RESEARCH ARTICLE

Colonization and distribution of segmented filamentous bacteria (SFB) in chicken gastrointestinal tract and their relationship with host immunity

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Abstract

Uncultivable segmented filamentous bacteria (SFB) reside in the gastrointestinal (GI) tract of mammals and can boost the host immunity. Immunoglobulin A (IgA) from mother's milk has been previously shown to be a key factor in regulating SFB colonization. Because neonatal chicken cannot acquire IgA from maternal milk, they are a good model to examine the role of IgA in SFB colonization. Here, we used the fluorescent *in situ* hybridization (FISH) and quantitative PCR (qPCR) to monitor the colonization and distribution of SFB in chickens aged from 2-day-old to 6-week-old. Early SFB colonization, which primarily occurred in the ileal mucosa (< 13 days old), was IgA independent. From the age of 17–42 days, there was an increase in IgA in the gut mucosa, which was correlated with a decrease in SFB. To examine the effect of probiotics and immunosuppression on SFB colonization, we treated the chickens by feeding them *Lactobacillus delbrueckii* or giving them a subcutaneous injection of cyclophosphamide (CTX). Feeding lactobacilli at birth rendered SFB colonization occurring 4 days earlier, while CTX treatment increases the SFB colonization through reducing the other non-SFB bacteria. Altogether, our data suggest that early colonization of SFB in chicken occurs independently of IgA and the population of SFB in the GI tract of chicken may be manipulated from birth via probiotic or CTX treatment.

Introduction

It has been well established that intestinal microorganisms are necessary for the development of the gut immune system. In germ-free (GF) animals, Peyer's patches (PP) are small and poorly developed in comparison with those of conventional animals (Rothkotter & Pabst, 1989). Moreover, the intraepithelial lymphocytes (IELs) and IgA-producing cells in the lamina propria are scarcely distributed (Moreau *et al.*, 1982; Umesaki *et al.*, 1993). Although approximately 500–1000 bacterial species inhabit the gut, few of them have been identified as essential in the promotion of maturation of host immunity. However, segmented filamentous bacteria (SFB) have the capacity for recapitulating the immune-inducing effect of the conventional microbiota (Ivanov *et al.*, 2008, 2009; Gaboriau-Routhiau *et al.*, 2009). In SFB-monoassociated mice, the immunological characteristics of the small intestine are almost converted to that in conventional mice without the assistance of other bacteria (Umesaki *et al.*, 1995). GF mice, after being solely colonized with SFB, show fully developed thymus-dependent IELs with an increase in IgA-producing cells and IgA titers (Klaasen *et al.*, 1993a, b; Umesaki *et al.*, 1993, 1995; Snel *et al.*, 1997; Talham *et al.*, 1999). These results indicate that SFB could stimulate the mucosal immune system to a greater extent than other autochthonous gut bacteria (Klaasen *et al.*, 1993a, b).

SFB were first discovered 30 years ago based on very distinct cell morphology (Savage, 1969; Davis & Savage, 1974; Chase & Erlandsen, 1976). Since then, SFB have been discovered in a wide range of vertebrates and invertebrates, including mouse, rat, monkey, pig, sheep, bovine, horse, zebra, dog, cat, guinea pig, frog, toad, duck, chicken, turkey, quail, rainbow trout, myriapod, termite, cockroach, beetle, and isopod (Hampton & Rosario, 1965; Savage, 1969; Fuller & Turvey, 1971; Davis & Savage, 1974; Pearson *et al.*, 1982; Klaasen *et al.*, 1992, 1993a, b; Urdaci *et al.*, 2001). So far, *in vitro* cultivation of SFB has been unsuccessful, but phylogenetic classification, based on the 16S rRNA gene sequences groups, placed this bacteria into the *Clostridiacaea* group and has been designated as '*Candidatus Arthromitus*' (Snel *et al.*, 1995). Several factors including age, anatomy position, diet composition, antibiotics, immunity status, and environmental stress have been reported to affect the populations and colonization of SFB in the small intestine (Davis & Savage, 1976; Klaasen *et al.*, 1990; Jiang *et al.*, 2001). Among them, luminal IgA seems to be the key driving force in the determination of SFB colonization (Davis & Savage, 1974; Glick *et al.*, 1978; Garland *et al.*, 1982; Koopman *et al.*, 1986; Klaasen *et al.*, 1990, 1991; Goodwin *et al.*, 1991; Jiang *et al.*, 2001). Using a mouse model, the colonization of SFB in the terminal ileum occurred in an age-dependent manner and was particularly influenced by the IgA concentration in maternal milk during the sucking period and in the luminal content produced by the pups after weaning (Jiang *et al.*, 2001).

SFB are also an indigenous bacteria that heavily inhabit the chicken gastrointestinal (GI) tract, where a holdfast appendix is produced by SFB to anchor to the intestinal epithelium cells (Yamauchi & Snel, 2000). An earlier study using a transmission electron microscope (TEM) indicated that SFB are phagocytized by the ileal epithelial cells after adherence to the chicken ileum, which is recognized as the first step in triggering the immunological response. However, unlike mammals, there is little maternal IgA influence in chicks because IgY is the predominant Ig isotype that transfers from the dam to chicks. IgA that exists mainly in albumen is 10–100 times lower than IgY in chicken yolks (Hamal *et al.*, 2006; Lammers *et al.*, 2010). Owing to this apparent low level of IgA, chicken are an alternative model to study the relationship between the development of host immunity and SFB colonization.

The correlation between the population of lactobacilli and ileal SFB is another interesting subject yet to be explored. Previous study demonstrated that oral administration of *Lactobacillus plantarum* to mice resulted in a significant decline of indigenous populations of SFB (Fuentes *et al.*, 2008). On the other hand, *Lactobacillus murinus* and SFB are the two taxa that exclusively coexisted with significantly higher populations in the Taconic mice (Ivanov *et al.*, 2009). Because *Lactobacillus delbrueckii* have been used as probiotics commercially on chicken farms (Xu, 2008; Liu *et al.*, 2009), it is interesting to know whether feeding *L. delbrueckii* to chickens affects the colonization and populations of SFB in the small intestine.

In this study, we used the fluorescent *in situ* hybridization (FISH) and quantitative PCR (qPCR) to examine the colonization and distribution of SFB in chicken GI tract. Following the development of chicken from 2-day-old young chicks to 42-day-old, we found that early colonization of SFB, which primarily occurs in the ileum, is IgA independent. Furthermore, we analyzed the impact of probiotic *Lactobacillus* and a known immunosuppressive agent, cyclophosphamide (CTX), on the colonization. The SFB colonization in the GI tract positively responds to these treatments.

Materials and methods

Bacterial strains and growth conditions

Lactobacillus delbrueckii was obtained from a commercial company in China (Shengda, Gansu, China). It was originally isolated from yogurt and has been used commercially as probiotics for chickens in China (Xu & Li, 2003). After being confirmed by 16S rRNA gene sequencing (Wang *et al.*, 2007), *L. delbrueckii* was grown in Man–Rogosa–Sharpe (MRS) broth (Hopebio, Qingdao, China) for 24 h at 37 °C under anaerobic conditions (MACS1000 Anaerobic cabinet, Don Whitley, UK). Ten fold serial dilutions were performed in phosphate-buffered saline (PBS) solution (0.1 M, pH 7.2; Oxoid, UK), and 100-mL aliquots of each dilution were plated onto MRS agar to determine the bacterial counts.

To test the specificity of the SFB probe, *L. plantarum* (CGMCC1.124), *Streptomyces caelestis* (CGMCC4.1687), and *S. bovis* (CGMCC1.1624) were obtained from China General Microbiological Culture Collection Center (CGMCC) and grown in potato dextrose agar (PDA) and Kenner Fecal *Streptococcus* Agar (KF) (HuaYi, Shanghai, China), respectively, according to the information provided by CGMCC.

Animals and experimental design

The distributions of SFB in the different anatomic sections of the chicken GI tract were investigated initially. A total of 102 one-day-old as-hatched chicks (commercial strain of Hubbard) were obtained from a local hatchery company (Zhenda, Hangzhou, China) and allotted into

three steer cages randomly. Maize- and soybean-based starter diets, without any antibiotic additives, were formulated based on the Feeding Standard of Chickens in China (NY-T 33-2004; Supporting information, Table S1) and provided *ad libitum* to all birds with water. Chicks were brooded initially at 31–33 °C in the first 5 days and with subsequent weekly reduction of 2–3 °C until the temperature reached 22–23 °C. Lighting was continuous, and the relative humidity was 60%. Two birds were randomly collected from each cage (total of six birds from three cages) at the age of 1, 3, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 19, 23, 27, 31, and 35 days and then were anesthetized by ether and killed by cervical dislocation. Sections of the ileum, ceca, and large intestine were aseptically removed and stored at −80 °C for further analysis.

In the second experiment, a total of 144 one-day-old as-hatched chicks were obtained from a local hatchery (Zhenda, Hangzhou, China) and allotted into three treatments randomly. All birds were free from commercial vaccinations and feed with maize- and soybean-based starter diet until the end of the experiment. To understand the relationship between the dynamics of SFB in the gut and the host immune status, birds in group 1 received 100 mg kg^{-1} body weight of CTX (Asta Medica, Prasfarma, Spain) by subcutaneous injection on days 1, 4, 8, 12, and 16, and the dose was reduced to 50 mg kg^{-1} body weight on day 27 and day 41. Birds in group 2 received 1×10^8 CFU mL^{-1} per bird of *L. delbrueckii* (LAC) daily in the drinking water. The control group (CL) without any treatment was assigned as group 3. Six chickens were randomly selected from each group on days 0, 2, 5, 9, 13, 17, 28, and 42. After recording the body weights, blood samples were collected by cardiac puncture, and subsequently, the ileal samples including mucosa and luminal contents were obtained for the enumeration of SFBs and for the detection of immune status.

All birds used in these experiments were treated according to the current regulations for laboratory animal management in China, and experiments were approved by the laboratory animal care and usage committee, Zhejiang Academy of Agricultural Sciences.

Sampling and data collection

In the first experiment, whole sections of ileum, ceca, and large intestine were collected from birds aged < 9 days. When birds were at an age of 10 days or older, sections of ileum (approximately 10 cm distant anterior to ileo-cecal junction), cecum (approximately 4-cm-long segments between the cecal tonsil and the tip), and large intestine (approximately 3 cm distant posterior to the ileo-cecal junction) were removed. After gently squeezing the digesta into Eppendorf tubes, the tissue remains were then opened and rinsed with PBS (0.1 M, pH 7.2) twice to remove the nontissue-associated bacteria. Three biopsies of 1 cm^2 (1 × 1 cm, wide × length, measured with a ruler) were subsequently taken from each section and homogenized individually with ice-cold PBS for 15 s using a beadbeater (BioSpec, Bartlesville, OK). After being centrifuged at 200 ***g*** for 10 min to remove the gross materials, 1-mL aliquots of supernatant were then collected for FISH analysis and qPCR detection. In a second experiment, three biopsies (1 cm^2 each) were removed from the terminal ileal (10 cm anterior from ileo-cecal junction) and homogenized after being rinsed twice with ice-cold PBS buffer, as described previously. The supernatants were collected and stored for FISH analysis and qPCR detection. Meanwhile, the ileal contents were collected and homogenized with ice-cold PBS buffer to make 10% slurries. Total lactobacillus population was detected by qPCR.

DNA extraction and qPCR

DNA from samples of the ileum, ceca, and large intestine was extracted and purified using a QIAamp DNA Stool Mini Kit following the manufacturer's instructions (Qiagen, Germany). DNA concentration was subsequently determined by NanoDrop ND-2000 (NanoDrop) and checked by electrophoresis.

For qPCR analysis, total bacterial populations of samples were amplified with universal primers Bac1114F (5′-CGG CAA CGA GCG CAA CCC-3′) and Bac1275R (5′-CCA TTG TAG CAC GTG TGT AGC C-3′) (Denman & McSweeney, 2006). The population size of the SFB was determined using SFB-specific primers 779F (5′-TGT GGG TTG TGA ATA ACA AT-3′) and 1008R (5′-GCG GGC TTC CCT CAT TAC AAG G-3′), as described previously (Snel *et al.*, 1995; Urdaci *et al.*, 2001). Total *Lactobacillus* in the ileal contents was measured using specific primers F-lac (5′-GCA GCA GTA GGG AAT CTT CCA-3′) and R-lac (5′-GCA TTY CAC CGC TAC ACA TG-3′) (Walter *et al.*, 2001). Amplification was carried out in a final volume of 20 μL containing 10 μL of SYBR Green Super Premix (SYBR Green Mix, Toyobo, Japan), 5 μL of DNA templates (10 ng μL^{-1}), and 0.2 μM of each primer, and reactions were performed in a Roche Light Cycler 2.0 (Roche, Mannheim, Germany). Thermocycling conditions were as follows: initial DNA denaturation at 95 °Cfor 10 min, 40 cycles of denaturation at 95 °C for 5 s, primer annealing at 58 °C for 5 s, and extension at 72 °C for 20 s. Following amplification, the melting curves were determined by auto dissociation protocol (65 °C).

Because SFB belonged to the uncultivated bacteria, a recombinant plasmid containing partial SFB 16S rRNA gene sequence was used as the template to construct the

standard curve. As described previously, about 230-bp SFB 16S rRNA gene was obtained by PCR from FISH-positive intestinal samples using SFB-specific primers 779F and 1008R and was subsequently connected with a PMD 18-T vector (TaKaRa, Japan). The number of SFB was calculated by standardization with a calibration curve of a dilution series of the recombinant plasmid (Lee *et al.*, 1996). For the measurement of total gut bacteria of the chickens, a recombinant plasmid was made by inserting partial 16S rRNA gene sequences (amplified using universal primers 1114F and 1275R from genomic DNA of *Lactobacillus*, *Enterococcus*, and *Pediococcus* and then mixed with the proportion 2:2:1) into PMD 18-T vectors (Sun *et al.*, 2008).

A 10-fold serial dilution series of the reference plasmids, ranging from 1×10^1 to 1×10^9 copies μL^{-1}, was used to construct the standard curves for SFB, lactobacillus, and total bacteria. The concentration of plasmid was measured using NanoDrop ND-2000, and the corresponding copy number was calculated using the following equation (Whelan *et al.*, 2003):

$$\text{DNA (copy)} = \frac{6.02 \times 10^{23}(\text{copy/mol}) \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660(\text{g/mol/bp})}$$

Each standard curve was generated by a linear regression of the plotted points. C_T values for each dilution were measured in triplicate using a qPCR and were plotted against the logarithm of their initial template copy numbers. From the slope of each standard curve, PCR amplification efficiency (E) was calculated according to the equation (Rasmussen, 2001): $E = 10^{[-1/\text{slope}]} - 1$.

In the current study, qPCR efficiency (E) was 1.998 for total bacteria, 2.009 for SFB, and 2.001 for lactobacillus with high linearity ($R^2 = 0.99$), calculated by LightCycler 4.1 software (Roche Diagnostics). All intestinal samples of the different groups were analyzed by qPCR in triplicate. Data were analyzed using an absolute quantification method (Yu *et al.*, 2005; Lee *et al.*, 2006). Results were expressed as the percentage of SFBs to the total bacteria in each sample.

Enzyme-linked immunosorbent assay (ELISA)

To measure total SIgA concentrations in the ileal mucosa, six chickens of each group were killed by cervical dislocation and intestinal secretions were obtained by scraping the ileum mucosa using a glass slide. Total SIgA was quantified by ELISA using a chicken secretary IgA ELISA detection Kit (USCNLIFE, Wuhan, China). The measurement was taken according to the manufacturer's instructions.

Peripheral blood leukocyte (PBL) counts

Blood samples were obtained by heart puncture with disposable syringes in ether-anesthetized chickens. The number of PBL in the blood was enumerated using a hemocytometer (Housser, Horsham, PA), as described previously (Jimenez-Valera *et al.*, 2003).

T-cell population in PBL

Lymphocytes were further separated from fresh peripheral blood and purified by density gradient centrifugation using Lymphocyte Separation Medium (Chicken) (Shang-Hai Lengton Bioscience Co., Ltd). Chicken peripheral blood mononuclear cells were stained with mouse anti-chicken CD3-SPRD (Clone CT-3, Cat. No. 8200-13; SouthernBiotech), mouse anti-chicken CD8α-PE (Clone CT-8, Cat. No. 8220-09; SouthernBiotech), and mouse anti-chicken CD4-FITC (Clone CT-4, Cat. No. 8210-02; SouthernBiotech). Lymphocytes were then gated and analyzed on a flow cytometer (guava easyCyteTM HT; Guava Technologies, Hayward, CA).

FISH analysis

FISH for the enumeration of SFB was performed as described by Franks *et al.* (Franks *et al.*, 1998). Briefly, samples of the different sections of chicken intestine were fixed in ice-cold 4% (w/v) paraformaldehyde solution for 24 h at 4 °C and then centrifuged at 10 000 ***g*** for 10 min. After discarding the supernatants, the cells were stored in 50% ethanol at −20 °C; 20 μL of each fixed sample was then spotted onto glass slides and allowed to air-dry for 1 h. An aliquot of 20 μL hybridization solution (0.9 M NaCl, 20 mM Tris/HCl, 0.1% SDS, pH 7.2) containing 5 ng μL^{-1} SFB-specific probe was spotted onto the air-dried cells of the glass slide and then incubated overnight at 50 °C in the dark (Zarda *et al.*, 1991). The SFB-specific probe (GGG TAC TTA TTG CGT TTG CGA CGG CAC) corresponding to position 801–827 in the 16S rRNA gene sequence of rat SFB (X87244) was Cy3-labeled. After hybridization, the slides were immersed in washing solution (0.9 M NaCl, 20 mM Tris/HCl, 0.01% SDS, pH 7.2) at 48 °C for 15 min, briefly rinsed with ice-cold distilled water, and air-dried. The slides were subsequently stained with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) for 15 min (Franks *et al.*, 1998; Harmsen *et al.*, 2002), washed again with 80% ethanol, and then rinsed with distilled water. The air-dried bacterial cells were counted under a biology fluorescence microscope (CX-51; Olympus, Japan). DAPI slides were visualized with the aid of a DM 400 filter, and probe slides with the aid of a DM575 filter.

Approximately 25 fields of view were covered with each sample, and one filament was considered as one counting unit. The numbers of SFB and total bacteria in each sample were determined using the following equation (Ogue-Bon *et al.*, 2010):

$$DF \times ACC \times 13903.65 \times 50$$

where DF is the dilution factor (DF = 1); ACC is the average cell count of 25 fields of view; 13903.65 represents the area of the well (0.785 cm^2) divided by the area of the field of view (5646.57 μm^2); and 50 takes the cell count back to per ml of sample (1000 μL per 20 μL).

Statistical analysis

All values for bacteria are presented as mean ± standard error (SE). Significance of differences between groups was assessed by *t*-tests using SPSS (version 13.0; SPSS Inc, Chicago, IL), and a two-tailed $P < 0.05$ was considered to be significant.

Results

Distribution and cell morphology of SFB in the GI tract of chickens

FISH and qPCR are quantitative methods commonly used in the enumeration of uncultivated bacterial populations (Urdaci *et al.*, 2001; Fuentes *et al.*, 2008). Therefore, at the beginning of the experiment, we compared the numbers of SFB in the digesta of the ileum using both methods. As shown in Table S2, the levels of total bacteria and SFB determined by FISH and qPCR were similar among all tested samples, although there was a slight increase in the SFB population detected by the qPCR method. Those results confirmed that both methods were reliable and reproducible. As FISH analysis is time-consuming, qPCR was chosen for the evaluation of SFB populations in the following experiments.

The counts of SFB and total bacteria in different sections of chicken intestine were quantified by qPCR and are presented in Table 1. Consistent with previous studies, the highest population of total bacteria was detected in the cecal section contents. For the population of SFB, the peak number was found in the section of the ileal mucosa, while the levels became undetectable in the samples of the cecal and large intestinal mucosa. It was interesting to note that SFB distributed in the digesta of the ileum, cecum, and large intestine. However, the percentage of SFB in the total bacteria peaked in the ileal mucosa (Table 1). This suggested that the ileal mucosa was the primary anatomic site for colonization and proliferation of SFB and subsequently resulted in the distribution of SFB to other parts of the GI tract. Additionally, the colonization of SFB in chicken gut was influenced by age. The highest SFB enumeration in total bacterial population was found in the samples obtained from ileal mucosa and digesta on day 9. As bird aged from day 9, the SFB population started to decline. The colonization patterns of SFB in the ileal mucosa also varied among individual chickens, particularly at the early age of 6 and 9 days, which resulted in a relatively larger standard deviation.

The morphology of the SFB in different anatomic sections was demonstrated by FISH hybridized with SFB-specific probe (Fig. 1). In line with the qPCR results, SFB was not observed in the mucosal samples of the cecum and large intestine (data not shown). Cells in the mucosa of the terminal showed typical long filamentous shapes with segments (Fig. 1a). However, bacteria obtained from the ileum digesta and the contents of large intestine showed a reduction in size (Fig. 1b and d), and only

Table 1. Enumeration of the SFB population in different sections of the chicken intestinal tract by qPCR*

	SFB/total bacteria (%)					
Age of birds (days)	IM†	IC	CM	CC	LM	LC
3	ND‡	ND	ND	ND	ND	ND
6	0.009 ± 0.003	0.001 ± 0.000	ND	§	ND	§
9	4.209 ± 0.843	0.151 ± 0.007	ND	0.001 ± 0.000	ND	0.003 ± 0.001
11	2.295 ± 0.222	0.094 ± 0.015	ND	0.001 ± 0.000	ND	0.003 ± 0.000
13	1.201 ± 0.169	0.0434 ± 0.010	ND	0.0015 ± 0.000	ND	0.004 ± 0.000
23	0.213 ± 0.055	0.001 ± 0.000	ND	§	ND	§
27	0.221 ± 0.068	0.003 ± 0.000	ND	§	ND	§

*Values represent means of six chickens ± SE and are expressed as % of SFB in the total bacteria (% Total bacteria).
†IM, ileal mucosa; IC, ileal contents; CM, cecal mucosa; CC, cecal contents; LM, large intestinal mucosa; LC, large intestinal contents.
‡Undetectable.
§SFB detectable, but the percentage is lower than 0.001%.

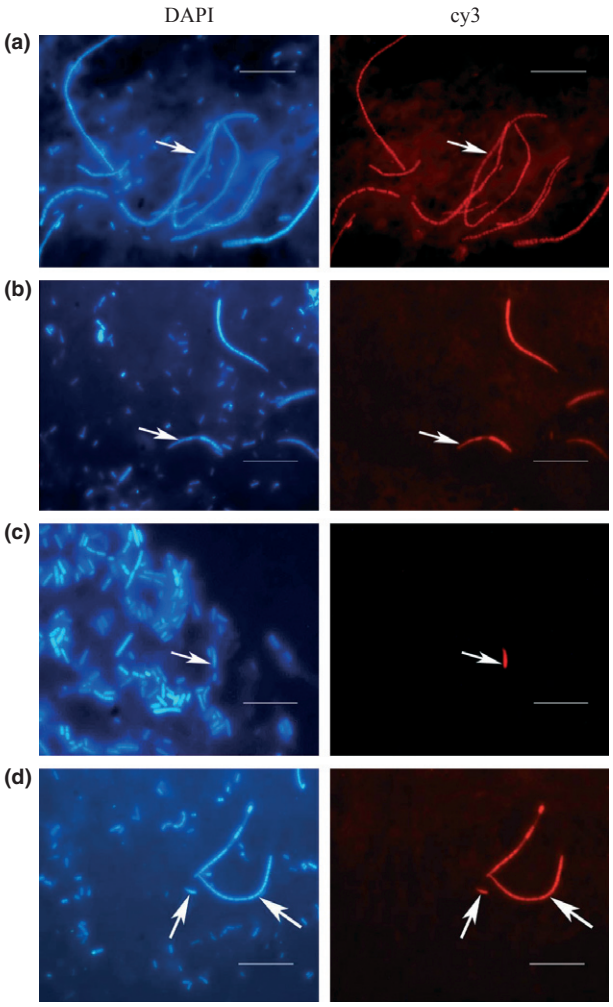

Fig. 1. Cell morphology of SFB in chicken ileal mucosa (a), ileal digesta (b), cecal digesta (c), and large intestinal digesta (d). Ileal mucosa (a), ileal digesta (b), cecal digesta (c), and large intestine digesta (d) were collected from broiler chickens at 9 days of age. Cell morphology was visualized by DAPI stain, and SFB were observed by hybridizing with a SFB-specific probe (cy3 labeled). The bar represents 10 μm (a, b, d) and 5 μm (c), respectively.

short rod-like cells were observed in the cecum (Fig. 1c). It was interesting to note that a mixture of various morphological cells from rod-like shapes to filamentous shapes was present in the digesta of the large intestine, but not in the digesta of the cecum.

Because the number of SFB in the ileal mucosa was significantly higher than that in other anatomic locations, the ileal mucosa was chosen to investigate the dynamics of SFB colonization with aging by FISH (Fig. 2). On day 6, 17% (one of the six) of the tested chickens showed positive colonization of SFB in the ileal mucosa and the numbers increased to 83% (five of the six) on day 8, eventually reached 100% (six of the six) on day 11, and

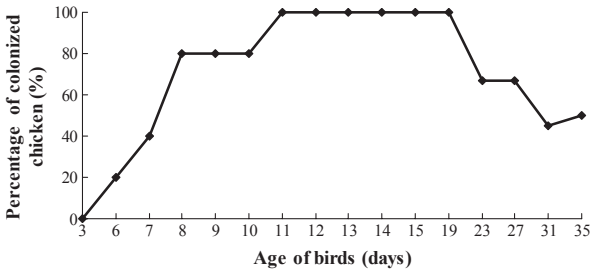

Fig. 2. Effect of age on SFB colonization in chicken ileal mucosa. A total of six birds were examined at each time point, and the percentage ratio represents the numbers of chickens colonized by SFB (qualified by FISH with SFB-specific probe) in the total number of tested birds.

lasted until day 19. However, on day 23, 67% (four of the six) of chicken were SFB positive. On day 31, only 33% (two of the six) of the chickens had SFB in their ileum mucosa.

The growth and immune status of chickens after being treated with *L. delbrueckii* and CTX

As SFB have the confirmed ability of modulating the host immunity, the influence of host immune conditions on the colonization of SFB was next examined after the treatment with *L. delbruckii* or CTX, an immunosuppressive agent in animals. The immune status of chickens in the current experiment was measured through growth performance, PBL numbers, numbers of CD4+, CD8+, and the ratio of CD4+ to CD8+ in blood. At the beginning of the experiment, a full dose of CTX (100 mg kg^{-1} body weight) was given to the birds to create an immunosuppression status; however, with a significant increase in mortality on day 17, the dose of CTX was reduced to half (50 mg kg^{-1} body weight) at the age of 27 and 41 days. The average body weights of chickens in the three treatments are shown in Fig. S1. There was no significant difference between the LAC group and the CL group, but a significant lower body weight was recorded in the CTX group. The PBL number in the blood was another indicator showing the immune status of the host. As shown in Fig. 3a, there was a significant decrease in the PBL count in the CTX group on day 5, day 9, day 13, and day 17, confirming the immunosuppression status of chickens. In addition, once the injection dose of CTX was reduced to half on day 27 and day 41, PBL counts resumed on day 28 and day 42. The impact of feeding *L. delbrueckii* and CTX treatment on the populations of CD4+, CD8+ and the ratio of CD4+ to CD8+ are shown in Fig. 3b–d. CTX treatments significantly decreased the populations of CD4+, CD8+ as well as CD4+/CD8+ ratio,

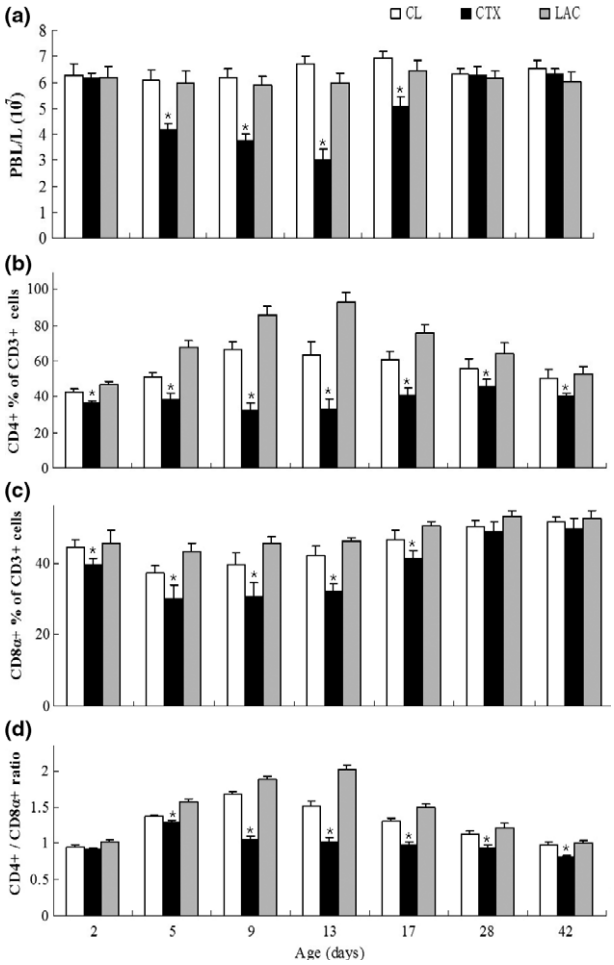

Fig. 3. Effects of feeding LAC and subcutaneously injecting CTX on the chicken blood PBL (a), CD4+ of % CD3+ cells (b), CD8+ of % CD3+ cells (c), and CD4+/CD8+ ratio (d) in peripheral blood lymphocytes. LAC, chickens were fed with *Lactobacillus delbrueckii* (1×10^8 CFU mL^{-1} per bird. per day); CTX, chickens were subcutaneously injected with CTX (100 mg kg^{-1} body weight on day 1, day 4, day 8, day 12, and day 16 and 50 mg kg^{-1} body weight on day 27 and day 41); CL, chickens were fed without any treatments. Values represent mean ± SE from six chickens per groups, and * represents that the difference is significant ($P < 0.05$).

further suggesting that the CTX treatment caused the immunosuppression in chickens.

The effects of *L. delbrueckii* and CTX treatments on the concentrations of SIgA in the ileal mucosa are shown in Fig. 4. In general, age significantly affected the SIgA levels in the surface of ileal mucosa. The concentration of SIgA remained at low levels until the chickens were 17 days of age and then started to increase significantly at 28 days of age in all tested groups. Subcutaneous injection of a full dose of CTX showed a slight inhibition of the SIgA level, although the inhibition effect became obvious on day 17

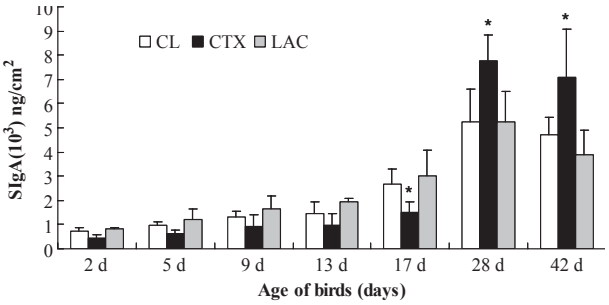

Fig. 4. Effects of feeding LAC and subcutaneously injecting CTX on SIgA in the ileal mucosa of chickens. LAC group, chickens were fed with *Lactobacillus delbrueckii* (1×10^8 CFU mL^{-1} per bird per day); CTX group, chickens were subcutaneously injected with CTX (100 mg kg^{-1} body weight on day 1, day 4, day 8, day 12, and day 16 and 50 mg kg^{-1} body weight on day 27 and day 41); CL group, chickens were fed without any treatments. Values represent mean ± SE from six chickens per groups, and * represents that the difference is significant ($P < 0.05$).

($P < 0.05$). Owing to the increase in mortality in the CTX group on day 17, we reduced the injection dose of CTX on day 27 and day 41. A half dose of CTX significantly increased the level of the SIgA in the ileal mucosa on day 28 and day 42 in comparison with the LAC and CL groups ($P < 0.05$).

Effects of treatments of *L. delbrueckii* and CTX on the SFB distribution in the mucosa of the ileum

The populations of total bacteria and SFB and the percentage of SFB are presented in Fig. 5a–c. In general, CTX treatment significantly decreased the numbers of total bacteria in the ileal mucosa, but has a minimal effect on the population of SFB, although the decline of SFB population was observed in all groups when the chickens aged. Moreover, the percentage of SFB in the ileal mucosa of all tested groups also showed in a time-dependent manner (Fig. 5b) that the peak time and size of SFB colonization in the ileal mucosa were significantly affected by the different treatments. For example, feeding lactobacilli significantly enhanced the SFB population in the ileum (Fig. 5c) and SFB reached the peak size in the LAC group on day 5. In fact, five of six birds in the LAC group on day 5 showed the positive colonization of SFB in the ileal mucosa. In contrast, the peak time of SFB in the ileal mucosa of the CTX group was postponed and the highest percentage of colonization (9.82% of total bacteria) occurred at the age of 13 days. In CL, the peak time for the colonization of SFB was on day 9. Additionally in all tested groups, population sizes of SFB started to decline from the age of 17 days.

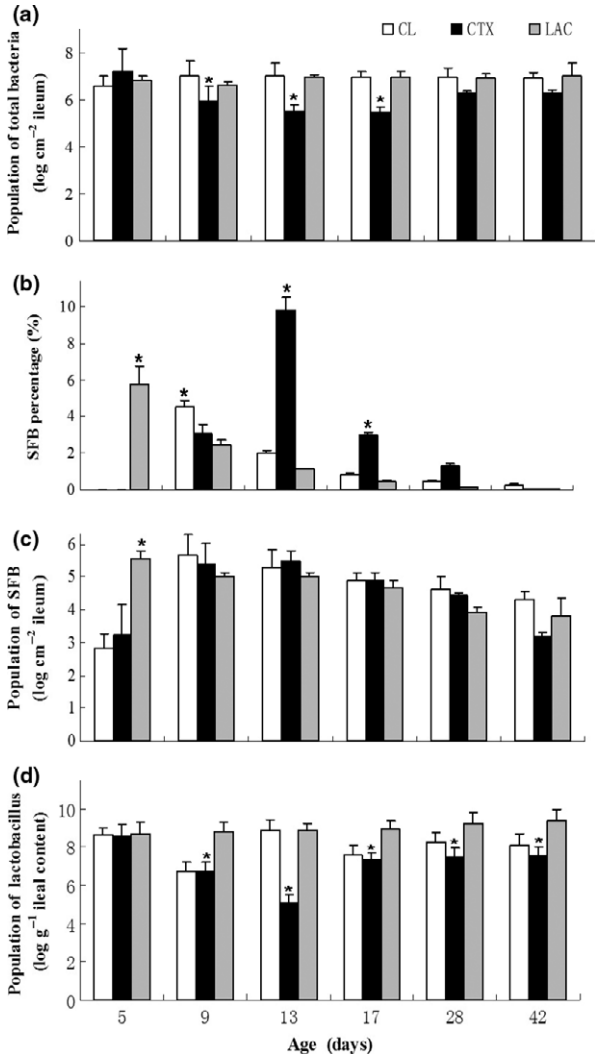

Fig. 5. Effects of feeding LAC and subcutaneously injecting CTX on the population of total bacteria (a), percentage of SFB in the total bacteria (b), population of SFB (c), and lactobacillus (d) in chicken ileal samples. LAC group, chickens were fed with *Lactobacillus delbrueckii* (1×10^8 CFU mL^{-1} per bird per day); in the CTX group, chickens were subcutaneously injected with CTX (100 mg kg^{-1} body weight on day 1, day 4, day 8, day 12, and day 16 and 50 mg kg^{-1} body weight on day 27 and day 41); CL group, chickens were selected as controls without any other treatments. Values represent mean ± SE from six chickens per groups measured by qPCR. Populations of SFB are expressed as the percentage of SFB to total bacteria, and * represents that the difference is significant ($P < 0.05$).

Populations of *Lactobacillus*

The total population of *Lactobacillus* in the ileal contents at each experimental time points was monitored by qPCR. In general, the level of lactobacillus population fluctuated along with the time points, but feeding *L. delbrueckii* enables the relatively stable maintenance of the total number of lactobacillus in the ileum. CTX treatment significantly decreased the number of lactobacillus, particularly on days 9 and 13 (Fig. 5d).

Discussion

SFB is an important colonic bacterium that has the ability to modulate the development of the host immune system. In the present study, the distribution and cell morphology of SFBs in chicken GI tracts were monitored by both qPCR and FISH methods, and the results demonstrated that the mucosa of terminal ileum is the primary site for SFB colonization. Subsequently, the colonization patterns in the chicken ileum are influenced by the factors including the host immune status and feeding additives. Feeding lactobacilli at birth rendered the first colonization 4 days earlier, while immunosuppression reagent CTX has little effect on the colonization of SFB. Instead, significant influence on the population of total bacteria is observed. According to the results presented in the current study, because SIgA production in the very young chicks has not been developed, there is no correlation between the SIgA productions and the SFB colonization in the early life stage of chicken. However when the endogenous IgA in the ileum of chicken increases significantly on day 28, the decline of SFB population is coincident with the increase in SIgA.

In concordance with previous studies, the varied cell morphology of SFB was observed in the different anatomic locations of the chicken GI tract. The typical filamentous shape was only detected in the chicken ileal mucosa, and the length was significantly reduced in the ileal digesta. Short rod was the major cell shape present in the digesta of the cecum, but mixed cell shapes including filamentous types and short rods were found in the digesta of the large intestine. Although SFB have still been unable to be cultivated *in vitro* to date, examination by a scanning electron microscope (SEM) and TEM suggested that there are three growth stages in the SFB life cycle, for example rod-shaped cells vacuolated with a head appearance in the first stage, a long and dense shape in the second stage, and spores in third stage (Sanford, 1991). In fact, short rod types of SFB were observed by FISH with a SFB-specific probe in the *in vitro* gut modeling system inoculated with human feces (Child *et al.*, 2006). Because chickens possess two ceca that form as blind sacs lying backwards along the terminal portion of the ileum, cecal villi present at the entrance of the chicken cecum form an interdigitating meshwork to exclusively allow fluid and fine particles to pass through (Duke, 1986), which leads to the longer retaining time for water-soluble materials occurring in the ceca (7–9 h) compared to the general transit time (4–5 h) for the large

particles that will bypass the ceca directly and excrete out (Vergara *et al.*, 1989). Hence, two possibilities may explain the difference in cell morphological shapes present in the cecum and large intestine. First, because of the longer retaining time associated with cecal digesta, SFB present in the ceca of chicken may have enough time to reach the growth stage of the short rod shape. Secondly, because of the mesh-like cecal villi present at the entrance of the ceca, only short rods will be allowed to enter the ceca.

The mucosa of the terminal ileum is considered as the primary site for SFB proliferation in chicken, which is evidenced by both FISH and qPCR detection. In concordance with previous studies, age plays a key factor in determining the SFB population, which is ascribed to immune system maturation along with aging (Koopman *et al.*, 1987; Sanford, 1991; Jiang *et al.*, 2001). In a mouse model, an increased number of SFB in the ileum was coincident with the gap period, posterior to weaning but anterior to the self-production of IgA, implying ileal IgA is the major factor in the intestinal tract affecting SFB localization (Ohashi *et al.*, 2006). Newly hatched chicks, as the neonatal equivalent of mammals, experience a critical transit period from innate and maternal immunity to endogenous adaptive immunity (Lammers *et al.*, 2010). The major protection of dams, unlike in mammals from milk, is derived from yolk, where IgY is the predominant Ig isotype transferred from the dam to her offspring. There is IgA detected in the yolk; however, the concentration of IgA in the yolk is 10–100 times lower than that of IgY (Hamal *et al.*, 2006). Previous studies reported that the endogenous IgA in the ileum of chicken increases significantly at the age of 21 days, which is in agreement with our current observations (Kaspers *et al.*, 1996; Hamal *et al.*, 2006; Lammers *et al.*, 2010). In the current study, the overall decline of SFB occurs at the age of 28 days, which is coincident with the increase in ileal SIgA, also suggesting that self-producing IgA in chickens could play an important role in the regulation of SFB colonization. However, because very young chicks produce little SIgA in the first 2 weeks, the fluctuation in the SFB proportion of total bacteria is not because of the change in SIgA concentration. In fact, other host physiological or environmental factors may also influence the SFB colonization in the ileum of chicken at early life stage.

CTX is reported to cause the depletion of B lymphocytes and suppresses humoral immunity (Corrier *et al.*, 1991). In addition, it temporarily affects the T-cell activity following the administration of high dose (Rouse & Szenberg, 1974), which is also evidenced in the current study by lowering SIgA concentration and lowering the ratio of CD4+ to CD8+ in the CTX-treated group. In the previous study of immunosuppression mouse model caused by CTX treatment, the proportion of SFBs in the ileal mucosa increase, which is proposed as a result from the decrease in the concentration of ileal SIgA (Jiang *et al.*, 2001; Suzuki *et al.*, 2004; Ohashi *et al.*, 2006). In contrast for the very young chicks, the SFB colonization is not affected by CTX treatment through SIgA production. Instead, we found that the immunosuppression status caused by the CTX treatment significantly affects the bacterial populations, which is evidenced by the decline of total bacterial population and Lactobacilla population (Fig. 5a and d). As a result, the decrease in total bacterial population causes the rise in the percentage of SFB in the ileal samples of CTX treatment group (Fig. 5b). Indeed, there is no significant change in the number of SFB in the ileal mucosa of CTX group (Fig. 5c). Likewise, pathogens such as salmonella invade the host through the M cell in PP and mainly cause the cell-mediated response, and CTX treatment has little effect on the population of salmonella in the ceca of chicken in the first 2 weeks of life (Corrier *et al.*, 1991).

In the present study, the effect of oral administration of *L. delbrueckii* on the colonization patterns of ileum SFB was interesting. There was no marked fluctuation in the host immune status after constantly feeding Lactobacilli, which was in line with previous report (Pouwels *et al.*, 1996; Sato *et al.*, 2009); however, oral feeding of *L. delbrueckii* after hatching significantly rendered the first colonization of SFB 4 days earlier. Previous studies showed that oral administration of *L. plantarum* to mice led to a significant decline of indigenous populations of SFB (Fuentes *et al.*, 2008), which was not consistent with our current observation. Given the fact that SFB colonization in the chicken ileum is age dependent, sampling times become a crucial factor affecting the detection. The discrepancy between current results and previous studies could be ascribed to the different sampling times, although the difference between the lactobacilli strains in the ability of immune modulation may also contribute. On the other hand, Ivanov *et al.* (2009) found that *L. murinus* and SFB were the only two phylotypes with the most abundant populations co-existing in Taconic mice, suggesting that a potential symbiosis may occur between these two taxa . It is also highly likely that feeding *L. delbrueckii* to chicken helps to create an anaerobic environment that seems to facilitate the SFB colonization. Obviously, future study is needed to explore the detailed relationship between the SFB localization and the presence of *Lactobacillus*.

In summary, current study demonstrates that the colonization of SFB in the very young chickens is influenced by multiple factors including the host immune status and the feed additives. Our results suggest that the colonization of SFB in the chickens can be manipulated from

birth via feeding *Lactobacillus*, which implicates a new potential beneficial effect from probiotics by promoting SFB colonization.

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Authors' contribution

N.L. and Y.Y. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effects of feeding LAC and subcutaneous injection of CTX on chicken body weights.
Table S1. The composition of diet used in this study.
Table S2. Comparison of SFB population in the ileal digesta of chickens using FISH and qPCR.

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