The ISME Journal (2010) 4, 367–376

www.nature.com/ismej

ORIGINAL ARTICLE

Exposure of different bacterial inocula to newborn chicken affects gut microbiota development and ileum gene expression

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The transition from a sterile gut environment to the development of microbiota in the newborns is not fully understood. The objective of this study was to investigate the impact of exposure to bacterial communities on the development of gut microbiota in the newly hatched chicken. A total of 90 as-hatched chicks were divided into three groups. Groups A and B were treated with inocula of the cecal origin, whereas group C was fed with sterile water. The major bacteria in Inoculum-I to treat group A included *Bacteroides* (20.7%), *Lachnospiraceae* (17.2%) and unclassified *Ruminococcaceae* (16.1%), whereas group B was introduced with Inoculum-II composed of *Prevotella* (37.9%), *Acidaminococcus* (16.1%) and *Dorea* (12.6%). Analyses of the ileal and cecal contents over a period of 15 days showed that Inoculum-I resulted in a higher rate of colonization than Inoculum-II, but the colonization was predominantly in the cecum. The influence of Inoculum-II on group B was similar to that of water on group C, showing only a marginal effect on colonization. Microarray analysis showed that each group presented a distinct pattern of gene expression in the ileum. In group A, the most obvious changes were noted in genes controlling the function of ion transport, cell cycle and chromosome maintenance, suggesting that the inocula influenced gene expression. Our findings indicate that initial exposure to different bacterial communities could lead to the development of distinct microbiota and gene expression in the gut. It is possible to manipulate the gut microbiota by feeding to a proper bacterial composition at an early age.

The ISME Journal (2010) **4,** 367–376; doi:10.1038/ismej.2009.128; published online 3 December 2009
Subject Category: microbe-microbe and microbe-host interactions
Keywords: microbiota; chicken; succession; intestine; chemostat

Introduction

The intestine of vertebrates is colonized by an abundant and diverse community of bacteria. These bacteria influence the health of animals through their effects on gut morphology, nutrition, pathogenesis and immune responses (Metchnikoff, 1903; Mead, 2000). It is also well established that a given intestinal environment is associated with a specific component of microbial species. For example, in the cecum, the predominant bacteria are obligate anaerobes including *Clostridiaceae*, *Fusobacterium*, *Lactobacillus* and *Bacteroides*, whereas facultative and microaerophilic bacteria such as *Lactobacillus, Streptococcus* and *Enterococcus* comprise the consortium of the ileum (Moran, 1982; Barrow, 1992; Csordas, 1995; Lu *et al.*, 2003). The alimentary tract of animals before birth is considered a sterile environment. Microorganisms from the maternal body and the surrounding environment rapidly gain access to the intestinal tract of the newborns on birth. The factors that determine the colonization rate and composition of the intestinal microbial species are not fully understood. Recent studies showed that there is no core microbiota shared by all individuals in humans, indicating that environmental exposure at an early stage of life may have an important role in shaping the intestinal microbiota (Thompson *et al.*, 2008; Turnbaugh *et al.*, 2009).

Intestinal bacterial colonization at a very early stage of life was thought to be a random process that takes several weeks to stabilize. All environmental

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Received 10 September 2009; revised 27 October 2009; accepted 29 October 2009; published online 3 December 2009

microorganisms, including commensals and pathogens, would have equal opportunities to colonize the gut during this period of time (Thompson *et al.*, 2008). To prevent pathogenic bacteria such as *Salmonella* and *Campylobacter* from colonizing, commercial competitive-exclusion products have been developed for the neonatal animals (Pivnick and Nurmi, 1982; Schneitz, 2005). These products are composed of a complex of microbial species rather than a simple mixture of a few species (Stavric and D'Aoust, 1993), and made as an undefined bacterial culture by a chemostat system in which a single cecum of the chicken was used as the source of the inoculation (Revolledo *et al.*, 2003; Schneitz, 2005). The compositions of bacterial species in these products are not constant because of the various compositions of the cecal microbiota, and thus inconsistent efficacy has been reported (Ferreira *et al.*, 2003; Schneitz, 2005). Therefore, it is desirable to understand the correlation between the compositions in the inocula to the subsequent colonization rate in the chicken gut, and the impact of inocula on the dynamic changes in the development of gut microbiota with regard to age.

The interaction between the intestinal microorganisms and host gene expression is crucial for the development of the immune system (Mazmanian *et al.*, 2005). It is well documented that early bacterial colonization patterns are associated with antibody production in the gut (Suzuki *et al.*, 2007), and that the variation of microbiota modestly influences the host gene expression (Rawls *et al.*, 2006). However, there is little information regarding interaction between the host gene expression and the structure of gut microbiota. In this study, the impact of environmental factors on the development of intestinal microbiota was assessed by the comparison of colonization rates in the ilea and ceca of as-hatched chickens fed with two inocula of different bacterial compositions. The effects of the inocula on the host responses were evaluated by comparison of gene expression profiles in the ileum. Our findings suggest that an early exposure to different environments, together with the host physiology, has a direct impact on the development of gut microbiota and gene expression in the newly hatched chicken.

Materials and methods

Animals and experimental design

The schematic diagram of the experimental design is shown in Supplementary Figure S1. A total of 90 as-hatched chicks (day 0, commercial lines of Hubbard) were obtained from a local hatchery (Zhejiang Zhenda Broilers Co. Ltd, Hangzhou, China) and randomly allotted to three cages. Birds in group A were inoculated with 0.2 ml of chemostat product 1 (Inoculum-I) by oral gavage on day 0 (within 4 h after hatch), and chemostat product 2 (Inoculum-II) was fed to group B. A volume of 0.2 ml of sterilized distilled water was used as a control to feed group C. All birds were fed maize and a soybean-based starter diet, according to the Feeding Standards of Chickens in China (NY-T 33-2004), throughout the duration of the experiment. The chicks were brooded in steel wire cages with housing conditions as described by Wang *et al.* (2008). No antibiotics or commercial vaccines were applied in the experiment.

All the animals used in this experiment were handled in strict compliance with the current regulations and guidelines concerning the use of laboratory animals in China, and approved by the Laboratory Animal Care and Usage Committee of Zhejiang Academy of Agricultural Sciences.

Sample collection

Five chickens were randomly selected, weighed, anesthetized with ether and killed by cervical dislocation on days 1, 2, 5, 7 and 15 post inoculation. A piece of ileum was removed from a position 1.0 cm away from the ileocecal junction and immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction. The rest of the ileum was opened and mucosal samples were scraped off using microscopic slides and then frozen immediately in liquid nitrogen. The cecal contents were squeezed into Eppendorf tubes and kept at −80 °C for bacterial genomic DNA extraction.

Chemostat system

A single-stage chemostat system, which is similar to the model described by Gibson and Wang (1994), was used to generate the inocula for this study. Temperature (37 °C) and pH (6.2) were controlled automatically. The systems were anaerobically maintained by continuously sparging with O_2-free N_2 into the medium reservoir and working vessel. The growth medium was Viande Levure broth (Genovese *et al.*, 2003), which contained (g l^{-1}): beef extract 2.4, tryptose 10, L-cysteine 0.6, glucose 2.5, yeast extract 5.0 and NaCl 5.0.

Two 90-day-old chickens hatched by hens in two separate villages were randomly collected and their cecal contents were used as the donor for chemostat inoculation. A portion of 10% slurries was made by homogenizing the cecal contents individually with anaerobic phosphate-buffered saline. The large particles were removed by passing through a 2 mm sieve twice before inoculation. The system was allowed to equilibrate for 16 h before the medium started to flow into the working vessel by the peristaltic pump. In this experiment, the system was operated with a retention time of 24 h, and ran at least 168 h to establish the steady-state condition. The steady-state condition was achieved only when consistent patterns of PCR-DGGE profiling were obtained from four individual samples collected at

24 h intervals. Then, the bacterial cells were collected from chemostat systems and cell pellets lyophilized. The final inocula used for chicks were obtained by resuspending the lyophilized culture with anaerobic PBS to make the final concentration of 10^8 CFU per ml.

DNA extraction, purification and PCR-DGGE analysis
DNA from samples of ileum mucosa, cecal contents and inocula was extracted as previously described (Leser *et al.*, 2000). PCR amplification, DGGE electrophoresis of PCR amplicons and visualization of DNA bands were conducted as previously described (Muyzer, 1999; Holben *et al.*, 2004). Primers 341f (5′-ATTACCGCGGCTGCTGG-3′) and 534r with GC clamps (5′-CGCCCGCCGCGCGCGGCG GGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGC AGCAG-3′) against the V3 region of the 16S rRNA genes (position 339–539 in the *E. coli* gene) were used. The similarity of PCR-DGGE profiling was analyzed using Quantity One software with a match tolerance of 2% (Version 4.6.1; BIO-RAD Laboratories Inc., Hercules, CA, USA). Principal component analysis (PCA) was performed on the band-matching matrix and Dice's similarity coefficient was calculated as previously described (Thompson *et al.*, 2008).

16S rRNA clone library analysis
For PCR-DGGE profiling analysis, DNA samples from individual chickens were used. In contrast, for 16S rRNA clone library analysis, pools of DNA from the ileum mucosa and cecal contents were made by adding equal amounts of DNA of three chickens from each group. 16S rRNA genes from the inoculum samples, cecal and ileum contents were amplified with universal primers 27f (5′-AGAGTTT GATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTG TTACGACTT-3′) as previously described (Lane, 1991). The PCR conditions, as well as production of 16S rRNA clone libraries and DNA sequencing, were conducted as described in Supplementary Materials and Methods. The DNA sequences that have a low homology (<95%) were submitted to the National Center for Biotechnology Information (NCBI) (accession number FJ795048–FJ795335). The unique sequences were further parsed into the analysis of operational taxonomic units (OTUs) on the basis of their genetic distance in a neighbor-joining tree using the DOTUR program (Schloss and Handelsman, 2005) with a similarity threshold of 98%. CLASSIFIER and LIBCOMPARE were applied for the identification of taxa and comparison between libraries, respectively. Library coverage was calculated as described previously (Good, 1953; Singleton *et al.*, 2001). Correlation coefficients between each library were constructed using SPSS (version 13.0) based on the number of OTUs and abundance of each OTUs.

Microarray assay and real-time PCR confirmation
The procedure of RNA isolation, double-stranded cDNA synthesis and cDNA labeling was performed after the suggestion of NimbleGen's protocol (NimbleChipTM Arrays User's Guide Gene Expression Analysis, http://www.nimblegen.com). A total of 55 366 sequences, including 4126 Refseq genes, Ensembl-predicted genes, Genscan-predicted genes and 3259 random probes, were spotted on the microarray chips. A total of 15 microarrays were used and each microarray had two replicate blocks. The procedures of prehybridization, hybridization, post-hybridization washing-up, scanning and data analysis were conducted according to the manufacturer's instructions and are provided in http://www.nimblegen.com. All microarray data have been submitted into the NCBI Gene Expression Omnibus (GEO) website (http://www.ncbi.nlm.nih.gov/geo/). The accession numbers are GSE15012 and GPL8231. Each of the 15 slides has been submitted, and the accession numbers range from GSM375477 to GSM375491.

The QT Model was applied to identify differentially expressed genes between the treatment group and age (Yang *et al.*, 2009, http://ibi.zju.edu.cn/software/qtmodel), and PCA analysis (Acuity 4.0) was applied for the clustering of gene expression. The functional annotation of the biological processes involving differentially expressed genes was carried out using Web Gene Ontology Annotation Plotting (WEGO, http://wego.genomics.org.cn/cgi-bin/wego/).

Fourteen genes, including ACTB, CCR2, CD36, FABP4, IFNG, IL10, IL4, IL8, MYD88, STAT3, TLR2, TMEM18, BRIP1, HSP70 and KCTD7, showing differential expression between test groups A, B and C during microarray analysis were selected for validation by real-time PCR. The primers and operation procedures are listed in Supplemental Materials and Methods and Supplementary Table S4. β-Actin was chosen as the reference gene. Results (fold changes) were expressed as $2^{-\Delta\Delta C_t}$ methods (Livak and Schmittgen, 2001).

Results

Preparation of inocula with different composition by growth of chicken gut contents in chemostat
Through DGGE analysis of the gut contents from adult chickens, we noticed that geographic location is an important determinant of microbiota in the chicken gut because similar patterns of bacteria seem to be shared among the animals of the same farm (Supplementary Figure S2). We chose to evaluate the impact of the exposure to different environments on the development of gut microbiota in the newly hatched chicken. Inocula with different compositions were used to simulate exposure to different environments. As the contents from a single cecum did not provide enough sample size for inoculation, two chemostat systems were

combined to produce a large quantity of stabilized inocula. The cecal contents of two individual adult chickens served as a starter culture to inoculate the two separate chemostats with the same growth media. At the steady state, the bacterial composition of these chemostat products were significantly different (Supplementary Figure S3), although the bacterial diversity, as manifested by OTU numbers, library coverage and Shannon index, showed no difference between the two libraries (Supplementary Table S1). The major phyla in the two inocula, which were distributed evenly, included *Bacteroidetes*, *Firmicutes* and *Proteobacteria* (33.3, 62.1 and 4.6% in Inoculum-I, and 39.1, 59.8 and 1.1% in Inoculum-II, respectively). However, the percentage of certain species was different between the two populations of chemostat, as measured by the percentage of each species in the library. These species were *Bacteroides fragilis* (20.7% in Inoculum-I versus 1.1% in Inoculum-II), *Prevotella albensis* (3.4% in Inoculum-I versus 37.9% in Inoculum-II), *Acidaminococcus* (0% in Inoculum-I versus 16.1% in Inoculum-II) and *Dorea* (0% in Inoculum-I versus 12.6% in Inoculum-II). The other major phylogenetic group in Inoculum-I that is remarkably different from Inoculum-II was *Lachnospiraceae Incertae Sedis*. *Subdoligranulum* showed equal populations in both inocula.

Inocula with different compositions influence the development of gut microbiota in the newly hatched chicken

As the two inocula showed a significant difference in bacterial composition (Supplementary Figure S3), we next investigated the effect of the inocula on the dynamic changes of bacterial community structures in the ileum and cecum of as-hatched chickens. Three groups of as-hatched chickens were inoculated with Inoculum-I (group A), Inoculum-II (group B) and sterile water (group C). At different time points, the ileal and cecal contents were collected and analyzed by PCR-DGGE band profiling and 16S rRNA sequence analysis (Supplementary Figure S4). In general, the community fingerprint similarity between individuals within a test group was significantly higher than among inter-groups (Supplementary Table S2). The community dynamics were further visualized by PCA analysis (Figure 1). Band profiles in the ilea of groups A, B and C were individually clustered but randomly distributed during the course of the experiment. In contrast, cecal patterns showed a relative stability over the time course, of which group A distanced from groups B and C and eventually groups B and C merged on day 15. These results suggest that initial inoculation has a determining role in shaping the gut microbiota profiles at a later stage of life; however, such impact on the ilea and ceca were different.

The effect of inocula on gut microbiota structures was also analyzed by 16S rRNA sequences, and then SPSS software (Version 13.0, IBM, New York, NY, USA) was applied to calculate the correlation coefficients. As shown in Table 1, the coefficients between inocula to ilea were higher than to ceca on day 1. Furthermore, Inoculum-I showed higher coefficients to the gut microbiota than did Inoculum-II, for example, 0.441 in the ileum on day 1, and 0.370 in the cecum on day 15 between Inoculum-I and group A. In comparison, we noted 0.125 in the ileum, and 0.077 in the cecum between Inoculum-II and group B at the same time point. Those results suggest that Inoculum-I resulted in a higher colonization rate than did Inoculum-II, particularly in the cecum. In addition, group B had a higher correlation coefficient than group C on day 15, indicating that groups B and C shared a large number of the same sequences, which was in agreement with the results of PCR-DGGE profiling.

The phylogenetic analysis of the bacteria composition in the ilea and ceca at each time point is shown in Figure 2. The predominant bacterial groups belong to *Enterobacteriaceae, Enterococcaceae*, *Prevotellaceae*, *Bacteroides*, *Lactobacillus* and *Clostridiales*. The major groups of bacteria in the ileum continuously shifted with an increase of the bird age, which is consistent with the changes of PCR-DGGE profiling patterns. On day 1, diversified *Bacteroides* and *Clostridiales* were identified in the ilea of groups A and C, whereas a high number of *Lactobacillus* was exclusively detected in group B. In all groups, *Enterococcus* and *Lactobacillus* emerged as the prevalent species on day 7, followed by *Bacteroides, Dialister* and *Porphyromonadaceae* on day 15. However, in ceca, the structures of microbiota were more stable. *Enterococcaceae* and *Enterobacteriaceae* were found in all groups on day 1, but particularly a higher number was associated with group C. *Bacteroides, Lachnospiraceae Incertae Sedis*, unclassified *Lachnospiracease* and unclassified *Ruminococcaecae* became the main phylogenic groups on days 7 and 15.

Inoculations with different bacterial community result in variations in gene expression of ilea

As different compositions of inocula affect the development of gut microbiota, we investigated whether the host gene expression can be influenced by different microbiotas. The ileum samples of these three groups of chickens were collected for microarray analysis. A total of 52 107 genes were included in microarray, in which 4126 belonged to the reference sequence genes and 47 981 belonged to the predicted genes. Among them, 86% of the reference genes and 41% of the predicted genes had expression values over three times higher than the background at each time point. The reliability of the assay was evaluated on the basis of the correlation coefficients of the absolute intensity

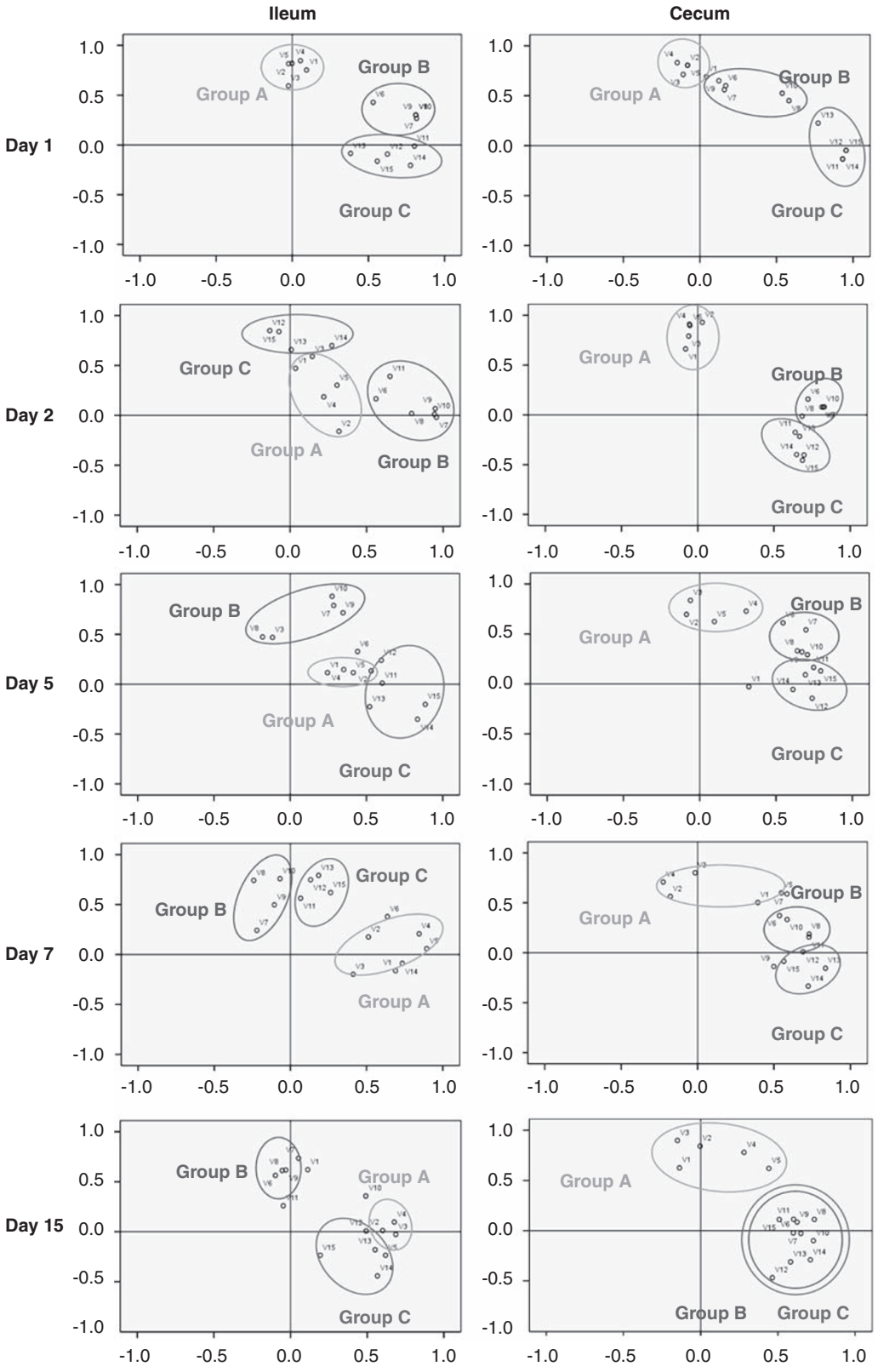

Figure 1 Principal component analysis (PCA) of PCR-DGGE profiling of ileal mucosa and cecal contents collected on days 1, 2, 5, 7 and 15. Three groups of as-hatched chickens were inoculated with chemostat-grown bacteria (2×10^7 CFU per chick). Groups A and B were inoculated with inocula I and II, respectively, on day 0. Distilled water was fed to group C to serve as the control. Ileal mucosa and cecal contents from five birds in each group per time point were collected for PCR-DGGE analysis. *X* axis represents component 1 and *Y* axis represents component 2.

Table 1 Correlation coefficients of 16S rRNA sequences of microbiota from ileum and cecum contents[a]

Site	*Age (days)*	*Correlation coefficients*[b]				
		Ino-I[c]*:A*[d]	*Ino-II*[e]*:B*[f]	*A:B*	*A:C*[g]	*B:C*
Ileum[h]	1	0.441	0.125	0.257	0.360	0.415
	7	0.235	0.238	0.844	0.018	0.165
	15	0.062	0.128	0.719	0.955	0.803
Cecum[h]	1	0.168	0.139	0.181	0.018	0.295
	7	0.122	0.127	0.416	0.283	0.852
	15	0.370	0.077	0.151	0.249	0.955

[a]Sequence with 98% similarity is regarded as the same OUT.
[b]Correlation coefficients were measured by proximities of SPSS.
[c]Represents Inoculum-I.
[d]Represents group A.
[e]Represents Inoculum-II.
[f]Represents group B.
[g]Represents group C.
[h]Pools of DNA, which were made by adding equal amounts of DNA extracted from three chickens in the same group.

between the intra-replicate blocks and the duplicate chips. The correlation coefficients were 0.97 for intra-replicate and 0.98 for duplicate chips. In general, the average log value of absolute intensities from the reference sequence genes was 10.89, compared with 7.38 from the predicted genes, indicating that there is a higher level of expression from the reference genes than from the predicted genes.

The QT Model analysis was applied to compare the difference of gene expression intensities with $P<0.05$ and FDR<0.05 as the cutoff value. Comparison of the different treatment groups, 4.4% (181 out of 4126) of reference genes and 2.4% (1132 out of 47 981) of predicted genes showed significantly different expression. The gene expression between group A to group C and group B to group C was different ($P<0.05$, comparison algorithm is described in Supplementary Materials and Methods), with 75 genes upregulated and 75 genes downregulated between group A and group C; 71 genes were upregulated and 51 were downregulated between group B and group C. A two-way hierarchical cluster analysis (HCA) was applied to the normalized data of reference genes. With a total of 1312 sequences that were detected with significantly differential expression, we saw three different expression patterns (Figure 3a). This was also noted with the known genes subset (Figure 3b). Furthermore, the reference sequence genes clearly separated all groups on day 1 and group A was distanced from groups B and C on days 7 and 15 (Figure 3c). Together, the HCA patterns and PCA results suggest that characteristic profiles of gene expression occurred in group A, particularly on days 7 and 15.

The functional annotation of 181 genes with different expression values between treatment groups was subsequently analyzed by the gene ontology (GO) program provided by WEGO. Only over-represented GO terms, which were designated as low *P*-value ($P<0.05$) with at least three genes associated with the term, are listed in Supplementary Table S3. The GO terms that were up- or downregulated in both groups A and B included the nucleus, ATP binding, integral to membrane and intracellular membrane-bounded organelle. Upregulated genes included cation and ion transmembrane transport and the G-protein-coupled receptor protein signaling pathway, whereas downregulated genes included chromosome organization, cytoskeleton, DNA repairs, cell cycles, organelle organization and post-translational protein modification. There were more GO terms associated with group A compared with group B (Supplementary Table S3), indicating again that host gene expressions were influenced by different inocula.

The upregulated genes in groups A and B included mitochondrial carrier protein (*SLC25A14*), monocarboxylate transporter (*SLC16A8*), positive ion transport (*KCNJ2*, *KCTD7* and *CNGA3*), G-protein-coupled receptors (*GPR126*, *GPR34*, *XCR1*, *EDG7*), steroid receptors (*GILZ*, *NR0B1*), frizzled receptors and Wnt signaling (*FZD4*) and cytokine receptors (*GHR*, *CRHR2*, *TNFAIP1*, *NFIL3*). Furthermore, genes (*CYP1A1*, *CYP1A4*) in the cytochrome P450 superfamily and heme oxygenase 1 (*HMOX1*) were also upregulated.

Downregulated genes in groups A and B included genes involved in cell division, such as chromosome segregation (*SMC2*, *SMC3*, *ZW10*), chromosome structure maintenance and transcriptional regulation (*MCM5*, *HDAC7A*, *SAP130*), alteration of RNA secondary structure (*DDX42*, *DDX27*, *DDX193*, *BRCA1*, *BRIP1*), DNA double-strand break repair (*ESF1P*), biogenesis of 18S rRNA (*ESF1P*) and microtubule stabilization (*NMSAP*, *KIF4A*, *KIF1*, *KTN*).

To validate the microarray results, we performed quantitative RT–PCR for 14 genes, and the results showed a positive correlation with the microarray data (Supplementary Figure S5).

Discussion

In a sterile neonatal gut, colonization by the environmental microbiota is the first step in the establishment of a diverse bacterial community. This study has shown that compositions of inocula significantly affect the structure of intestinal microbiota, and that gut anatomy can also influence the colonization rates. In this study, we used newly hatched chickens to evaluate the impact of different environments on the development of microbiota. One advantage of using the chicken as a model to study microbiota of newborns is the relatively negligible influence from the maternal microbiota. With the two inocula generated by the chemostat growth of chicken cecal contents, Inoculum-I showed a higher ability of colonization, but only in the cecum. In contrast, the lower rate in the colonization of inoculum-II has resulted in the profiles of cecal microbiota in group B being more

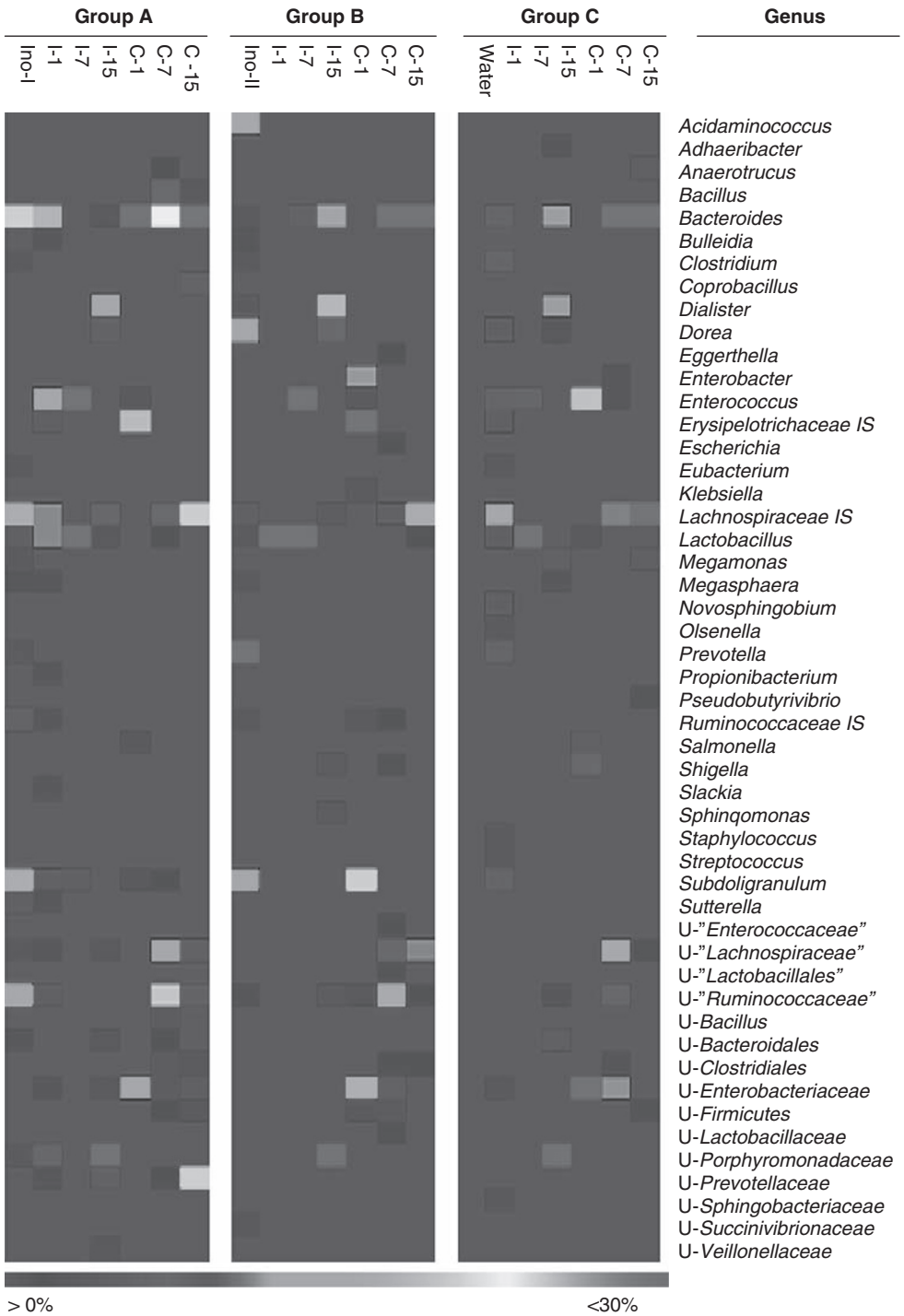

Figure 2 Relative abundances of taxonomic groups represented by 16S rRNA gene sequences from each group. The heat map shows the relative abundances of operational taxonomic units (OTUs) from each sample. Color intensity for all panels is the percentage (0–30%) of OTUs in each library. Each column represents one sample. Ino-I, Inoculum-I; Ino-II, Inoculum-II; I-1, I-7, I-15, ileum mucosa collected on days 1, 7 and 15, respectively; C-1, C-7, C-15, cecal contents collected on days 1, 7 and 15, respectively. Each row represents the alphabetically arranged genus. Sequences classified into family and order taxonomic level are listed at the bottom of the heat map. In the genus names, U represents unclassified and IS represents incertae sedis. A full colour version of this figure is available at *The ISME Journal* online.

similar to the control group. These results suggest that bacterial succession in the gut at a very early stage of life can be influenced by the bacterial composition of inocula and that host also exerts a selective pressure in the process of colonization.

Turnbaugh *et al*. (2009) reported previously that no core microbiota is shared by all individuals. This finding is in general agreement with our current results. Two chickens randomly selected from separate geographic areas possessed different

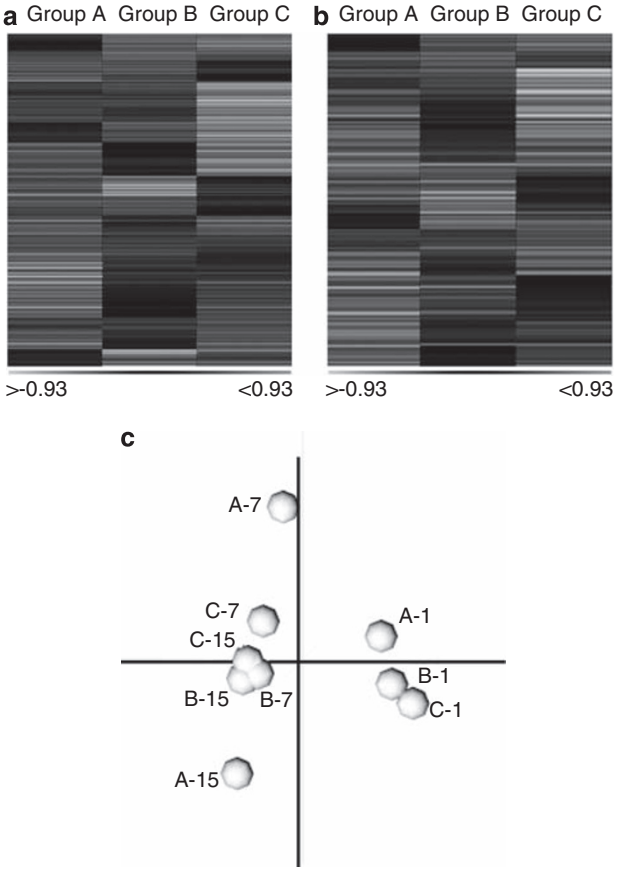

Figure 3 Profiles of gene expression of ileum samples from groups A, B and C. (**a**, **b**) Cluster image of differentially expressed reference genes among groups A, B and C. Genes expressed at higher levels are represented as red, lower levels as green. A, for 1312 sequences spotted on microarray, including known and predicted genes; B, 181 known genes. (**c**) The principal component analysis (PCA) of the gene expression profiles of reference genes. A-1, A-7, A-15 are group A on days 1, 7 and 15, respectively; B-1, B-7, B-15 are group B on days 1, 7 and 15, respectively; C-1, C-7, C-15 are group C on days 1, 7 and 15, respectively. *X* axis represents component 1 and the *Y* axis represents component 2. A full colour version of this figure is available at *The ISME Journal* online.

structures of microbiota in the cecum with less than 20% of similarities at the genus level, as evaluated by both 16S rRNA clone sequences and pyrosequencing (Lei F & Wang X, unpublished data). Owing to the marked difference of microbiota between individual chickens, we chose to use chemostat to enrich the gut microbiota in this study. The chemostat system used here produced two inocula with different compositions. Although the chemostat products only represent 13–17% at the genus level and about 7% at the species level, the predominant groups of bacteria of the cecum are preserved (Lei F & Wang X, unpublished data).

Previous studies used both culture-dependent and -independent methods to show an increase in the diversity of the bacterial community in the ileum and cecum as a bird ages (Knarreborg *et al.*, 2002; van der Wielen *et al.*, 2002; Hume *et al.*, 2003; Gong *et al.*, 2008). Our data agree with these observations. A higher similarity index between the inocula to the ilea on day 1 indicates that bacteria colonized the ileum after oral administration, and then transited and proliferated in the cecum with the onset of time. This has been evidenced by a higher correlation coefficient between the inocula and cecum on day 15 (Table 1). As we can only detect the predominant groups of bacteria from a mixed community by the techniques used in this study, the lower similarity index between the inocula to cecum on days 1 and 7 could be partially attributed to the populations below the detection level. In addition, the different dynamic patterns of microbiota in the ilea and ceca suggest that the two anatomic regions have distinct bacterial community structures and can exert different selective processes for bacterial colonization. Inoculum-I has a higher colonization rate, as evidenced by the high similarity index in the cecum on day 15 and a relatively weak colonization rate in the ileum. As the inocula used in the current experiments were derived from the cecal contents, it is tempting to hypothesize that to obtain the higher colonization rate in the ileum, the ileal contents should be used as inocula.

In our study, the different colonization rates of two inocula were apparently attributed to the bacterial composition in the inocula. The gut physiology seems to favor the bacterial composition of Inoculum-I over Inoculum-II. *Bacteroides*, *Lachnospiraceae* and *Ruminococcaceae* in Inoculum-I were the most prevalent bacteria in the chicken ceca, which were similar to what were described in previous studies (Zhu *et al.*, 2002; Lu *et al.*, 2003). However, a recent report states that *Prevotella* was also one of the predominant groups (Callaway *et al.*, 2009). It is unclear why the high percentage of *Prevotella* in Inoculum-II could not lead to a high rate of colonization in group B. The unusually high population of *Acidaminococcus* in Inoculum-II, which was only detected in the gut of ruminants and associated with peptides and amino acids utilization, may contribute to the low colonization rate. Further study will be conducted to investigate the mechanism of different colonization rates exerted by *Bacteroides* and *Prevotella*. The exclusive population of *Lactobacillus* detected in the ileum of group B on day 1 is most likely because of the impact generated by the bacterial composition of Inoculum-II. The fluctuation in the numbers of *Lactobacillus*, particularly in the ileum, has been previously reported to be sensitive to the environmental stimuli and strongly influenced by diet components (Lu *et al.*, 2003; Wise and Siragusa, 2007). Whether these conditions are similar to those used in this study needs to be further investigated.

From the gene expression data, a total of 1312 genes showed differential expression between treatment groups, most of them annotated as 'unknown function', and only 181 genes have been assigned into GO terms. Compared with the control group C, genes having functions related to ion transport were

the most upregulated in groups A and B, suggesting that there is a possible mechanism of the gene regulation. Alteration of the gut microbiota may directly influence the production of short-chain fatty acid in the gut, and later can led to a more acidified environment to accelerate ion absorption (Cummings 1984; Binder and Mehta 1989). Indeed, the intake of probiotics in feed can enhance calcium absorption by increasing the gene expression of monocarboxylate transport 1 and chloride channel calcium activated 5 (Shima *et al.*, 2008).

It was previously reported that sodium butyrate induces cell cycle arrest in MDBK cell and blocks DNA replication (Li and Elsasser, 2005). Genes associated with cyclins, cylin-dependent kinases, histone deacetylase, helicases, chromosomal structure proteins and kinesins are downregulated in MDBK cells after treatment with sodium butyrate (Li and Li, 2006). That is consistent with our results. In this study, a large number of downregulated genes in groups A and B are related to the cell cycle and transcription regulations, including the process of chromosome segregation, alteration of RNA secondary structure, transcriptional regulation, DNA double-strand break repair, chromosome structure maintenance and so on. Several genes, such as *MCM5*, *HDAC7A* and *SAP130*, observed in our study are identical to those downregulated genes in the previous work (Li and Li, 2006), confirming again that short-chain fatty acids produced by gut microbiota influence the host gene expression. Surprisingly, during the analysis of our microarray data, we did not find a significant number of differentially expressed genes related to innate immunity. One possible explanation could be the fact that our inocula are derived from the cecal contents of adult chicken, which contain a large number of commensal bacteria. As ileum is considered as the primary site where the gut mucosal immunity takes place (Lillehoj and Trout, 1996), it was chosen to examine the potential beneficial effects of different bacterial inocula. However, the lack of the host gene expression data from the cecum somewhat limits the impact of this study. However, the ileum gene expression pattern is consistent with bacterial colonization patterns in the ceca of groups B and C (Figures 1 and 3c). This supports that Inoculum-I, unlike Inoculum-II that is slightly more effective than the water control in regulating gene expression in the ileum, is superior in promoting gut colonization and shaping microbiota development.

In conclusion, this study shows that the succession of the microbiota community in the animal intestine is a process that was strongly affected by the bacterial compositions of the inocula and gut physiological habitat. The influence of bacterial inoculations at a very early stage of life can be correlated with the host gene expression, although on a limited scale. Therefore, as the intestinal microbiota structure of individuals are related to disease vulnerability and the harvest of energy, it is possible to use an artificial inocula with the properly tailored bacterial composition to manipulate the gut microbiota of young animals.

Acknowledgements

This research was supported by the Science and Technology Department of Zhejiang Province through the Agricultural Privilege Project (2006C22053) as well as the Zhejiang Academy of Agricultural Science through the Cooperation Project. The collection of chickens from commercial chicken farms was made possible by the support of Mr Weiwu Zhang, the Hangzhou Academy of Agricultural Sciences. QT model analysis for microarray data was performed by Dr LongJiang Fan and Ms Chengyin Ye from Zhejiang University. We also thank Qun Fang, TingWu Yang, Weilan Wang and Xi Yang for technique and bio-information support. Finally, we thank Dr Hongwei Yu and Mr Ryan Withers from Marshall University for editing this paper.

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