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ORIGINAL ARTICLE

Comparative analysis of the distribution of segmented filamentous bacteria in humans, mice and chickens

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Segmented filamentous bacteria (SFB) are indigenous gut commensal bacteria. They are commonly detected in the gastrointestinal tracts of both vertebrates and invertebrates. Despite the significant role they have in the modulation of the development of host immune systems, little information exists regarding the presence of SFB in humans. The aim of this study was to investigate the distribution and diversity of SFB in humans and to determine their phylogenetic relationships with their hosts. Gut contents from 251 humans, 92 mice and 72 chickens were collected for bacterial genomic DNA extraction and subjected to SFB 16S rRNA-specific PCR detection. The results showed SFB colonization to be age-dependent in humans, with the majority of individuals colonized within the first 2 years of life, but this colonization disappeared by the age of 3 years. Results of 16S rRNA sequencing showed that multiple operational taxonomic units of SFB could exist in the same individuals. Cross-species comparison among human, mouse and chicken samples demonstrated that each host possessed an exclusive predominant SFB sequence. In summary, our results showed that SFB display host specificity, and SFB colonization, which occurs early in human life, declines in an age-dependent manner.

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Introduction

Segmented filamentous bacteria (SFB) are indigenous gut commensal bacteria. They attach themselves to the intestinal epithelium using an apparatus with a holdfast structure (Hampton and Rosario, 1965; Savage, 1969; Klaasen *et al.*, 1992). SFB have been detected under light microscopy and scanning electron microscopy in the gastrointestinal tracts of a wide range of vertebrates and invertebrates (Savage, 1969; Davis and Savage, 1974; Pearson *et al.*, 1982; Klaasen *et al.*, 1992, 1993a; Urdaci *et al.*, 2001). The very distinct filamentous morphology with spore-forming properties enabled these bacteria to be first observed a century ago (Savage, 1969; Davis and Savage, 1974; Chase and Erlandsen, 1976). However, *in vitro* cultivation has so far been unsuccessful.

The significance of the contribution of indigenous microbiota to the development of the immune system in the animal intestine has long been recognized, and SFB are critical to this process. Germ-free animals mono-associated with SFB develop about the same numbers of Th1, Th17 and T-reg cells as animals colonized by complex microbiota (Ivanov *et al.*, 2008, 2009; Gaboriau-Routhiau *et al.*, 2009). Colonization induces the expression of

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a variety of innate and adaptive immune genes in mice. SFB mono-associated mice show increased numbers of lymphoid cells and immunoglobulin-A-secreting (IgA-secreting) cells, and elevated IgA titers in the serum and small intestine (Klaasen *et al.*, 1993b; Umesaki *et al.*, 1995, 1999; Suzuki *et al.*, 2004).

Attempts to transfer SFB from one host species to another have failed, indicating that these bacteria are strongly host-selective and -specific (Tannock *et al.*, 1984; Allen, 1992). Phylogenetic analysis based on 16S rRNA sequences show that SFB are most closely related to the genus *Clostridium*, and they were previously designated '*Candidatus Arthromitus*' (Snel *et al.*, 1995). The whole SFB genome was recently determined using DNA isolated from the feces of SFB mono-associated mice and single cell sequencing of five individual mouse isolates. Its taxonomic classification was confirmed as a unique member of *Clostridiales* (Prakash *et al.*, 2011; Sczesnak *et al.*, 2011; Pamp *et al.*, 2012). The mouse SFB genome lacks a number of basic metabolic pathways found in free-living bacteria, which suggests that SFB require the unusual nutritional supplements from environmental factors, which must be derived from hosts or other commensals. This lack of basic metabolic pathways may also contribute to the strong immune-modulatory effects of these bacteria. Chemotaxis may drive the bacteria to adhere tightly to the surfaces of epithelial cells.

Although it is known that SFB are distributed in the gastrointestinal tracts of a wide variety of vertebrates and invertebrates, information regarding their presence and distribution in the human gastrointestinal tract is sparse. Klaasen *et al.* (1993a) observed filamentous bacteria attached to the surfaces of the ileal mucosa using a light microscope. However, the identification of SFB sequences in the genome-wide sequences of 263 human gut metagenomes failed. This supports the conclusion that SFB are not present in human fecal samples (Prakash *et al.*, 2011; Sczesnak *et al.*, 2011). There are few genes in the human gut that are similar to mouse and rat SFB genes (Kuwahara *et al.*, 2011; Prakash *et al.*, 2011; Sczesnak *et al.*, 2011). The issue of whether SFB inhabit the human gut and their taxonomic relationship with their counterparts in other hosts remains to be elucidated. In this study, we used PCR techniques to determine the distribution of SFB in human, mouse and chicken intestinal samples. The phylogenetic relationships between human, mouse and chicken SFB were then analyzed by sequencing.

Materials and methods

Origin of samples

A total of 251 healthy human volunteers (142 male and 109 female), ranging from 1 day to 72 years of age, were included in the current study. Exclusion criteria included recent antibiotic treatment, frequent gastrointestinal disorders and metabolic diseases. The study was carried out according to the Helsinki declaration, and informed written consent was obtained from all the human subjects. Details regarding age, gender and location are listed in Supplementary Table S1. About 2 g fresh fecal samples were collected immediately after defecation, placed in an ice box and then stored at $-80\,^{\circ}$C for further analysis. The current study was approved by the Ethics Committee of Zhejiang Academy of Agricultural Sciences.

The distal ends of the small intestine (approximately 5 cm anterior to the ileocecal junction) and cecal contents were collected from 48 ICR mice obtained from the animal research center at Zhejiang University. The samples were collected when the mice were 0, 7, 14, 21, 28, 35, 42 and 49 days old. At each point of time, ileal mucosal and cecal samples were collected from three female mice and three male mice. Six chicken samples (Hubbard strain), including terminal ileal mucosal and cecal contents, were collected when the chickens were 0, 7, 14, 21, 28 and 35 days old. The chickens were obtained from the animal house at the Zhejiang Academy of Agricultural Sciences. After collection, all samples were kept at $-80\,^{\circ}$C for further analysis. All the animals used in the current experiment were handled using strict compliance with current regulations and guidelines concerning the use of laboratory animals in China. The procedures were approved by the Laboratory Animal Care and Usage Committee of Zhejiang Academy of Agricultural Sciences.

DNA extraction

Bacterial genomic DNA was extracted using a QIAamp DNA Stool Mini Kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). The concentration of extracted DNA was determined using a NanoDrop ND-2000 (NanoDrop Technologies, Wilmington, DE, USA), and its integrity and size were confirmed by agar gel electrophoresis (1.0%). In order to determine the quality of the DNA, primers of 341 F (5′-ATTACCGCGGCTGCTGG-3′) and 534 R (5′-CCTACGGGAGGCAGCAG-3′) were used to amplify the V3 region of the bacterial 16S rRNA gene.

PCR amplification, cloning and sequencing

Oligonucleotides targeted to SFB 16S rRNA gene regions were used as PCR primers. Two primer pairs of 779 F (5′-TGTGGGTTGTGAATAACAAT-3′) with 1008 R (5′-GCGGGCTTCCCTCATTACAAGG-3′) and 779 F with 1380 R (5′-GGTTAGCCCACAGGCTTCGG-3′) were used in the current study (Snel *et al.*, 1995; Urdaci *et al.*, 2001). A standard PCR was performed in a total volume of 25 μl containing 2.5 μl dNTP (2.5 mmol), 2.5 μl 10 × EX Taq buffer, 1 μl of each primer (10 μmol),

0.3 μl EX Taq polymerase (TaKaRa, 5 U μl^{-1}) and 40 ng of prepared nucleic acid. Thirty cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s were performed in a DNA thermal cycler (ABI Veriti 96-Well; (Applied Biosystems, Grand Island, NY, USA)). This was followed by incubation at 72 °C for 5 min. Amplification products were separated using TAE-agarose gel electrophoresis (1.0%) and purified using a Biospin Gel Extraction Kit (Bioer Technology Co., Ltd, Hangzhou, China). Amplification products were then cloned into pMD18-T vector (Takara Biotechnology (Dalian) Co., Ltd, Dalian, China) according to the manufacturer's instructions to produce a shotgun library. Clones from each library were randomly selected and sent for sequencing with M13 primer at Beijing Genomics Institute (China). Ten high-quality sequences were obtained per library.

Phylogenetic analysis of 16S rRNA sequences

A total of 250 16S rRNA gene sequences (average length 620 bp) generated by SFB-specific primers (779 F and 1380 R) were obtained from human fecal samples. Mouse gut samples yielded 180 sequences and chicken samples yielded 130. Sequence alignment was performed using ClustalX1.83. Considering the presence of technical error during the sequencing, DNAStar software and the MegAlign program (DNASTAR, Inc., Madison, WI, USA) were used to identify the operational taxonomic units at a 99.5% cutoff level. Then 16S rRNA gene sequences from the 10 most closely related *Clostridium* species were downloaded from GenBank using BLAST (Basic local alignment search tool) (Altschul *et al.*, 1997) and the phylogenetic tree was constructed using a MEGA 4.1 neighbor-joining algorithm.

Quantitative PCR

Quantification of bacterial DNA was performed using an ABI PRISM 7500 Real-Time PCR Detection System (Applied Biosystems) according to the manufacturer's instructions. A 20 μl amplification reaction was performed with 10 μl Thunderbird SYBR qPCR (quantitative PCR) Mix (Toyobo Co., Ltd, Osaka, Japan), 0.04 μl 50 × ROX reference dye, 0.5 μM of each primer, 1 μl DNA template (20 ng μl^{-1}), and distilled water. Amplifications were performed with the following temperature profiles: one cycle at 95 °C for 1 min, 40 cycles at 95 °C for 15 s, at an appropriate annealing temperatures for 35 s and 72 °C for 35 s. Fluorescence was measured after the extension phase of each cycle. Melt curve analyses were performed by slowly heating the PCR mixtures from 55 °C to 95 °C. These served as end point assays and were used to confirm PCR specificity. Ten pairs of primers were selected for quantification of the total number of bacteria, *Bacteroides – Prevotella* group, *Bifidobacterium*, *Clostridium* clusters I, XI and XIVab, *Enterobacteriaceae*, *Enterococcus faecalis*, *Lactobacillus* and SFB. The primer sequences and annealing temperatures for each pair of primers are shown in Supplementary Table S2. The quantitative measurement of unknown samples was achieved using standard curves made from known concentrations of plasmid DNA containing the respective amplicons for each set of primers (Fite *et al.*, 2004).

Results

Distribution and diversity of SFB in the intestines of humans, mice and chickens

The specificity of the two sets of SFB-specific primers was evaluated before investigation of the distribution of SFB in human, mouse and chicken intestinal samples. Phylogenetic analysis showed that sequences obtained from both pairs of primers were identical to the published SFB sequences, suggesting that these primers were reliable. In order to determine whether the partial sequence was enough to represent the full-length sequence in phylogenetic analysis, we used full-length published SFB sequences as templates to find homologous sequences in GenBank using BLAST. We obtained a total of 52 homologous sequences when the scale of max identity was set at ⩾95% and query coverage at ⩾80%. Among these sequences, three were derived from human skin, dog duodenum and gorilla feces, respectively, and the rest were from mice. We conducted a similar search with partial sequences between 779 F and 1380 R. This resulted in two phylogenetic trees, one formed by full-length sequences and the other by partial sequences. As shown in Supplementary Figures S1 and S2, the topology of the two trees was similar, suggesting that the sequences generated from primers 779 F and 1380 R are accurate enough for phylogenetic analysis. For this reason, primers 779 F and 1380 R were used in our evaluations of the distribution and diversity of SFB.

Among the 251 human fecal samples collected, 51 (20.32%) were found to be SFB-positive under PCR detection. The samples positive for SFB were found to be distributed in an age-dependent manner. As shown in Table 1, 25.0% of fecal samples collected from infants under 6 months of age were SFB-positive. This increased to 78.6% among infants between 7 and 12 months of age. This value was lower in older subjects. Only 18.2% of samples taken from subjects over 25 months old were SFB-positive. In general, SFB were not detected in subjects over 36 months old. However, 6 out of the 20 adults between the ages of 20 and 31, whom we evaluated, had SFB in their feces. The same was true for 4 out of the 24 volunteers who were 41 to 51 years old. Of the 21 – 30 years old subjects who were positive for SFB, 5 out of 6 worked in a laboratory in which SFB research was performed, and dealt with infected mice and chickens on a daily basis.

The distribution of SFB among mouse and chicken intestinal samples was also age-dependent

Table 1 Distribution of SFB in human fecal samples

Age[a]	*Total (F/M)*	*Positive (F/M)*	*Positive ratio (F/M)%*
1–180 days	8 (4/4)	2 (1/1)	25.0 (25.0/25.0)
7–12 months	14 (6/8)	11 (4/7)	78.6 (66.7/87.5)
13–18 months	29 (10/19)	17 (6/11)	58.6 (60.0/57.9)
19–24 months	16 (6/10)	7 (4/3)	43.8 (66.7/30.0)
25–30 months	11 (1/10)	2 (1/1)	18.2 (100/10.0)
31–36 months	11 (5/6)	2(2/0)	18.2 (40.0/0)
3–5 years	25 (15/10)	0	0
10–11 years	27 (15/12)	0	0
15–20 years	28 (9/19)	0	0
21–30 years[b]	20 (10/10)	6 (4/2)	30.0 (40.0/20.0)
31–40 years	21 (8/13)	0	0
41–50 years	24 (12/12)	4 (2/2)	16.7 (16.7/16.7)
51–60 years	13 (6/7)	0	0
61–75 years	4 (2/2)	0	0
Total	251 (109/142)	51 (24/27)	20.3 (22.0/19.0)

Abbreviations: F, female; M, male
[a]Age is given based on the time that fecal samples collected.
[b]Seventeen of the twenty samples from volunteers aged 21–30 years old were collected from three different laboratories. Five of six SFB-positive samples were from individuals who dealt with infected mice and chickens at the time of the SFB experiments.

(Supplementary Figures 3A and 3B). In general, SFB first appeared in the ileum early during the host animal's life. For example, four of the six mouse ilea were SFB-positive when the mice were 7 days old. These numbers dropped to 50% at 2 and 3 weeks of age. All of the mice were SFB-positive at 4 weeks of age and the bacteria persisted in the ileum until 7 weeks. In contrast, SFB appeared in chicken ilea during the first week of life, reaching to a peak in both the ileum and cecum at 2 weeks, after which they started to decline. Only 50% of chickens were SFB-positive at 5 weeks of age.

For analysis of SFB diversity, clones were randomly picked for each 16S rRNA library generated from SFB-positive gut samples. They were sequenced, and 10 high-quality sequences were obtained per library. A total of 250 partial 16S rRNA sequences, 620 bp in length (positions 779–1380, corresponding to the 16S rRNA sequence of *Escherichia coli*) were generated from 25 human fecal samples. Likewise, 180 sequences were obtained from 18 mice and 130 sequences from 13 chickens. All sequences were submitted to NCBI under the accession numbers JQ361161–JQ361480. Three sequences obtained from humans and chicken were omitted, as their similarity to the published SFB sequences was below 90%. The analysis of operational taxonomic units was performed using 99.5% identity as a cutoff value. The number of operational taxonomic units per individual varied from one to five. The results are listed in Supplementary Table S3.

Phylogenetic analysis of SFB sequences found in humans, mice and chickens

A phylogenetic tree was constructed to better illustrate the relationships between SFB colonization among the different hosts. A 620 bp length of the 16S rRNA gene sequences (equivalent to the primers 779F and 1380R) was then generated from 15 published SFB 16S rRNA full-sequences (SFB from mice, rats, chickens and trout). Ten of the most closely related *Clostridium* sequences (*C. algidicarnis*, *C. beijerinckii*, *C. butyricum*, *C. diolis*, *C. frigidicarnis*, *C. gasigenes*, *C. mesophilum*, *C. perfringens*, *C. putrifaciens*, *C. tertium*) were also trimmed to 620 bp. Of the 557 SFB sequences obtained in the current study, we only preserved the unique sequences in each tested intestinal sample. There were 137 unique sequences found in human feces, 109 in mice and 71 in chickens. As shown in Figure 1, SFB sequences separated from the closely related *Clostridium* species and clustered together according to their origins. Human SFB formed a new branch, and most of the chicken SFB sequences (65 out of 71) clustered together with published chicken SFB sequences to form another branch. However, mouse SFB were separated into two groups. Out of the 109 mouse sequences, 41 clustered together with human SFB, all of which were from ileal samples. The remaining 68 distinct mouse sequences grouped with published mouse SFB sequences to form a separate branch (Figure 1 and Supplementary Figure S4).

The SFB population and its relationship with other indigenous bacteria in human fecal samples

The populations of human SFB in the fecal samples of SFB-positive infants were also assessed using qPCR with SFB-specific primers. Among the 33 tested samples, SFB counts remained relatively stable, with an average of 7.22 ($\log_{10}$ of bacteria per g feces), ranging from 6.16 to 8.23 (Supplementary Table S4). In order to determine whether the presence of SFB were related to the populations of other bacteria in the human colon, the total bacterial population and eight indigenous bacteria, including *Bacteroides*, *Bifidobacterium*, *Clostridium* clusters I, XI and XIVab, *Lactobacillus*, *Enterococcus* and *Enterobacter* were assessed in 67 fecal samples using qPCR analysis with specific primers. We divided the fecal samples of infants under 3 years of age into two groups according to the presence (group 1) or absence (group 2) of SFB. As shown in Supplementary Table S5, the total bacterial population was significantly higher in group 1 than in group 2 ($\log_{10}$ of bacteria per gram feces, 13.98 vs 13.28, $P<0.05$). The same was true for the numbers of *Bacteroides* and *Lactobacillus* ($\log_{10}$ of bacteria per gram feces, 12.67 vs 11.40 and 8.60 vs 7.90, respectively, $P<0.05$). We also noted that the relative abundance of *Bacteroides – Prevotella* was higher in SFB-positive infants, but no significant difference was observed with other indigenous bacteria.

SFB morphology

To further confirm the presence of SFB in human, mouse and chicken gut samples, SFB were

visualized using fluorescent *in situ* hybridization (FISH) with a SFB-specific probe. As shown in Supplementary Figure S5, filamentous cells were detected in mouse ileal mucosa and in cecal and colon contents (Supplementary Figure S5 B1-6). However, the SFB found in biopsies of human ilea were rod-shaped with a change to coccoid in feces (Supplementary Figures S5A1-4 and A5-6).

Discussion

Based on the published whole-genome sequence of mouse SFB, several efforts have been made to detect fragments of the SFB genome in human metagenomic data sets. These projects include one database generated from 124 individuals (Qin *et al.*, 2010) and another generated from 139 individuals (Sczesnak *et al.*, 2011). All searches were negative, although Kuwahara *et al.* (2011) reported 227 sequence reads of mouse SFB that were highly homologous to human sequences. This finding could be ascribed to the fact that all individuals involved in the previous human metagenomic studies were adults between 18 and 69 years old (Qin *et al.*, 2010). In the current study, we detected the presence of SFB in human fecal samples using SFB-specific primers. The results showed the colonization of humans to be age-related. SFB were present exclusively in the feces of children under 3 years of age, showing peak numbers in children between 7 and 12 months of age (Table 1). The age-dependent colonization of SFB in humans is consistent with patterns observed in other animal species, such as mice, rats and chickens (Koopman *et al*, 1987; Davis and Savage, 1974; Blumershine and Savage, 1978).

The age-dependent patterns of SFB colonization in humans could be ascribed to the maturation of the host immune system over time. It has been proposed that intestinal secretory IgA (SIgA), which either originates from breast milk or is self-produced by

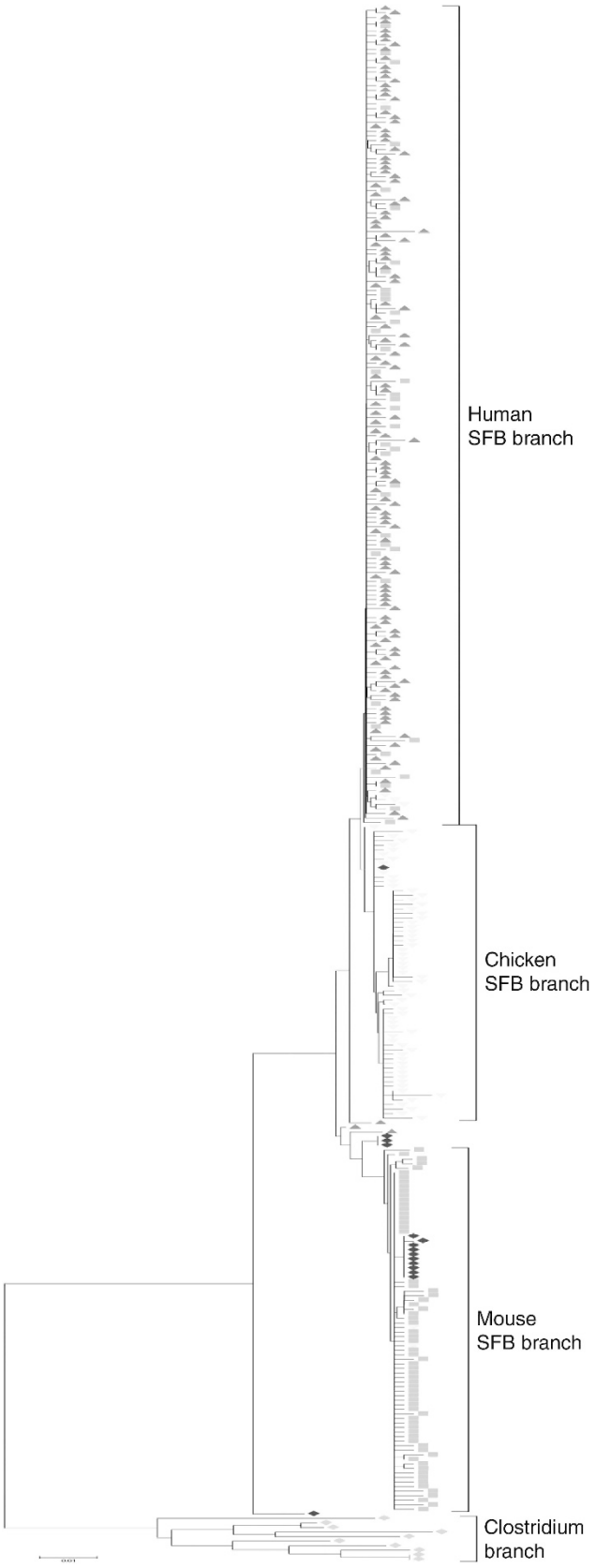

Figure 1 Phylogenetic relationships among the SFB (segmented filamentous bacteria) of human, mouse and chicken origin as shown by 16S rRNA sequencing. Five hundred and fifty-seven sequences corresponding to SFB 16S rRNA genes between primers 779F and 1380R were obtained from human, mouse and chicken samples. In each sample, the same sequences were deleted except one, which was preserved for further analysis. Fifteen published SFB sequences and ten closed *Clostridium* sequences were downloaded from the NCBI GenBank. In all, 342 sequences were aligned using ClustalX1.83. These were then used to construct a phylogenetic tree using MEGA 4.1 and a neighbor-joining algorithm. The red upward-facing triangles represent sequences of human fecal samples; the green squares represent mouse samples; the yellow downward-facing triangles represent chicken gut samples; the blue spindles represent published SFB sequences downloaded from NCBI and the cyan spindles represent the close *Clostridium* sequences downloaded from NCBI. The details of the human SFB branch, chicken SFB branch, mouse SFB branch and *Clostridium* branch are listed in Supplementary Figure S4. The color reproduction of this figure is available at *The ISME Journal* online.

the B cells of the host, affects the SFB colonization profoundly (Ohashi *et al.*, 2006; Liao *et al.*, 2012). For mice, the peak number of SFB in the ileum coincides with the window between weaning and the initiation of SIgA self-production. The high level of SIgA in mouse breast milk is considered as one of the key factors causing the decrease in the number of SFB (Jiang *et al.*, 2001). A similar phenomenon can be observed in human infants. It has been reported that breast milk contributes significantly to the levels of fecal SIgA in neonates at the age of 1 month. The level of fecal SIgA gradually decreases until the infant reaches the age of 5 months (Maruyama *et al.*, 2009). The concentration of fecal SIgA not only remains relatively low but also relatively stable until the age of 24 months (Dion *et al.*, 2004). SFB colonization in infants remains relatively low during the first 6 months, increases significantly between 7 and 12 months, and then declines after 24 months. The inverse relationship between changes in the concentration of intestinal SIgA and in the SFB population suggests that the intestinal SIgA may affect SFB colonization. However, as we did not measure the levels of fecal SIgA or record each infant's feeding regimens (breastfeeding vs formula feeding), it is reasonable to hypothesize that the increase in the SFB population after the infants reached 6 months of age may be due to the decline in the concentration of SIgA in the breast milk, and the subsequent disappearance of the SFB population in subjects over 3 years of age may be due to the result of increased self-produced SIgA when the host is matured.

Highly restricted host specificity is one of the distinguishing characteristics of SFB. Previous studies have clearly indicated that the SFB isolated from the intestines of rats cannot colonize mice or chickens (Tannock *et al.*, 1984; Allen, 1992). Phylogenetic analysis of 16S rRNA sequences suggested that among all the tested human, mouse and chicken samples, only one distinct SFB species per organism was present. This is consistent with previous studies. However, one predominant sequence (HP1) was detected in the all human samples with 100% similarity. Likewise, two predominant sequences (MP1 and MP2) were detected in mice, and three predominant sequences (CP1, CP2 and CP3) were detected in chickens. Analysis of the 620 bp length of 16S rRNA sequences showed the human predominant sequence (HP1) to be identical to the mouse predominant sequence (MP2) (Supplementary Figure S6). Further comparative analysis of the whole SFB genome sequence is needed to determine the mechanisms underlying host specificity. Pamp *et al.* (2012) recently found five SFB colonies in the gut of the same mouse, demonstrating that the single-nucleotide polymorphisms are present not only in 16S rRNA gene but also in whole genomic level, suggesting the existence of distinct SFB lineages within the same mouse colony. In the current study, phylogenetic analysis showed the presence of 16S rRNA sequence polymorphisms in the same human fecal sample. As increasing numbers of studies have fostered the accumulation of SFB genome sequence information generated from different hosts, the significance of SFB sequence polymorphisms and host specificity may be revealed in the near future.

Ivanov *et al.* (2009) reported that the abundance of 479 taxa to be significantly different between SFB-positive and -negative mouse stains ($P<0.05$). A higher population of *Lactobacillus* is particularly associated with SFB-positive Taconic B6 mice. Liao *et al.* (2012) reported that feeding *Lactobacillus* in drinking water can enhance the earlier colonization of SFB in neonatal chickens. In the current study, qPCR results showed the populations of the total bacteria and other indigenous bacteria to be higher in SFB-positive infants than in SFB-negative infants. However, the relative abundance of these bacteria showed no increase with the exception of *Bacteriodies.* This suggests that SFB colonization does not significantly change the distribution of bacterial clades. The relationship between the presence of SFB and other bacteria, particularly *Lactobacillus*, needs further investigation.

In summary, the current study indicates that the colonization of SFB in the human colon is age-related. Infants between 7 and 12 months of age showed the highest colonization rates.

Conflict of interest

The authors declare no conflict of interest.

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