0.06	0 (0.076)
B. breve (ATCC15700)	Control	5.11	0.68	0.37	1.00±0.04	0 (0)
	NeuAc 0.01	4.67	1.33	0.69	1.86±0.11*	0 (0.014)
	NeuAc 0.1	4.43	1.91	0.97	2.62±0.21*	0 (0.145)
	NeuAc 1	4.91	0.93	0.41	1.10±0.24	0 (0.948)
	SL 0.01	4.73	1.37	0.53	1.43±0.16*	0 (0)
	SL 0.1	4.52	1.69	0.54	1.45±0.09*	0 (0.068)
	SL 1	4.59	1.53	0.75	2.02±0.28*	0 (0.412)
	GMP 0.01	4.47	1.98	0.98	2.64±0.33*	0 (0)
	GMP 0.1	4.99	0.92	0.46	1.29±0.14*	0 (0)
	GMP 1	5.15	0.56	0.39	1.05±0.09	0 (0.076)
B. bifidum (ATCC29521)	Control	4.52	2.23	0.95	1.00±0.08	0 (0)
	NeuAc 0.01	4.44	2.34	1.18	1.24±0.06*	0 (0.014)
	NeuAc 0.1	4.40	2.39	1.22	1.28±0.10*	0 (0.145)
	NeuAc 1	4.52	2.25	1.14	1.20±0.18	0 (0.948)
	SL 0.01	4.64	2.18	0.92	0.96±0.08	0 (0)
	SL 0.1	4.56	2.20	1.00	1.05±0.06	0 (0.068)
	SL 1	4.35	2.41	1.35	1.42±0.13*	0 (0.412)
	GMP 0.01	4.33	2.46	1.30	1.37±0.12*	0 (0)
	GMP 0.1	4.54	2.27	1.23	1.29±0.10*	0 (0)
	GMP 1	4.61	2.03	0.95	1.00±0.99	0 (0.076)
B. infantis (ATCC15697)	Control	5.24	0.56	0.27	1.00±0.10	0 (0)
	NeuAc 0.01	5.56	0.27	0.28	1.04±0.03	0 (0.014)
	NeuAc 0.1	5.29	0.45	0.34	1.25±0.18	0 (0.145)
	NeuAc 1	5.48	0.29	0.28	1.04±0.14	0 (0.948)
	SL 0.01	5.65	0.20	0.22	0.81±0.03	0 (0)
	SL 0.1	5.49	0.27	0.25	0.92±0.05	0 (0.068)
	SL 1	5.61	0.24	0.24	0.88±0.05	0 (0.412)
	GMP 0.01	5.11	0.73	0.53	1.96±0.15*	0 (0)
	GMP 0.1	5.61	0.22	0.30	1.11±0.08	0 (0)
	GMP 1	5.70	0.18	0.26	0.96±0.05	0 (0.076)

[a] In control, bacteria was cultivated in Tomarelli broth without NeuAc-containing substances. NeuAc, SL, or GMP was added to the contro broth at final concentrations of 0.01, 0.1, and 1 mg/ml.

[b] Values are the means of two experiments, each of which was assayed in duplicate.

[c] The growth promotion rate was calculated by dividing the activity of the material-containing broth by the average acidity of the control broth Values are the means±SD of four assays. Marked values (*) differ significantly from the value in control at $p<0.05$. The statistical significance was estimated by Student's *t*-test.

[d] Values represent NeuAc concentration in broth after cultivation and those in parentheses before cultivation. Values below the detection limit of the commercial kit were shown as zero.

produce a neuraminidase capable of splitting NeuAc(α2-3)Gal linkages, and the resulting core oligosaccharide becomes available for its gorwth requirements.[18] The strains used in this study also seem to produce a neuraminidase, because no NeuAc was detected in the broth after cultivation. The extent of decrease in pH and acidity depended on the growth of bifidobacteria that fermented lactose as indicated by the optical density, and had no relation to NeuAc consumption. These findings suggested that NeuAc was The growth of *B. breve* was stimulated by the addition of NeuAc, and its optimum concentration was 0.1 mg/ml. The growth of *B. bifidum* in NeuAc-containing broth was also superior to that in the control broth. SL promoted the growth of *B. breve* and *B. bifidum* in a dose-dependent manner, while no use of SL by these bacteria occurred in the fermentation tests. This finding suggested that the growth-promotion by SL in this case was due to an activity of its molecule but SL did not work as a nutrient. On the other

SL, their preference for SL as the carbohydrate source seemed to be extremely low when lactose-enriched broth containing a trace of SL was used. The promoting activity of GMP on the growth of *B. breve*, *B. bifidum*, and *B. infantis* was inversely proportional to its concentration in the broth. Because the content of NeuAc in this amount of GMP was extremely low (approximate 0.6 μg/ml), NeuAc in GMP might be not responsible for the growth of *B. breve*, *B. bifidum*, and *B. infantis*. Azuma *et al.*[19] reported that GMP derived from human milk κ-casein was effective as a bifidus growth-promoting factor even at concentrations as low as 0.05 mg/ml. Because the activity of GMP was drastically decreased by further hydrolysis with Pronase, they concluded that the polypeptide portion of GMP as well as its sugar portion might be significant as a bifidus growth-promoting factor. Unlike the other strains, the growth of *B. longum* was not promoted by NeuAc, SL, or GMP, while the bacteria effectively fermented NeuAc (Table I).

This study demonstrated the growth-promoting effects of NeuAc-containing substances on *B. breve*, *B. bifidum*, and *B. infantis*. Human milk contains 3 to 6 mg/ml of oligosaccharides.[20] As for the sialylated components in human milk, the content of SL is about one mg/ml. Cow's milk contains only a small amount of oligosaccharides with SL (0.03 to 0.06 mg/ml) as the major component. Pakkinen *et al.*[21] and Korhonen *et al.*[22] have shown that sialylated oligosaccharides abolished the binding activity of *E. coli* that causes meningitis and neonatal sepsis in infants. Although further studies are required to clarify the mechanism of the bifidus growth-promotion by NeuAc-containing substances, fortification with NeuAc-containing substances such as SL of infant formula may provide formula-fed infants with a function that human milk possesses.

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