Research Article

Bifidobacterium Supplementation of Colostrum and Breast Milk Enhances Weight Gain and Metabolic Responses Associated with Microbiota Establishment in Very-Preterm Infants

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What Is It About?

To investigate the effect on growth outcomes in very-preterm infants (VPIs), supplementation with *Bifidobacterium* along with mother's colostrum and breast milk was performed on 35 VPIs. Seventeen infants allocated to the bifidobacterial group, in comparison to 18 in the placebo group, showed significantly greater cumulative weight gain by 8 weeks, associated with significantly higher fecal bacterial counts including bifidobacteria and short-chain fatty acids, and nominally higher concentrations of $n-3$ polyunsaturated fatty acids. The conclusive findings demonstrate that *Bifidobacterium* supplementation of maternal colostrum and breast milk establishes a beneficial microbiota profile, leading to favorable metabolic responses that improve growth in VPIs.

Abstract

Background: Postnatal growth restriction in very-preterm infants (VPIs) may have long-lasting effects. Recent evidence suggests that developmental problems in VPIs are related to abnormalities in intestinal microbial communities. ***Objective:*** To investigate the effect on growth outcomes in VPIs of supplementation with *Bifidobacterium* along with mother's colostrum and breast milk. ***Methods:*** A randomized controlled study was performed on 35 VPIs, born between 24 and 31 weeks of gestation with birth weights <1,500 g. The patients received either

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daily *Bifidobacterium breve* supplementation (Bifid group) or vehicle supplement only (placebo group). Parenteral nutrition was initiated with glucose, amino acids, and fatty acids for all of the infants soon after birth. Each infant received their own mother's colostrum within 24 h of birth, and breast milk on subsequent days. Fecal bacteria, organic acids, pH, bile acids, and plasma fatty acids were analyzed. ***Results:*** Seventeen infants were allocated to the Bifid group and 18 to the placebo group; the birth weights and gestational ages did not differ significantly between the two groups. Compared to the placebo group, the Bifid group showed significantly greater and earlier weight gain by 8 weeks; significantly higher total fecal bacterial counts, including bifidobacteria; higher levels of total fecal short-chain fatty acids and nominally (but not significantly) higher concentrations of plasma n–3 fatty acids; and lower levels of total fecal bile acid. ***Conclusions:*** Bifidobacterial supplementation of maternal colostrum and breast milk yielded the establishment of a beneficial microbiota profile, leading to favorable metabolic responses that appeared to provide improved growth in VPIs.

Introduction

Preterm infants are predisposed to serious health complications in both the short and the long term. These effects are due to prematurity and nutrition affecting maturation of the gut microbiota, the gastrointestinal tract, and the immune system. These consequences of prematurity subsequently influence growth and development of the infants [1–3]. Therefore, preterm infants would benefit from enhanced weight gain, which affects growth and organ development. For this purpose, breast milk is the preferred source of nutrition for preterm infants [4, 5].

Organic acids (short-chain fatty acids and lactic acid) are the end products of bifidobacterial fermentation, particularly of human milk oligosaccharides (HMOs). In the gut, these organic acids can be absorbed into the circulation, where these acids serve as both microbiota-generated calories and important regulatory molecules, such that the host has been estimated to receive 6–10% (or more in infants) of their energy from organic acids [6]. Bile acids are formed by the microbiota from host cholesterol, and these molecules also serve as signaling molecules that bind to distinct receptors [7] that have major impacts on metabolism.

The composition of the gut microbiota thus may be taken to represent a very important contributor, particularly in low-birth-weight infants, to normal growth and development. On this basis, our aim was to investigate the effects of *Bifidobacterium* supplementation concomitant with early feeding of mother's colostrum and breast milk in very-preterm infants (VPIs), focusing in particular on growth outcomes. We also analyzed the levels of fecal bacterial and related fecal parameters in these infants.

Subjects and Methods

Study Design and Participants

In this blinded, randomized, controlled study, we recruited infants born between 24 and 31 weeks of gestation and with body weights <1,500 g who had been admitted to the NICU (neonatal intensive care unit) of the Okinawa Prefectural Nanbu Children's Medical Center.

The clinical information gathered on each participant included diet and antibiotic administration. The infants were alternately allocated to either of two groups within 24 h of birth,

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receiving either *Bifidobacterium breve* supplementation (Bifid group) or vehicle supplement only (placebo group).

Nutritional Management

Parenteral nutrition (PN), by which the infants were provided with glucose (8%; glucose infusion rate: 3–4 mg/kg/day), amino acids (1–1.5 g/kg/day), and fatty acids (Intralipos; high content of n–6: ~0.5–1 g/kg/day), was initiated for all the infants within the first few hours of life. Each infant received their own mother's colostrum as a trophic feeding within 24 h of birth and breast milk on subsequent days; these feedings were augmented with PN according to the infant's tolerance. PN was stopped when infants tolerated 120 mL/kg/day of enteral feeding and showed sustained growth, defined by gaining at least 15 g/kg/day during the preceding 72 h. All infants were fed their mother's breast milk fortified with HMS-1 (Morinaga, Tokyo, Japan) and medium-chain triglyceride oil. When a mother was not able to supply a sufficient volume of her own breast milk, the infant was nourished partially with preterm formula (GP-P; Morinaga).

Bifidobacterium *Supplementation*

The infants were provided with once-daily supplementation with *B. breve* (BBG-01) or an equivalent volume of placebo. BBG-01, a probiotic supplement provided by Yakult Honsha Co., Ltd. (Tokyo, Japan), was packaged as a powder, freeze-dried with corn starch, in a 1-g sachet containing not less than 10^9 viable cells (CFU [colony-forming units]) of *B. breve*-strain Yakult bacteria [8]. The vehicle supplement, which consisted of corn starch alone, was provided as an identical powder in identical sachets. The content of each sachet was suspended in 4 mL of water and the corn starch was allowed to settle; 1 mL of the resulting supernatant (containing 2.5×10^8 CFU of the probiotic) was administered via a gastric feeding tube. The placebo was formulated and administered using the same procedure. Dosing was initiated within several hours after birth and continued once daily during the hospital stay. Antibiotics were used primarily to prevent infections due to tracheal intubation or use of a central venous catheter.

Fecal specimens for bacterial analysis were collected on day 1 (meconium), as well as 1, 2, 4, and 8 weeks after birth. RNA [9] and DNA [10] were isolated from the fecal specimens using the previously described methods. Bacterial counts were determined using the Yakult Intestinal Flora-SCAN® (YIF-SCAN® [8]; Yakult Honsha Co., Ltd.), a technique that employs the reverse-transcription quantitative PCR [9]. For enumeration of the *B. breve* strain Yakult, a strain-specific quantitative PCR technique was used [11]. The fecal specimens were tested for fecal organic acid concentrations and pH using the previously described methods [12]. Fecal bile acid profiles were analyzed using liquid chromatography-electrospray ionization-tandem mass spectrometry [13]. Plasma levels of polyunsaturated fatty acids (PUFAs) such as n–3 (eicosapentaenoic acid and docosahexaenoic acid) and n–6 (arachidonic acid [AA]) were measured with capillary gas chromatography [14] (SRL, Inc., Tokyo, Japan) using blood obtained from the umbilical cord at birth and at 4 weeks of age.

Primary outcomes included body weight gain and the composite of the measured fecal and plasma outcomes during 8 weeks postpartum. Secondary outcome measures were incidences of serious clinical complications such as necrotizing enterocolitis (NEC) or sepsis.

Statistical Analysis

Statistical analyses were performed with SPSS statistics software version 22 (SPSS Japan, Inc., Tokyo, Japan). For between-group comparisons, the two-tailed Mann-Whitney U test (for fecal microbiota and organic acid levels) or the two-tailed, unpaired Student t test (for fecal pH, fecal bile acids, and plasma fatty acids) was used to compare differences in continuous

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Table 1. Baseline and early characteristics of the study participants

	BBG-01 group	Placebo group
Participants (male/female)	17 (6/15)	18 (7/10)
Gestational age, weeks	28.1±3.1	28.2±3.3
Birth weight, g (ELBWI:LBWI, *n*)	1,049±302 (8:9)	1,002±289 (9:9)
Cesarean delivery	14 (82.3%)	15 (83.3%)
5-min Apgar score <6*	6.4±1.6	4.7±1.9
Artificial ventilation	14 (82.3%)	16 (88.8%)
Duration of ventilation, days*	11.9±14.1	30.7±21.1
Intrauterine infection		
Chorioamnionitis	3 (17.6%)	5 (27.8%)
PROM >24 h	4 (23.5%)	2 (11.1%)
IgM >10	1 (5.9%)	0 (0%)
Antibiotics		
Antibiotics in newborns	Ampicillin + amikacin	Ampicillin + amikacin
Early nutrition characteristics		
Parenteral nutrition started	Within a few hours after birth in all	Within a few hours after birth in all
Enteral feeding started	Trophic feeding on 1st day in all	Trophic feeding on 1st day in all
Type of milk received		
Breast milk plus formula, mixed, *n* (% of all infants)	3 (17.6%)	4 (22.2%)
Exclusive breastfeeding	14 (82.4%)	13 (72.2%)
Fortification		
Added breast milk fortifier started, day of life	12.9±4.5	13.9±3.2
Added MCT oil started, day of life	10.5±4.0	11.4±3.8

Values are presented as *n* (%) or mean ± SD unless stated otherwise. ELBWI, extremely-low-birth-weight infant; IgM, immunoglobulin M; LBWI, low-birth-weight infant; MCT, medium-chain triglyceride; PROM, premature rupture of membranes; SD, standard deviation. * Significant difference ($p < 0.05$).

variables; the two-tailed Fisher exact probability test was used to compare differences in categorical variables. $p < 0.05$ was considered statistically significant. Unless otherwise indicated, data are presented as mean ± SD.

Results

Between June 1, 2015, and September 30, 2017, 35 VPIs (17 extremely-low-birth-weight [ELBW] and 18 very-low-birth-weight [VLBW] infants) were recruited to the study; 17 were allocated to the Bifid group and 18 to the placebo group (Table 1). The baseline and early clinical characteristics of the included infants and details on the feedings received in the first few weeks of life were similar between the two groups (Table 1).

The Bifid group exhibited "catch-up" weight gain, resulting in a significantly larger cumulative body weight gain by 8 weeks ($p < 0.05$) (Fig. 1) compared to the placebo group. Similarly, the average daily caloric intake by the BBG-01-fed infants was significantly higher at 1, 2, and 4 weeks compared to the placebo group at the respective time points (Fig. 1). This trend continued at 8 weeks (122.6 vs. 119.0 kcal/kg/day for the Bifid and the placebo group, respectively).

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Fig. 1. Change in weight and caloric intake following birth. The BBG-01-fed group (Bifid group) exhibited a significantly greater body weight gain associated with higher caloric intake (compared to the placebo group) by 8 weeks of life (two-tailed, unpaired Student's t test, * $p < 0.05$). Data are presented as mean ± SD.
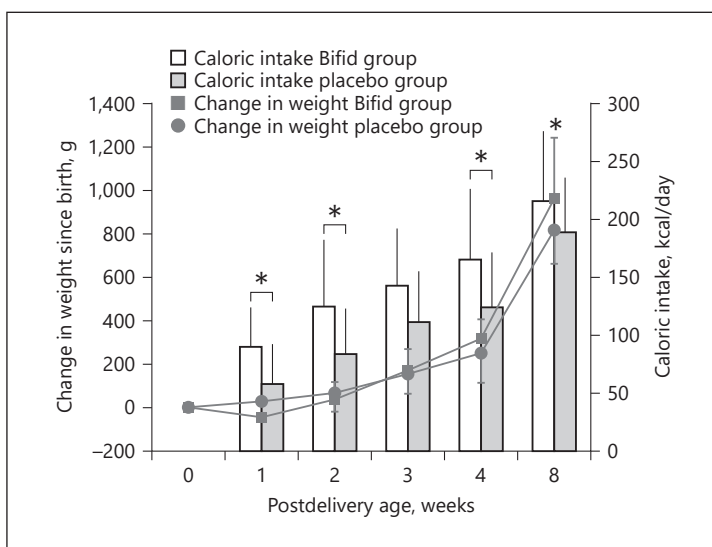
Sepsis was observed in 3 infants (16.7%) in the placebo group and no infants in the Bifid group, but this difference did not achieve significance. No infants in either group developed NEC.

Fecal Microbiota

The fecal microbiota analysis results are shown in Table 2. The total bacterial counts in meconium were similar in the two groups. In the meconium of the two groups, almost no obligate anaerobes, including *Bifidobacterium*, were found, but facultative anaerobes such as *Lactobacillus* were detected. At 1 week of life, the total fecal bacterial count in the Bifid group was significantly elevated to 10^9 cells/g feces (compared to the count in the placebo group at the same time point), and *Bifidobacterium* was the predominant genus among the bacteria in the Bifid group. However, the total bacterial count in the placebo group did not reach 10^{10} cells/g feces by 8 weeks of life. The colonization of the placebo group with BBG-01 was suspected to represent contamination that presumably occurred during the formulation and administration processes.

Fecal Organic Acid Concentrations and pH

The total organic acid concentrations in the meconium did not differ significantly between the two groups, but these values were significantly lower than those in healthy term infants [12]. In the Bifid group, from 1 to 8 weeks of life, the fecal concentrations of total organic acid, and those of lactic and acetic acids, were significantly elevated (Table 3), but the fecal pH values were significantly lower, compared with the values in the placebo group at the respective time points. The concentrations of the other organic acids were low or undetectable in the fecal samples from both groups.

Plasma PUFA Concentrations

The concentrations of plasma $n–6$ PUFAs and $n–3$ PUFAs in the infants of the two groups exhibited nominal increases from the first day through 4 weeks of life (Table 3). A comparison of the 4-week values between the two groups indicated that the concentrations of all of these fatty acids (with the exception of AA) were nominally higher in the Bifid group.

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Table 2. Changes in fecal microbiota counts and prevalence in preterm infants during the first 8 weeks after birth

	Meconium		Week 1		Week 2		Week 4		Week 8	
	BBG-01 (14)* count[a], %	placebo (14) count, %	BBG-01 (11) count, %	placebo (12) count, %	BBG-01 (16) count, %	placebo (15) count, %	BBG-01 (17) count, %	placebo (18) count, %	BBG-01 (15) count, %	placebo (15) count, %
Total bacteria	6.9±2.5 (79)	7.0±2.2 (86)	9.4±1.4[b] (100)	8.3±1.7 (100)	9.9±0.7 (100)	8.8±1.9 (100)	10.0±0.6[b] (100)	9.3±0.9 (100)	10.1±0.5 (100)	9.7±0.8 (100)
Obligate anaerobes										
Clostridium coccoides group	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)
Clostridium leptum subgroup	ND (0)	5.3±0.1 (14)	ND (0)	5.3 (17)	5.4 (6)	5.3 (7)	6.4 (6)	5.5 (6)	5.8±0.3 (27)	5.9±0.6 (20)
Bacteroides fragilis group	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)
Bifidobacterium	7.7±2.1 (21)	ND (0)	9.0±1.5 (91)[cc]	9.9 (8)	9.6±1.0 (100)[cc]	10.5±0.5 (27)	9.4±1.1 (100)[cc]	8.7±2.4 (22)	9.4±1.7 (93)[cc]	9.4±1.1 (60)
Atopobium cluster	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)
Prevotella	ND (0)	ND (0)	5.2 (9)	5.4 (8)	5.3 (6)	ND (0)	5.6 (12)	ND (0)	ND (0)	5.5 (7)
Clostridium perfringens	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)
Clostridium difficile	ND (0)	ND (0)	ND (0)	7.8 (8)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	5.2 (13)
Facultative anaerobes										
Enterobacteriaceae	7.8±1.7 (36)	7.8±2.0 (50)	8.8±1.2 (64)	7.6±1.9 (83)	8.0±1.4 (63)	8.1±1.5 (67)	8.8±1.4 (88)	8.2±1.9 (89)	9.2±1.1 (87)	9.3±0.9 (93)
Enterococcus	6.8±3.2 (29)	9.2 (14)	8.3±1.4 (55)	8.2±2.0 (50)	7.9±2.0 (56)	7.7±2.2 (33)	8.5±1.5 (59)	7.9±2.1 (67)	7.0±1.9 (53)	7.7±1.7 (80)
Staphylococcus	5.5±1.5 (64)	5.5±1.7 (64)	7.6±1.4 (100)	7.0±1.5 (100)	7.3±1.6 (100)	7.6±1.6 (100)	7.5±1.0 (100)	7.3±1.0 (100)	7.2±1.0 (100)	6.9±1.3 (100)
Lactobacillus	3.3±0.7 (21)	3.3 (14)	4.6 (9)	3.8 (17)	3.5±1.1 (25)	4.1 (13)	3.9 (6)	4.4±1.2 (22)	3.9±0.5 (20)	4.8±2.1 (27)
Aerobes										
Pseudomonas	4.2 (7)	ND (0)	5.7 (18)	4.2 (8)	4.2 (13)	4.0 (7)	4.4±0.5 (18)	5.1±0.7 (17)	4.1 (7)	5.7±0.8 (20)
Administered bacteria										
Bifidobacterium breve strain Yakult	7.1 (7)	ND (0)	8.5±0.8 (91)[cc]	ND (0)	8.8±0.8 (100)[cc]	9.7 (13)	8.7±0.7 (100)[cc]	9.2 (6)	8.4±1.0 (87)[cc]	9.1±0.7 (27)

Preterm infants with a birth weight <1,500 g. ND, not detected. * For all columns: number of samples examined (%; bacterial detection rate). [a] For all columns: log₁₀ cells/g feces, mean ± SD (SD is not presented for bacteria detected in 2 subjects or fewer). [b] $p < 0.05$ (vs. placebo), two-tailed non-parametric Mann-Whitney U test. [cc] $p < 0.01$ (vs. placebo), two-tailed Fisher's exact probability test.

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Table 3. Changes in fecal organic acid levels, pH, total bile acids, and plasma fatty acids in preterm infants during the first 8 weeks after birth

Organic acids	Meconium BBG-01 (16)* count[a], %	Meconium placebo (15) count, %	Week 1 BBG-01 (14) count, %	Week 1 placebo (14) count, %	Week 2 BBG-01 (17) count, %	Week 2 placebo (17) count, %	Week 4 BBG-01 (17) count, %	Week 4 placebo (18) count, %	Week 8 BBG-01 (15) count, %	Week 8 placebo (17) count, %
Total organic acids in feces, µmol/g feces	16.2±13.5 (100)	19.0±21.5 (93)	30.3±21.2[b] (100)	9.4±8.5 (100)	32.9±24.4 (100)	19.6±24.8 (100)	36.7±20.0[b] (100)	23.3±14.5 (100)	52.3±29.8[b] (100)	35.9±27.4 (100)
Succinic acid	3.3 (13)	8.9±15.3 (20)	1.8±3.1 (43)	1.4 (14)	1.5±1.7 (76)	2.2±2.1 (41)	5.0±3.6 (76)	3.6±2.8 (72)	4.4±3.5 (80)	5.1±3.3 (94)
Lactic acid	2.8±1.8 (56)	1.3±0.6 (40)	8.0±4.2[b] (71)	2.1±0.4 (29)	10.6±8.7 (76)	8.0±11.8 (65)	6.3±4.5 (88)	6.4±5.6 (72)	10.8±17.6 (100)	7.4±8.1 (82)
Formic acid	2.2 (13)	0.6±0.5 (40)	3.2±3.0 (57)	0.5±0.2 (36)	2.6±1.5 (53)	1.9±1.5 (47)	1.5±1.8 (29)	0.8±0.6 (28)	1.5±1.6 (53)	1.6±1.5 (35)
Acetic acid	14.6±13.5 (88)	16.2±18.6 (93)	21.9±15.7[b] (100)	8.1±8.7 (100)	22.0±16.6 (100)	12.6±14.2 (100)	25.6±15.2[b] (94)	14.5±10.3 (100)	35.1±18.1[bb] (100)	22.2±18.6 (100)
Propionic acid	18.3 (6)	ND (0)	ND (0)	1.2 (7)	1.5±0.2 (18)	0.9 (6)	4.2±4.7 (59)	2.7±2.3 (50)	2.9±2.2 (73)	3.2±2.1 (71)
Butyric acid	ND (0)	ND (0)	ND (0)	0.1 (7)	ND (0)	ND (0)	4.8 (6)	ND (0)	ND (0)	ND (0)

Bile acids	Meconium BBG-01 (9)	Meconium placebo (10)	Week 4 BBG-01 (16)	Week 4 Placebo (10)	Week 8 BBG-01 (12)	Week 8 Placebo (10)
Total bile acids in feces, µmol/g	8.64±2.6	8.99±5.1	0.27±0.2[b]	2.78±1.3	0.26±0.36[b]	2.19±0.9

Fatty acids in plasma	Umbilical cord BBG-01 (16)	placebo (18)	Week 4 BBG-01 (13)	placebo (17)
DGLA, µg/mL	36.7±18	31.7±13	51.8±9[b]	46.4±21
AA, µg/mL	156.2±41	164.9±61	172.2±46	179.4±83
EPA, µg/mL	7.5±4	5.8±2	18.1±10	15.8±7
DHA, µg/mL	57.5±26	48.9±17	72.0±26	66.7±22

Preterm infants with a body weight <1,500 g. ND, not detected. * For all columns: number of samples examined (%: bacterial detection rate), [b] $p < 0.05$, [bb] $p < 0.01$ (vs. placebo), non-parametric two-tailed Mann-Whitney U test, two-tailed unpaired Student's t test.

Fecal Bile Acid Concentration

The values of total bile acid in the Bifid group at 4 and 8 weeks of life were significantly lower than those in the placebo group at the respective time points (Table 3).

Discussion and Conclusion

We here present comprehensive data from a double-blind, randomized, controlled study of *B. breve* supplementation concomitant with early feeding with maternal colostrum and breast milk during the first 8 weeks postpartum in ELBW and VLBW infants with birth weights <1,500 g.

VPIs are known to suffer from various deficiencies in intestinal functions leading to increased susceptibility to inflammatory diseases [1–3]. Colostrum contains a larger range of growth factors, anti-inflammatory components, and anti-infective components than mature milk [15]. Higher concentrations of specific HMOs, including α1,2 fucosylated oligosaccharides, in human colostrum may be linked to protection of newborns from NEC-related pathogens [5, 16]. Therefore, it would be greatly desirable to initiate trophic feeding using the mother's colostrum in ELBW and VLBW infants, as was done in the present study; notably, neither NEC nor sepsis developed in the *B. breve* supplementation group.

We found that the fecal *Bifidobacterium* in the Bifid group became the dominant species among the fecal bacteria from 1 week of life, an effect that persisted, together with increasing commensal bacterial numbers, until 8 weeks of life. Consistent with the dominance of *Bifidobacterium*, the total fecal organic acid concentrations were significantly elevated in the Bifid group. The significantly larger cumulative weight gain in the Bifid group by 8 weeks of life was consistent with the observation of higher caloric intake in infants provided with *Bifidobacterium* supplementation. Notably, the increased number of bacteria in the distal intestine is expected to facilitate the fermentation of undigested carbohydrates (e.g., HMOs) to produce additional short-chain fatty acids, which in turn can be utilized for de novo synthesis of lipids and glucose [17–19] for use as the main energy source by the host. The concentrations of all of the measured plasma n–3 and n–6 PUFAs (with the exception of AA) exhibited an upward trend from birth to 4 weeks of life, an effect that was nominally greater in the Bifid group than in the control group; this observation may contribute, at least in part, to the higher caloric intake by the *Bifidobacterium*-supplemented infants.

Bacteria in the small intestine generate deconjugated bile acids, which enhance fat digestion and absorption. Bile acids then are absorbed through high-affinity active transport in the distal ileum, known as an enterohepatic circulation, but some amount of bile acid escapes into the large intestine and is lost in the feces. This bile acid metabolism appeared to result in a significant decrease in the total fecal bile acid concentration in the Bifid group, probably due to more efficient enterohepatic circulation of the increased levels of deconjugated bile acids [20]. Indeed, bile acids now are appreciated to serve as versatile signaling molecules that bind to distinct receptors known as TGR5 and FXR, both of which have a major impact on host metabolism, including energy and glucose homeostasis [21].

The above-mentioned results of the present study establish a causal relationship between the state of microbial community development and improved growth in infants, in particular VPIs.

Our study had at least two limitations. First, this study was a single-center study where the hospital staff and the included patients and their parents may not have been free from bias related to administration of BBG-01 or placebo. Second, the sample size and the duration of this study may not have been sufficient to fully evaluate the effects of BBG-01 supplementation. However, our results demonstrated that early bifidobacterial (BBG-01) supplemen-

tation, together with parenteral support and early enteral feeding with the infants' own mothers' colostrum and breast milk, may be associated with improved short-term growth outcomes, highlighting the benefits of microbiota establishment in preterm infants. While BBG-01 supplementation appeared to provide considerable energy from organic acids, as well as the beneficial effects of fatty acids and bile acids, these effects alone may not be sufficient to explain the infants' "catch-up" in cumulative weight gain. However, to our knowledge, there are no reports of adverse safety issues associated with BBG-01 supplementation [22].

In conclusion, bifidobacterial supplementation, concomitant with early feeding with maternal colostrum and breast milk, yielded the establishment of a beneficial microbiota profile. The associated changes in fecal organic acid levels, fecal pH, and bile acid levels appeared to provide improved growth in preterm infants.

Acknowledgments

Statement of Ethics

Written parental consent was obtained for all participants. All research was performed according to the guidelines of the Helsinki Declaration, and the trial protocol was approved by the institutional ethics committees of both the Children's Medical Center and the Juntendo University Hospital. This clinical trial was performed following registration with the UMIN Clinical Trials Registry (http://www.umin.ac.jp/ctr/; registration No. UMIN000005412).

Disclosure Statement

T. Takahashi, H. Tsuji, and T. Asahara are salaried employees of Yakult Honsha Co., Ltd.

Funding Sources

Author Contributions

T. Oshiro: data collection, analysis, and interpretation; Y. Yamashiro: study design, manuscript editing, and supervision; T. Takahashi: supplying resources and manuscript drafting. All other authors analyzed the measurements of the investigated subjects.

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