Bifidobacterium longum subsp. *longum* [...]

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To investigate the influences of host genotype and environment on *Bifidobacterium longum* subsp. *longum* inhabiting human intestines at the strain level, six pairs of twins, divided into two groups (children and adults), were recruited. Each group consisted of two monozygotic (MZ) twin pairs and one dizygotic (DZ) twin pair. Child twins had been living together from birth, while adult twins had been living separately for 5 to 10 years. A total of 345 *B. longum* subsp. *longum* isolates obtained from 60 fecal samples from these twins were analyzed by multilocus sequence typing (MLST), and 35 sequence types (STs) were finally acquired. Comparison of strains within and between the twin pairs showed that no strains with identical STs were observed between unrelated individuals or within adult DZ twin pairs. Eight STs were found to be monophyletic, existing within MZ twins and child DZ twins. The similarity of strain types within child cotwins was significantly higher than that within adult cotwins, which indicated that environment was one of the important determinants in *B. longum* subsp. *longum* strain types inhabiting human intestines. However, although these differences between MZ and DZ twins were observed, it is still difficult to reach an exact conclusion about the impact of host genotype. This is mainly because of the limited number of subjects tested in the present study and the lack of strain types tracing in the same twin pairs from birth until adulthood.

The human intestine is a complex microbial ecosystem. Recent research has shown that gut microbes control or influence many metabolic pathways in the human host (1, 2, 3). Bifidobacteria, which are naturally present in the colonic microbiota, represent up to 25% of the cultivable fecal bacteria in adults and 80% in infants (4). The interest in bifidobacteria has grown significantly, mainly because their presence has been associated with a healthy microbiota, which has led to their widespread use as functional ingredients in food and pharmaceutical products (4, 5, 6). Considering the rapidly increasing number of commercial and technological strains, it has been reported that probiotic characteristics such as antioxidant potential are strongly strain dependent (7). The *FAO/WHO Guidelines for the Evaluation of Probiotics in Food* reports the following: "The current state of evidence suggests that probiotic effects are strain specific" (8). Therefore, the health effects of probiotics as well as successful colonization and studies of their mechanism of action should be linked to a specific strain rather than be at species level. However, it is not clear what kinds of *Bifidobacterium* strains colonize a person, and little is known about the factors that exert an influence on the various strains.

The drivers of intestinal bacteria are multiple and can be categorized as environment associated and host associated. Environment-associated factors include diet, geographical location, and the maternal source in early life (9, 10, 11, 12). A recent study implies that the first bacteria entering the intestine of an infant may be derived from his/her mother's placenta (9). Dietary intake and lifestyle habits were also demonstrated to be associated with the composition of the stool microbiota in humans (13). The impacts on the intestinal bacterial community of host-associated factors, including disease, gender, age, and host genotype, have been discussed previously (14, 15, 16, 17, 18). These studies mainly focused on the general composition of intestinal flora using 16S rRNA gene analysis, with the classification at genus level and only occasionally to species level.

Bifidobacterium longum subsp. *longum* is the most common species of *Bifidobacterium* and can be detected in the intestinal tracts of both adults and infants (4, 19). In the present study, in order to describe the amounts of *Bifidobacterium* spp. and the strain diversity of *B. longum* subsp. *longum* in human hosts, as well as to explore the effects of environment and host genotype on the *B. longum* subsp. *longum* strain types inhabiting the intestines of different people, two groups of child and adult twin pairs were set up as twins living in the same environment or in different environment, respectively. Moreover, both monozygotic (MZ) and dizygotic (DZ) twin pairs were selected in each group to provide a model for dissecting the influences of host genotype and environmental exposures. Feces from each subject were collected for enumeration and isolation of *Bifidobacterium*, and a recently established method, multilocus sequence typing (MLST), was adopted

A 1 Information on twins participating in the study

Twin pair	Subjects	Age[a]	Twin type
CMA	CMA1, CMA2	3 yr	MZ
CMB	CMB1, CMB2	6 mo	MZ
CDC	CDC1, CDC2	2 yr	DZ
AMD	AMD1, AMD2	32 yr	MZ
AME	AME1, AME2	30 yr	MZ
ADF	ADF1, ADF2	25 yr	DZ

[a] Age at the beginning of this study.

for the analysis of *B. longum* subsp. *longum* isolates from these twins.

MATERIALS AND METHODS

Twin participants and sample collection. Six pairs of twins were recruited for the current study. The three pairs in the child group were all under 3 years old, and the three pairs in the adult group were between 25 and 35 years old. Each group consisted of two MZ twin pairs and one DZ twin pair. Child twins had been living together from birth until the end of the present study, while adult twins had been living separately for 5 to 10 years. All the participants were healthy and reported no use of antibiotics or other drugs influencing the gut microbiota for at least 6 months. They consumed a mixed diet without any restrictions, except that the consumption of probiotics was restricted 2 weeks before fecal sampling. Information on the twin participants is shown in Table 1. The first letter, "C" or "A," in the code name means child or adult, respectively; the second letter, "M" or "D," means monozygotic or dizygotic twins, respectively; and the third letter, "A" to "F," represents different twin pairs. The numbers "1" and "2" represent the two individuals in a twin pair.

Approximately 2 g of fecal samples was collected with a sterile spatula and transferred to a sterile centrifuge tube containing 25 ml anaerobic transport medium. All the samples were kept at 0 to 4°C and delivered to the laboratory within 24 h for subsequent procedures. The anaerobic transport medium (per 1000 ml) contained 0.225 g KH_2PO_4, 0.225 g NaCl, 0.225 g $(NH_4)_2SO_4$, 0.0225 g $CaCl_2$, 0.0225 g $MgSO_4$, 0.5 g L-cysteine hydrochloride, 0.001 g resazurin, 0.5 g agar, 10 g Lab Lemco powder, 100 ml glycerol, and 2.1 ml 8% Na_2CO_3 (11). Five fecal samples were collected from each participant at time zero (time of the first sampling) and at 2, 4, 6, and 8 months thereafter. A total of 60 samples were obtained and analyzed.

The study was approved by the Research Ethics Committee of Shanghai Jiao Tong University, China. Written informed consent was obtained from all participants or their legal guardians.

Enumeration and isolation of *Bifidobacterium*. The centrifuge tubes containing 25 ml anaerobic transport medium for sample collection were weighed before and after sampling, and recorded as F_a and F_b, respectively. F_w is the weight of the sample ($F_w = F_b - F_a$). Each sample was homogenized with a vortex mixer to make a fecal suspension. The fecal suspensions were serially diluted with phosphate-buffered saline (PBS) (pH 7.2), and then 1 ml was inoculated onto *Bifidobacterium* genus-specific TOS propionate agar (Merck, Germany) containing 50 μg/ml mupirocin at an appropriate dilution series and incubated anaerobically (GENbag Anaer; Bio Mérieux) at 37°C for 72 h (11). Plates that displayed 30 to 300 colonies were selected for calculation of the total cell count of *Bifidobacterium* (recorded as B) in the samples: $B = X \cdot 10^n \cdot (F_w + 25)/F_w$, where X is the number of colonies on the plate and n is the corresponding dilution series.

Ten to 15 colonies were randomly selected from each plate, transferred into 15-ml tubes containing 10 ml sterile TOS broth, and then incubated anaerobically at 37°C for 24 to 48 h. The cell pellets were collected and stored in 20% glycerol for subsequent analysis.

DNA extraction and identification of *Bifidobacterium* isolates. Genomic DNA of isolates from the fecal samples was extracted as previ-

ously described (20). Briefly, overnight cultures were harvested by centrifugation, washed, and lysed. Then DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with sodium acetate solution and cold ethanol.

Identification of the isolates at species level was carried out by PCR and sequencing of the partial 16S rRNA gene. The genus-specific primers (5′-ATA ATG CGG CCG CAC GGG CGG TGT GTRC-3′ and 5′-TAA TAG CGG CCG CAG CMG CCG CGG TAA TWC-3′) were used to amplify a 900-bp fragment of the partial 16S rRNA gene as previously described (21). All the amplifications were performed with the T100 thermal cycler (Bio-Rad), and the nucleotide sequencing was done by the BGI Company (Shanghai, China). The resulting sequences were used to search for identical sequences deposited in the GenBank database using the BLAST algorithm, and the identities of the isolates were determined on the basis of the highest scores.

Amplification of housekeeping genes and sequencing. Ten genes proposed in several references describing the *Bifidobacterium* MLST scheme were initially evaluated in this study (data not shown) (11, 22, 23), and seven loci of housekeeping genes were finally selected for analysis of the *B. longum* subsp. *longum* isolates obtained. Six genes were based on the *B. longum* subsp. *longum* genome NC_004307 already described: *purF*, encoding amido phosphoribosyl transferase; *rpoB*, encoding a beta subunit of RNA polymerase; *fusA*, encoding GTP-binding elongation factor EF-G; *ileS*, encoding isoleucyl-tRNA synthetase; *clpC*, encoding a protease; and *gyrB*, encoding the beta subunit of DNA gyrase. The *dnaJ1* gene was based on the genome of *Bifidobacterium breve* UCC2003, encoding a chaperone protein. Primers for amplification were chosen as previously described and are shown in Table 2 (11, 22, 23).

The PCR was carried out in 50-μl final volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate (dNTP), 0.5 U *Taq* DNA polymerase (TaKaRa, Dalian, China), 0.4 μM each primer, and 10 ng DNA template. The PCR amplification program consisted of an initial heating step at 94°C for 10 min, 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min, and a final extension step at 72°C for 4 min. The amplicons were separated on a 1.2% agarose gel, followed by ethidium bromide staining. The PCR fragments were purified using a PCR purification spin kit (Qiagen, Germany) and were subsequently sequenced by the BGI Company (Shanghai, China).

Data analysis. Sequence data were edited using Chromas 2.4.1 software, and then aligned in MEGA5.0 software using CLUSTAL W. Analyses of allele sequences and MLST were conducted in Software BioNumerics 6.0, eBURST v3, and Sequence Type Analysis and Recombinational Tests version 2 (START 2.0). The statistical test was performed in the software SPSS version 20 to assess the significance of the differences between twin groups.

Nucleotide sequence accession numbers. All of the allele sequences were deposited in the GenBank database under accession numbers KP420237 to KP420284.

RESULTS

Enumeration of bifidobacteria in the intestinal tracts of child and adult twins. To observe the number of bifidobacteria present in the intestines of participants in this study, a viable cell count was carried out for all 60 fecal samples (Table 3). The count varied from 8.77 log CFU/g (5.9×10^8 CFU/g; CMB2, 0 month) to 10.50 log CFU/g (3.02×10^{10} CFU/g; CDC1, 6 months) in children and from 8.02 log CFU/g (1.05×10^8 CFU/g; ADF1, 4 months) to 9.38 log CFU/g (2.40×10^9 CFU/g; AME1, 6 months) in adults. The mean value and the standard deviation of the bifidobacterial cell count, as well as the variation range between the largest and the smallest amounts in one subject at different sampling times, were calculated and are displayed in Table 3. For the three adult twin pairs, the amount of bifidobacteria in the feces remained stable

A   2 Primers for amplification of housekeeping genes

Target gene	Primer	Sequence	Amplified position on gene	Amplicon size (bp)	Gene position on template genome	Template
purF	Fu	5′-CATTCGAACTCCGACACCGA-3′	400–1376	976	962003–963514	*B. longum* NC_004307
	Fr	5′-GTGGGGTAGTCGCCGTTG-3′				
dnaJ1	Ju	5′-GAGAAGTTCAAGGACATCTC-3′	99–639	540	191597–192610	*B. breve* UCC2003
	Jr	5′-GCTTGCCCTTGCCGG-3′				
rpoB	Rbu	5′-GGCGAGCTGATCCAGAACCA-3′	1135–2192	1,057	1083395–1086958	*B. longum* NC_004307
	Rbr	5′-GCATCCTCGTAGTTGTAsCC-3′				
fusA	Au	5′-ATCGGCATCATGGCyCACATyGAT-3′	40–824	784	931890–934013	*B. longum* NC_004307
	Ar	5′-CCAGCATCGGCTGmACrCCCTT-3′				
ileS	Lu	5′-ATCCCGCGyTACCAGACsATG-3′	253–1042	789	2210396–2213707	*B. longum* NC_004307
	Lr	5′-CGGTGTCGACGTAGTCGGCG-3′				
clpC	Cu	5′-GAGTACCGCAAGTACATCGAG-3′	991–1735	744	9047–11656	*B. longum* NC_004307
	Cr	5′-CATCCTCATCGTCGAACAGGAGGAAC-3′				
gyrB	Yu	5′-AAGTGCGCCGTCAGGGCTT-3′	1109–1601	492	1618399–1620489	*B. longum* NC_004307
	Yr	5′-GTGTTCGCGAAGGTGTGCAC-3′				

when sampled at 2-month intervals. However, the count varied much more in the children. Twin pair CMB possessed a bifidobacterial count of less than 10^9 CFU/g at the beginning of the study, which increased up to 10^{10} CFU/g after 8 months. The variation range for participant CMB1 (aged 6 months when the research started) reached 1.21 log CFU/g (approximately 16-fold). Twin pairs CMA and CDC, who were already over 2 years old when sampled, showed a relatively stable amount of bifidobacteria. The mean counts of bifidobacteria in children were 10 times higher than those in adults, although they remained stable in adults while varying widely in children as they grew. No significant difference was observed between DZ twins and MZ twins concerning the similarity of bifidobacterial counts within twin pairs.

Strain isolation and identification. A total of 577 *Bifidobacterium* isolates were obtained, with 30 to 49 isolates from each participant. The identities of these isolates were determined on the

basis of partial 16S rRNA gene sequence Blast searching in the GenBank database. Finally, 345 isolates were identified as *B. longum* subsp. *longum*, 58 isolates as *B. breve*, 52 isolates as *B. adolescentis*, 26 isolates as *B. longum* subsp. *infantis*, 47 isolates as *B. animalis*, and 19 isolates as *B. bifidum*, and 30 isolates could not be identified precisely. These 345 isolates of *B. longum* subsp. *longum* were subsequently analyzed by MLST. The origin and distribution of these *B. longum* subsp. *longum* isolates are shown in Table 4.

Housekeeping gene amplification and sequence analysis. All seven housekeeping genes were successfully amplified for 345 *B. longum* subsp. *longum* isolates, and the sequences of the fragments of the expected sizes were determined. The fragment sizes of the amplicons varied from 492 bp (*gyrB*) to 1,057 bp (*rpoB*). Original peaks on the sequencing charts were scanned to remove those nucleotides with low specificity on both sides. The remaining precise sequences, ranging in length from 387 bp (*dnaJ1*) to 860 bp

A   3 Enumeration of bifidobacteria in twins' feces at different sampling times

Subject	Log CFU/g At mo:			
	0	2	4	6	8	Mean ± SD	Variation range[a]
CMA1	9.63	9.82	9.21	9.81	9.41	9.63 ± 0.26	0.61
CMA2	9.46	9.65	9.72	9.39	9.54	9.57 ± 0.13	0.33
CMB1	8.89	9.81	9.91	9.93	10.10	9.95 ± 0.48	1.21
CMB2	8.77	9.38	9.66	9.72	9.95	9.64 ± 0.45	0.57
CDC1	10.40	10.00	10.30	10.50	10.30	10.32 ± 0.19	0.64
CDC2	10.30	10.70	10.30	10.20	10.40	10.40 ± 0.19	0.40
AMD1	8.71	8.82	8.57	8.66	8.79	8.72 ± 0.10	0.25
AMD2	8.73	8.88	8.49	8.58	8.51	8.66 ± 0.16	0.39
AME1	9.20	9.07	9.08	9.38	9.23	9.21 ± 0.13	0.31
AME2	8.83	8.35	8.61	8.77	8.62	8.67 ± 0.19	0.48
ADF1	8.62	8.56	8.02	8.25	8.31	8.40 ± 0.24	0.60
ADF2	8.57	8.34	8.54	8.42	8.10	8.42 ± 0.19	0.47

[a] The difference between the largest and the smallest amounts of bifidobacteria in one subject at different sampling times.

A   4 Origin and distribution of *B. longum* subsp. *longum* isolates used for MLST analysis

Subject	No. of isolates from samples at mo:					Total
	0	2	4	6	8	Total
CMA1	3	2	10	3	5	23
CMA2	2	3	8	3	7	23
CMB1	14	11	8	5	8	46
CMB2	9	4	5	3	7	28
CDC1	5	7	7	4	10	33
CDC2	4	9	6	1	6	26
AMD1	6	3	6	7	1	23
AMD2	8	6	1	6	4	25
AME1	4	5	5	6	5	25
AME2	10	3	6	5	3	27
ADF1	9	6	9	8	5	37
ADF2	5	10	4	6	4	29
Total						345
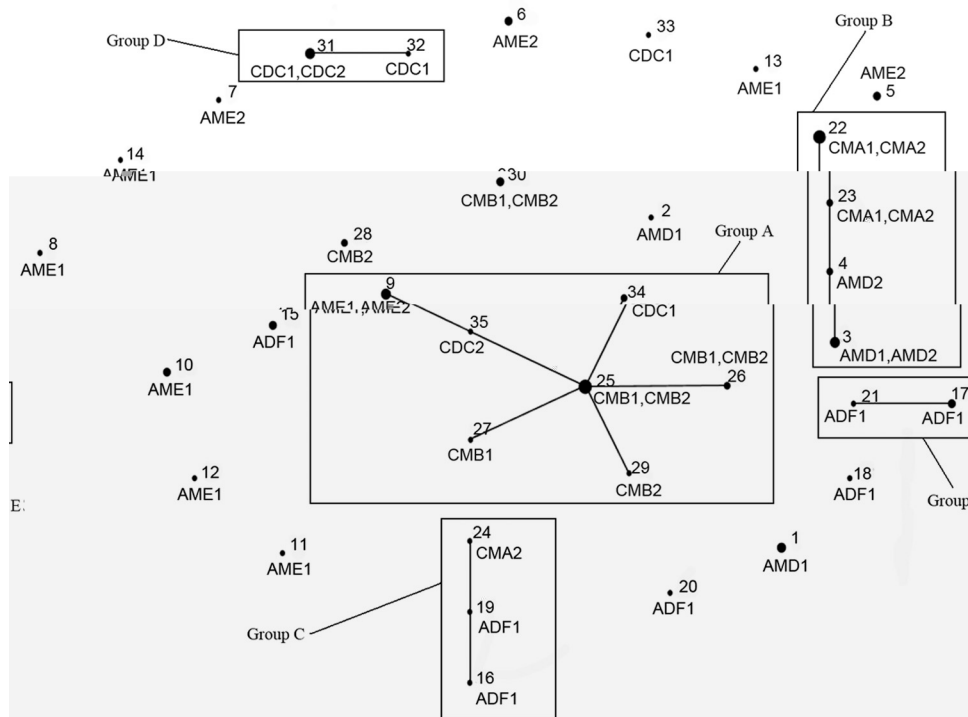
FIG 1 An E-Burst diagram of 82 *B. longum* subsp. *longum* individual strains based on STs was generated in eBURST v3. Each filled circle corresponds to a ST, and the circle area denotes the number of individual strains sharing the same ST. The designations of individuals from whom the ST was obtained are indicated to illustrate the relationships between different STs and the hosts.

(*rpoB*), were submitted for MLST analysis. Finally we submitted a total of 4,116 bp (25.36% coverage of the seven complete coding sequences [CDSs]) for the MLST protocol, whereas *B. longum* subsp. *longum* has a genome that is approximately 2.26 Mb long.

The sequences obtained from the seven genes of all isolates were imported into BioNumerics software (version 6.0). Polymorphic sites and the guanine-cytosine content for each locus were analyzed and determined to evaluate the reliability of this MLST set (see Table S1 in the supplemental material). Every unique sequence of each locus was assigned an allele number. The number of alleles per locus ranged from 4 (*clpC*) to 10 (*ileS*) (see Table S1 in the supplemental material). The allelic profiles of all isolates then were determined (data not shown).

Identification of STs and individual strains. Each unique allele profile could be perceived as a sequence type (ST) and was assigned a unique ST number in the software START 2.0. By combining the allelic profiles of the seven gene loci, 35 distinct STs were identified in the 345 isolates (see Table S2 in the supplemental material). The isolates that generated the same STs and were isolated from the same participant at the same sampling point were defined as the same individual strains (9); consequently, out of the 345 *B. longum* subsp. *longum* isolates, we finally obtained 82 individual strains.

After that, an e-Burst analysis was conducted using the software e-BURST version 3.0. A diagram showing the origins and relationships of different STs is given in Fig. 1. A total of 35 STs were divided into 5 groups (A to E) and 17 single STs. Among the 14 STs from child twins, 11 STs were clustered in four groups and 3 were identified as single STs; among the 21 STs from adult twins, 7 STs were clustered in four groups and 14 were identified as single

STs. It seems that the sequence types from children are more likely to cluster together (especially in group A), while sequence types from adults displayed lower relatedness with each other.

Comparison of the *B. longum* subsp. *longum* strains present in twins. Genetic relatedness among the 82 individual strains was assessed with a phylogenetic tree generated from the allelic profiles using the neighbor-joining method (Fig. 2). The results demonstrated that *B. longum* subsp. *longum* strains present in the intestines of different participants displayed high diversity and specificity. In this research, no identical ST was observed in unrelated individuals; i.e., no identical strains were found in participants belonging to different twin pairs. The number of STs isolated from one individual participant ranged from two to seven, and no significant difference was observed in the number of STs between the children and adults. By comparing the identity of STs between two participants within the cotwins of both groups, a total of eight STs was found to be monophyletic, existing within cotwins (ST22 and ST23 within CMA twins; ST25, ST26, and ST30 within CMB twins; ST31 within CDC twins; ST3 within AMD twins; and ST9 within AME twins).

The STs and the number of isolates identified as same individual strains in every sample are displayed in Table 5. In child twin pairs, both MZ and DZ, there were more identical *B. longum* subsp. *longum* isolates within cotwins (100% and 91.30% in twins CMA1 and CMA2, 95.65% and 92.85% in twins CMB1 and CMB2, and 57.58% and 88.46% in twins CDC1 and CDC2). In the adult twin group, the two MZ twin pairs possessed fewer monophyletic isolates than the child twin pairs (26.09% and 44% in twins AMD1 and AMD2 and 32.00% and 18.52% in twins AME1 and AME2). Moreover, in the adult DZ twins ADF1 and ADF2, a
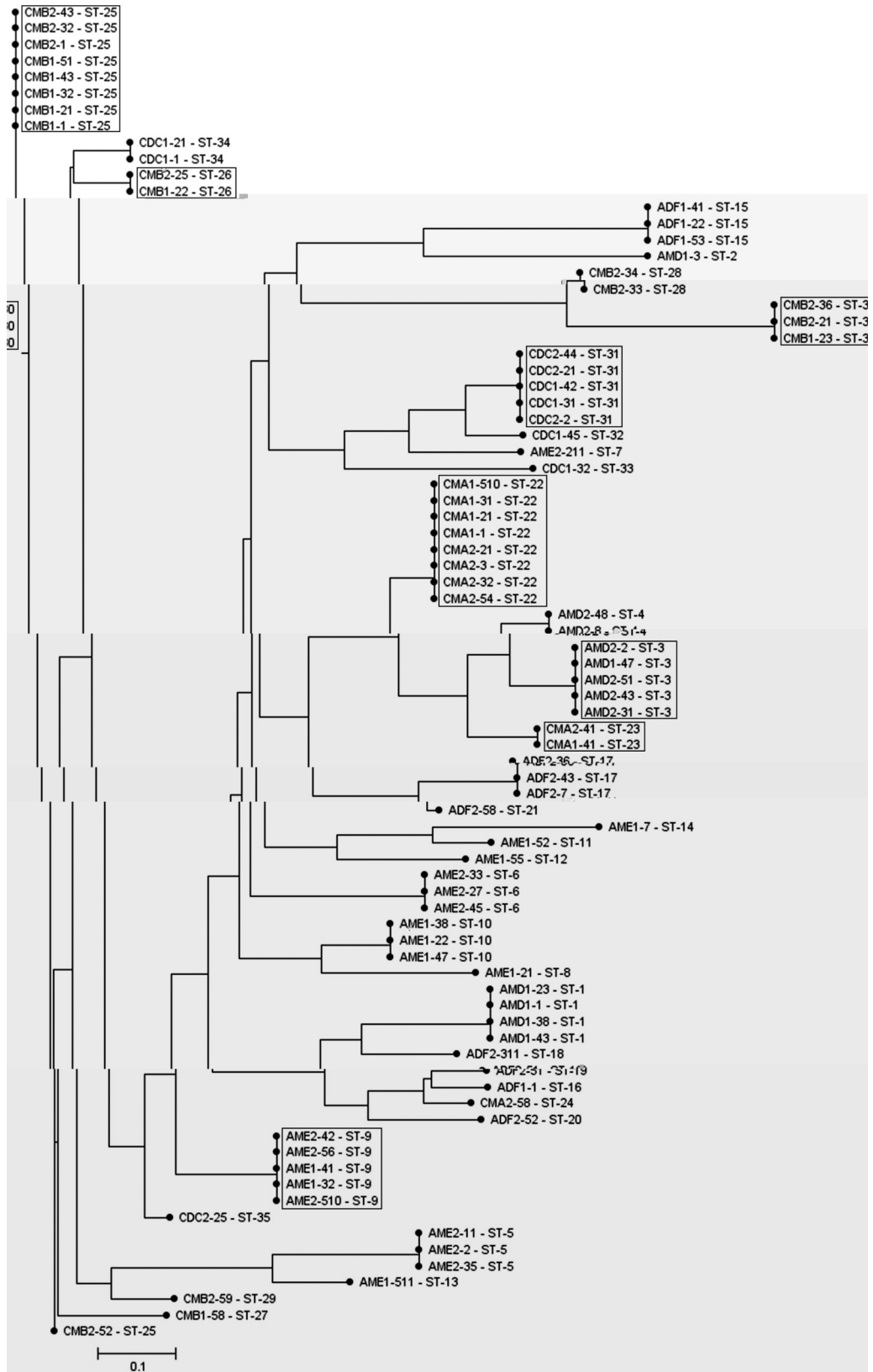
I   2 A phylogenetic tree of 82 individual strains was generated using phylogenetic analysis with START 2.0 software based on a neighbor-joining algorithm. Individual strains found monophyletically present within cotwins are marked with blocks.

A

5 Sequence type diversity and numbers of isolates identified as having the same STs

Subject	ST(s) (no. of isolates) at mo:					Total[b]	Rate of overlapping isolates (%)[a]
	0	2	4	6	8		
CMA1	ST22 (3)	ST22 (2)	ST22 (10)	ST23 (3)	ST22 (5)	ST22 (20), ST23 (3)	100
CMA2	ST22 (2)	ST22 (3)	ST22 (8)	ST23 (3)	ST22 (5), ST24 (2)	ST22 (18), ST23 (3), ST24 (2)	91.30
CMB1	ST25 (14)	ST25 (5), ST26 (5), ST30 (1)	ST25 (8)	ST25 (5)	ST25 (6), ST27 (2)	ST25 (38), ST26 (5), ST27 (2), ST30 (1)	95.65
CMB2	ST25 (9)	ST26 (1), ST30 (3)	ST25 (3), ST28 (1), ST30 (1)	ST25 (3)	ST25 (6), ST29 (1)	ST25 (21), ST26 (5), ST28 (1), ST29 (1), ST30 (4)	92.85
CDC1	ST34 (5)	ST34 (7)	ST31 (6), ST33 (1)	ST31 (3), ST32 (1)	ST31 (10)	ST31 (19), ST32 (1), ST33 (1), ST34 (12)	57.58
CDC2	ST31 (4)	ST31 (7), ST35 (2)	ST31 (6)	ST31 (1)	ST31 (5), ST35 (1)	ST31 (23), ST35 (3)	88.46
AMD1	ST1 (4), ST2 (2)	ST1 (3)	ST1 (5), ST3 (1)	ST1 (3), ST3 (1)	ST3 (1)	ST1 (15), ST2 (2), ST3 (6)	26.09
AMD2	ST3 (4), ST4 (4)	ST4 (6)	ST3 (1)	ST3 (2), ST4 (4)	ST3 (4)	ST3 (11), ST4 (14)	44.00
AME1	ST9 (1), ST14 (3)	ST10 (1), ST8 (4)	ST10 (2), ST9 (3)	ST9 (4), ST10 (2)	ST11 (2), ST12 (1), ST13 (2)	ST8 (4), ST9 (8), ST10 (5), ST11 (2), ST12 (1), ST13 (2), ST14 (3)	32.00
AME2	ST5 (10)	ST6 (2), ST7 (1)	ST5 (2), ST6 (4)	ST6 (3), ST9 (2)	ST9 (3)	ST5 (12), ST6 (9), ST7 (1), ST9 (5)	18.52
ADF1	ST16 (9)	ST15 (5), ST16 (1)	ST15 (6), ST16 (3)	ST15 (8)	ST15 (4), ST16 (1)	ST15 (23), ST16 (14)	
ADF2	ST17 (5)	ST17 (8), ST18 (2)	ST17 (3), ST18 (1)	ST17 (6)	ST19 (1), ST20 (1), ST21 (2)	ST17 (22), ST18 (3), ST19 (1), ST20 (1), ST21 (2)	

a

total of 66 isolates were acquired, but no identical isolate was found among them.

The statistical *t* test was performed in SPSS to assess the differences in the rate of overlapping isolates between different twin groups (Fig. 3). The rate of overlapping isolates between the child group and adult group was significantly different ($P = 0.001$). Differences between MZ and DZ twins in both the child and adult groups were also calculated. Interestingly, the rate of overlapping isolates within child MZ cotwins was not significantly different from that within the child DZ cotwins ($P = 0.09$); however, the rate within adult MZ cotwins was significantly different from that within adult DZ cotwins ($P = 0.02$).

DISCUSSION

It has been suggested that the host genotype, the physiology of the host, and environmental factors may affect the composition and function of the bacterial community in the intestine (16). However, opinions vary, and understanding of the main factors exerting an impact on the diversity of the intestinal inhabitants at strain level is currently deficient. This is mainly because methods used in previous studies, such as randomly amplified polymorphic DNA-PCR, ribotyping, and pulsed-field gel electrophoresis (PFGE), were considered not robust enough for strain delineation and phylogenetic analysis of large numbers of isolates (24, 25, 26). Kohara et al. (24) carried out PFGE on human fecal *B. longum* isolates and revealed that 12 participants harbored strains of unique PFGE types or subtypes over a 68-week period. Aires et al. (25) compared three human *B. longum* isolates with the sequenced model strain *B. longum* NCC2705 by PFGE and two-dimensional gel electrophoresis, and 37 proteins were found to differ between these strains. McCartney et al. (27) used ribotyping and PFGE to differentiate between *Bifidobacterium* strains in two participants during a 12-month period, finding that subject 1 and subject 2 harbored different *Bifidobacterium* strains, and strains common to both participants were not detected. However, these assays were less convenient for large numbers of isolates and could not be standardized between laboratories. More recently, MLST has been used as a novel method to discriminate *Bifidobacterium* isolates

(11, 21, 22, 28). MLST is a technique for accurately distinguishing different isolates within species. It involves determining the sequences of several housekeeping genes, which provides unambiguous genotype nomenclature that can easily be shared between laboratories and provides precise information on strain evolution (7, 29, 30, 31, 32, 33). Therefore, in the current study we adopted the MLST protocol for strain-level classification and observation of intraspecies diversity. All the allele sequences have been submitted to the GenBank database for global overview and comparison. This will provide a better understanding of the strain diversity of *Bifidobacterium* spp. in human hosts.

The number of animal trials and clinical studies designed to investigate the interactions among the intestinal microbiota, the host, and the environment has increased rapidly (4). Even so, it is difficult to design an experiment to distinguish the influences of environment and host genotype because of the wide differences between laboratory animals and humans, as well as the complexity and specificity of individuals. Also, it is impractical to carry out a long-term follow-up of human intestinal microbiota from birth to adulthood because of the limited experiment period. Twins have been recruited as models in some previous studies on this topic. Zoetendal et al. (16) studied the bacterial community using fecal 16S rRNA gene amplicons from three pairs of adult MZ twins by denatured gradient gel electrophoresis (DGGE); their results showed greater similarity within than between twin pairs. In contrast, fecal profiles of a married couple, who lived in the same environment, showed as low similarity as that for unrelated individuals, indicating that the host genotype has an important effect on the bacterial composition (16). Research on child twins was also carried out by Stewart et al. (14); comparison of identical twin pairs, fraternal twin pairs, and unrelated control pairs showed that the highest level of similarity was found in identical twins, indicating that host genetics influenced the composition of the dominant eubacterial population in children. Florin et al. (34) recruited adolescent twin pairs and their families to ascertain the relative contributions of genetics and environment in determining methane emission; the results implied that shared and unique environmental factors are the main determinants of microbial methane emission. Another study, by Hansen et al. (35), in adult female MZ and DZ twin pairs demonstrated that MZ twins have greater concordance for carriage and levels of methanogens in their fecal microbiota than DZ twins. Several studies have also adopted twin models to determine the impact of diet, obesity, or body mass index (BMI) on intestinal flora (13, 15, 36, 37, 38, 39). Most of the above-mentioned studies have focused on the bacterial composition, except for one report on methanogenic strains. Controversial results were suggested for the effects of host genotype and environment on intestinal bacterial composition or methanogenic strains. The current study for the first time used the twin model to investigate the influences of host genotype and environments on *B. longum* subsp. *longum* strain types, rather than on bacterial composition, in children and adults.

In this study, more identical STs were detected within child cotwins living in a shared environment than within adult twin pairs living in a separated environment. In addition, the rate of overlapping isolates within child cotwins was significantly higher than that within adult cotwins, who had experienced shared delivery, feeding, and living conditions in their early lives but had been living separately for at least 5 years before this study started. This indicated that environmental exposure could be an impor-

tant determinant on strain types of *B. longum* subsp. *longum* inhabiting the intestinal tracts. However, although the differences were observed between MZ and DZ twins, it is still difficult to draw an exact conclusion about the influences of the host genotype on *B. longum* subsp. *longum* strains types inhabiting human intestines. This is mainly because of the limited number of subjects in the present study and the lack of strain type tracing in the same twin pairs from birth until adulthood. Moreover, it could not be ascertained how the occurrence of identical strains within adult MZ cotwins rather than adult DZ twins originated in the present study.

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