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Spatial and Temporal Variation of the Intestinal Bacterial Community in Commercially Raised Broiler Chickens During Growth

P.W.J.J. van der Wielen, D.A. Keuzenkamp, L.J.A. Lipman, F. van Knapen, S. Biesterveld*

Centre for Veterinary Public Health and Environmental Protection, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 2, 3508 TD Utrecht, The Netherlands

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ABSTRACT

The objective of this study was to determine whether host, compartment, or environmental specific factors play an important role in the establishment of the intestinal microflora in broiler chickens during growth. This objective was addressed using a 16S rDNA approach. PCR-amplicons from the V6 to V8 regions of the 16S rDNA of intestinal samples were separated by denaturing gradient gel electrophoresis (DGGE). The number of bands in all intestinal compartments increased when broilers grew older, indicating that the dominant bacterial community becomes more complex when chickens age. Each chicken had a unique banding pattern for all locations in the intestinal tract, irrespective of the age of chickens. This suggests that host-related factors affect the establishment of the dominant bacterial community. Banding patterns of intestinal compartments within one chicken were different from each other for broilers older than 4 days, except for both ceca which were highly similar. In 4-day-old broilers, banding patterns from crop, duodenum, and ileum were very similar. We conclude that (unknown) host specific factors play an important role in the development of the intestinal bacterial community in each broiler chicken. Furthermore, compartment-specific factors play an important role in the bacterial development of each intestinal compartment within one chicken.

Introduction

The microflora in the gastrointestinal tract of broiler chickens plays an important role in nutrition, detoxification of certain compounds, growth performance, and protection against pathogenic bacteria [13, 18, 28]. The bacterial community of crop, duodenum, ileum, cecum,

**Present address:* Campina Melkunie, Hogeweg 9, 5301 LB Zaltbommel, The Netherlands.
Correspondence to: Paul W.J.J. van der Wielen at present address: Laboratory of Microbial Ecology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands; Tel: +31 50 3632169; Fax: +31 50 3632154; E-mail: P.W.J.J. van-der.Wielen@biol.rug.nl

and colon has mainly been studied by plate count analysis [6, 13, 14, 19, 20, 22, 26]. However, the use of these conventional microbiological methods has some major limitations. Bacteria difficult to cultivate or nonculturable bacterial species, e.g., due to unknown growth conditions, stress imposed by cultivation procedures, or obligate interactions with the host or other bacteria, will be missed. Furthermore, selective media have not been developed for all bacterial groups present in the intestinal tract and the strictly anoxic conditions some bacteria require cannot be met in the laboratory. Estimates of the culturability of the predominant bacterial community by comparing direct microscopic counts with total plate counts showed that only 10 to 50% of the intestinal bacterial community of broiler chickens (aged 1 day to 6 weeks) can be cultured [5–7, 14, 20]. This indicates that insight into interactions occurring between host and bacteria, as well as the influence of environmental factors on microbial communities in the intestinal tract of broiler chickens, is still lacking.

With the availability of molecular techniques based on 16S rDNA or rRNA, more insight into the microbial structure of different ecosystems has been obtained [reviewed by 1, 27, 29]. Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis are methods used to analyze microbial communities on a species level, based on sequence-specific separation of 16S rDNA amplicons [reviewed by 15]. Recently, these methods have been used to describe bacterial diversity in human feces [30] and in the intestine of pigs [23, 24]. These studies showed that every human or pig had its own specific bacterial community, which was stable during a 6-month period. Furthermore, each intestinal compartment within a pig had its own unique profile with the highest similarity to the closest other compartment. It remains uncertain whether these conclusions can be extended to the intestinal tract of broilers because the intestines of chickens with crop, two ceca, and a short colon differ from the intestinal tracts of pigs and humans.

The objective of this study was to determine whether host, compartment, or environmental specific factors play an important role in the establishment of the intestinal microflora in broiler chickens during growth. This was done by determining the similarity coefficients of DGGE banding patterns of intestinal samples between different chickens and between intestinal compartments within one chicken.

Materials and Methods

Chickens and Management

Broiler chickens were housed in a commercial farmhouse in Leusden, The Netherlands. The flock contained 17,000 broilers (Cobb breed). Three broiler chickens were obtained from this flock at days 1, 4, 7, 11, 15, 22, 28, and 39. Broilers were fed water and feed *ad libitum*. Broilers received a standard commercial feed for broiler chickens with growth-promoting or anti-coccidial antibiotics. From day 1 to day 10, feed contained nicarbazin (10 mg kg^{-1}) and avilamycin (10 mg kg^{-1}); from day 11 to day 33, feed contained avilamycin (9 mg kg^{-1}) and salinomycin (64 mg kg^{-1}); and from day 34 to day 40, feed contained avilamycin (6 mg kg^{-1}). After transportation to the laboratory, broilers were euthanized by cervical dislocation. The gastrointestinal tract was isolated, and the whole content of crop, duodenum, distal ileum (taken just before the cecal opening), and each cecum, respectively, was homogenized in mL 0.05 3 M potassium phosphate buffer (pH 7); 1 mL aliquots were stored overnight at −80°C.

DNA Isolation

After thawing, samples were centrifuged (2 min; 15,800*g*). Supernatant was discarded and the pellet was washed twice with TE buffer (10 mM Tris/HCl; 1 mM EDTA; pH 8.0) and resuspended in 500 μL TE buffer. DNA was isolated within 24 h of sampling as described by Simpson et al. [23] but with slight modifications. In short, cells were lysed with lysozyme at a final concentration (FC) of 900 μg mL^{-1} followed by sodium dodecyl sulfate (SDS; FC 0.83% w/v) and proteinase K (FC 10 units mL^{-1}) treatment. RNase treatment (FC 10 units mL^{-1}) was used to remove RNA from the samples. The lysate was deproteinized repeatedly with phenol/chloroform/isoamylalcohol (25:24:1) and chloroform/isoamylalcohol (24:1). DNA was precipitated with isopropanol, washed and resuspended in 200 μl TE buffer, and stored at −80°C.

PCR Amplification

Universal bacterial primers U968-GC (5′ CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401 (5′ CGG TGT GTA CAA GAC CC) [17] were used to amplify the V6 to V8 regions of the bacterial 16S rDNA since it has been shown that this region is a powerful tool for analysis of complex microbial communities in fecal samples [30]. The GC clamp in primer U968-GC creates PCR products suitable for separation by DGGE [16].

PCR was performed with Ready-to-Go PCR beads (Pharmacia Biotech, Roosendaal, The Netherlands). PCR mixtures of 25 μL contained 1.5 U *Taq* DNA polymerase, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 2.5 μg bovine serum albumin, 200 μM of each DNTP, 5 pmol of each primer, and 1 μL of 100 times diluted DNA. PCR mixtures were covered with mineral oil. Samples were amplified in a DNA Thermal Cycler 480 (Perkin-

Elmer, Oosterhout, The Netherlands) using the following program: 94°C for 10 min; 35 cycles of 94°C for 1 min, 56°C for 1 min, 68°C for 2 min; and finally 68°C for 8 min. Product size and amount were checked on a 1.5% (wt vol) agarose gel containing ethidium bromide.

DGGE Analysis and Similarity Index Calculations

Parallel DGGE was performed with the Bio-Rad D-code System (BioRad, Veenendaal, The Netherlands). Electrophoresis was performed in a 0.8 mm polyacrylamide gel (6% [wt/vol] acrylamide, 0.1% bisacrylamide) with TAE buffer (40 mM Tris base; 5 mM sodium acetate; 1 mM EDTA; pH 8.0) as the electrophoresis buffer at a fixed voltage of 80 V for 24 h at 60°C. A gradient of denaturants from 30 to 65% (100% denaturant corresponds to 7 M urea and 40% deionized formamide) was applied parallel to the electrophoresis running direction. PCR samples and standards were applied to gels in aliquots of 20 μL per lane. After completion of electrophoresis, DGGE gels were silver-stained with a DNA Silver Staining Kit (Pharmacia Biotech) in a Hoefer Automated Gel Stainer (Pharmacia Biotech). All samples were run twice on two different gels.

Background subtraction on gel images was performed as was described by Simpson et al. [23]. Band patterns were converted to peak profiles of densitometric curves with the gel analyzing program Image Master ID Elite (Pharmacia Biotech). Total numbers of bands were counted using these peak profiles. Gel patterns were further analyzed with the gel pattern analyzing program Molecular Analyst 1.12 (Bio-Rad). Similarity indices between patterns were calculated with Pearson product moment correlation coefficient. This coefficient takes band position and band density into account and is best suited to compare banding patterns [10]. A coefficient of 100 indicates that the densometric curves of the banding patterns of intestinal samples are identical in the three chickens. A coefficient of −100 indicates that the densometric curves of the banding patterns are the opposite (e.g., a valley in one curve corresponds to a peak in the other curve).

Data Analysis

The number of bands and the calculated similarity coefficients were analyzed statistically to determine differences between intestinal compartments. ANOVA using General Linear Models with Bonferonni post-hoc testing was used to determine statistical differences between crop, duodenum, ileum, and left and right cecum. All statistical procedures were calculated using SPSS 10.0 software.

Results

Development of the Intestinal Microflora

To study whether host, compartment or environmental specific factors play an important role in the establishment of the intestinal microflora in broiler chickens, intestinal samples from individual chickens were analyzed. Amplicons of the variable region V6-V8 were analyzed by DGGE, resulting in complex profiles Fig. 1. Intestinal samples of 1-day-old broilers showed no bands in DGGE gels except for the crop where a low number of bands were detected Fig. 2. This does not imply that bacteria are not present in duodenum, ileum, and both ceca of 1-day-old broilers, but the bacterial numbers are probably below the detection limit. This is confirmed by the very low numbers of total bacteria ($\sim 10^6$ cfu mL^{-1}) which could be counted from the caeca of 1-day-old broiler chickens as colony-forming units on agar plates [26]. The number of bands in the DGGE profiles increased in all parts of the intestinal tract as broilers aged from 1 to 11 days old Fig. 2. As broilers aged from 11 to 28 days, number of bands in crop and duodenum decreased but thereafter increased again ($p < 0.05$ and $p < 0.1$, respectively). In the ileum, band numbers did not increase or decrease when chickens aged from 11 to 28 days ($p > 0.1$). For both ceca, the number of bands did not increase or decrease either after chickens were 11 days of age ($p > 0.1$). Furthermore, the number of bands was higher in the caeca compared to the other three parts of the intestinal tract ($p < 0.001$) Fig. 2.

Similarity Indices between Chickens

Pearson product moment correlation coefficients between the banding patterns of intestinal samples of three chickens of the same age were calculated to determine whether these chickens had the same dominant bacterial community in their intestines. This approach resulted in three different similarity coefficients for the comparison of three chickens of the same age (between banding pattern of intestinal samples of chickens 1 and 2, chickens 1 and 3, and chickens 2 and 3), which were averaged. Mostly, the similarity coefficient fluctuated around a value of 50 for every intestinal compartment irrespective of the age of the broilers Fig. 3. Furthermore, standard deviations were high when similarity coefficients were averaged in this way. This indicates that the similarity coefficient between the first chicken and the second gave a very different value than when the first or second chicken was compared with the third chicken. There were no statistical differences ($p > 0.1$) observed when the similarity coefficients were compared between intestinal compartments Fig. 3. These results suggest that the dominant bacterial community in crop, duodenum, ileum, and both ceca of each chicken is

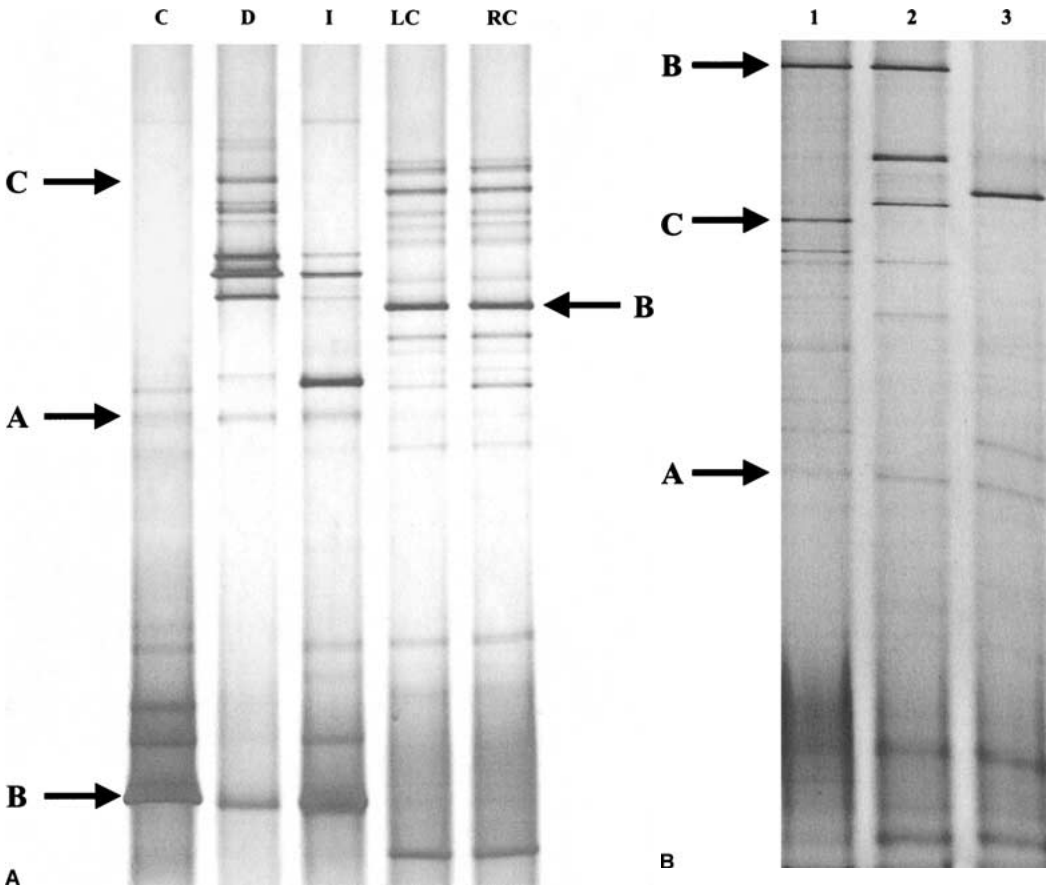

Fig. 1. DGGE-profiles of the V6-V8 regions of 16S rDNA from crop, duodenum, ileum, left and right cecum (C, D, I, LC, RC) of an 11-day old chicken (A) and from the ileum of 3 different chickens (1, 2, 3) aged 39 days (B). Arrows: A, band present in all samples, B, band present in more than one sample, C, band present in only one sample.

different from other chickens throughout the life of broilers.

Similarity Indices between Intestinal Compartments

To determine the similarity between samples from different intestinal compartments within one chicken, similarity coefficients were determined between banding patterns of different parts of the intestinal tract within one chicken (e.g., crop versus duodenum, ileum, right cecum, and left cecum). The coefficients obtained were averaged for three chickens of the same age and plotted as a function of the compartment Fig. 4. Similarity coefficients between banding patterns of crop and duodenum or crop and ileum were significantly higher than the similarity coefficient between crop and each cecum ($p < 0.001$) (Fig. 4A). The similarity between banding patterns of duodenum and crop or duodenum and ileum were also significantly higher than the similarity between duodenum and each cecum ($p < 0.001$) (Fig. 4B). Similarity coefficients between ileum and duodenum were significantly higher than between ileum and each cecum ($p < 0.01$). However, the similarity coefficient between ileum and crop was not significantly different from that between ileum and each cecum. During the first 2 weeks of life, the similarity coefficient between banding patterns of ileum and each cecum was much lower than the similarity between ileum and crop or duodenum. However, after 2 weeks the similarity between the banding patterns of ileum and each cecum had increased and was at the same level as between ileum and duodenum or crop (Fig. 4C). The similarity between banding patterns of the two ceca was very high. This value was significantly higher than the similarity between the ceca and other intestinal compartments ($p < 0.001$). In 28-day-old chickens the similarity between both ceca was much lower. However, at that specific age one of the three chickens showed an abnormally low similarity of banding patterns between both ceca, which

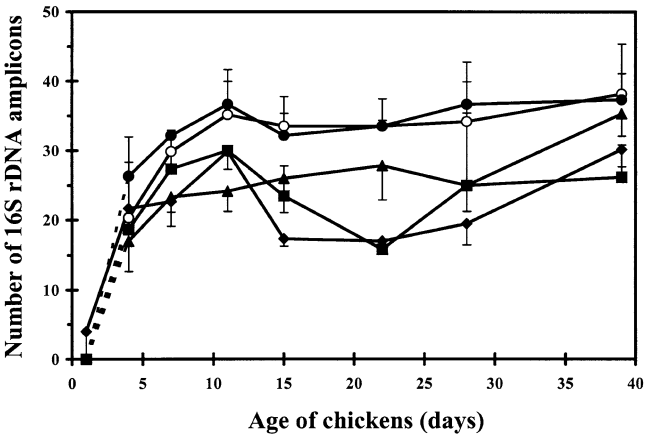

Fig. 2. Number of 16S rDNA amplicons in crop (◆), duodenum (■), ileum (△), left cecum (●), and right cecum (○) during growth of broilers. Results are given as mean of three chickens ± standard deviation.

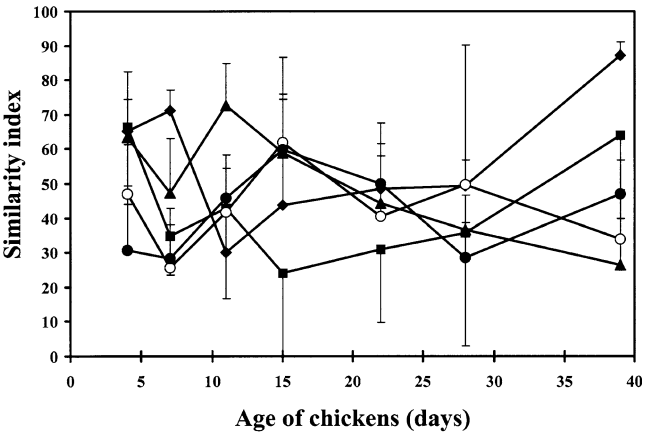

Fig. 3. Pearson product moment correlation coefficients of similarity between banding patterns of the dominant intestinal bacterial community in three chickens of the same age. Symbols as in Fig. 1. Results are given as mean of three chickens (i.e., average value of similarity coefficient between chickens 1–2, 1–3, and 2–3) ± standard deviation.

resulted in the low average similarity coefficient and a high standard deviation (Fig. 4D). Furthermore, it was observed that none of the bands were present in all five compartments except in 4- and 7-day-old broilers. This indicates that no bacterium was capable of dominantly colonizing the whole intestinal tract of chickens. However, some bands were present in crop, duodenum, and ileum but absent in the cecum (data not shown).

In 4-day-old broilers, banding patterns between crop, duodenum, and ileum showed high similarity coefficients with each other with values approaching 100 (Fig. 4). Thereafter, these similarity values decreased when broilers age to 11 days old, after which they remained stable. The similarity coefficient between crop, duodenum, or ileum and each cecum was also higher in 4-day-old chicken compared to older broilers.

Discussion

In this study, the development of the bacterial community in four parts of the intestinal tracts of broiler chickens was studied by DGGE analysis of 16S rDNA amplicons. We observed that the number of bands in the profiles increased when broiler chickens grew older. This might indicate that the diversity of the dominant bacterial community in the intestinal tract also increases when broilers grow older. For the ceca, this has also been observed using direct microscopic observations or cultivation of the dominant bacterial community [3, 4, 6, 26]. For crop, duodenum, and ileum, it has been observed with classical microbiological methods that lactobacilli dominated within 3 days and remained the dominant bacterial community with other groups being present around 10^7 per gram content or lower [4, 13, 22]. Only 1 to 3 *Lactobacillus* species seemed to be dominant in these parts of the intestinal tract [9, 13]. This seems in contrast to our observation where high numbers of bands were observed in these parts, suggesting that several bacterial species might dominate these parts of the intestinal tract.

Despite the fact that chickens of the same age were raised under the same conditions, received the same feed, and lived in contact with each other, they showed different banding patterns. This shows that every chicken has its own unique dominant intestinal bacterial community and suggests that host-specific factors are important in the establishment of the intestinal bacterial community. In contrast to these findings, it was concluded from a study with %G + C-profiling of the dominant intestinal bacterial community of chickens that chickens from the same age raised under same conditions had the same dominant intestinal bacterial community [2]. However, these authors did not compare intestinal samples from two different chickens but compared two replicate samples after intestinal samples from two chickens were pooled. We made direct comparisons between samples of different chickens without having these samples pooled. Furthermore, DGGE of 16S rDNA amplicons gives information about specific

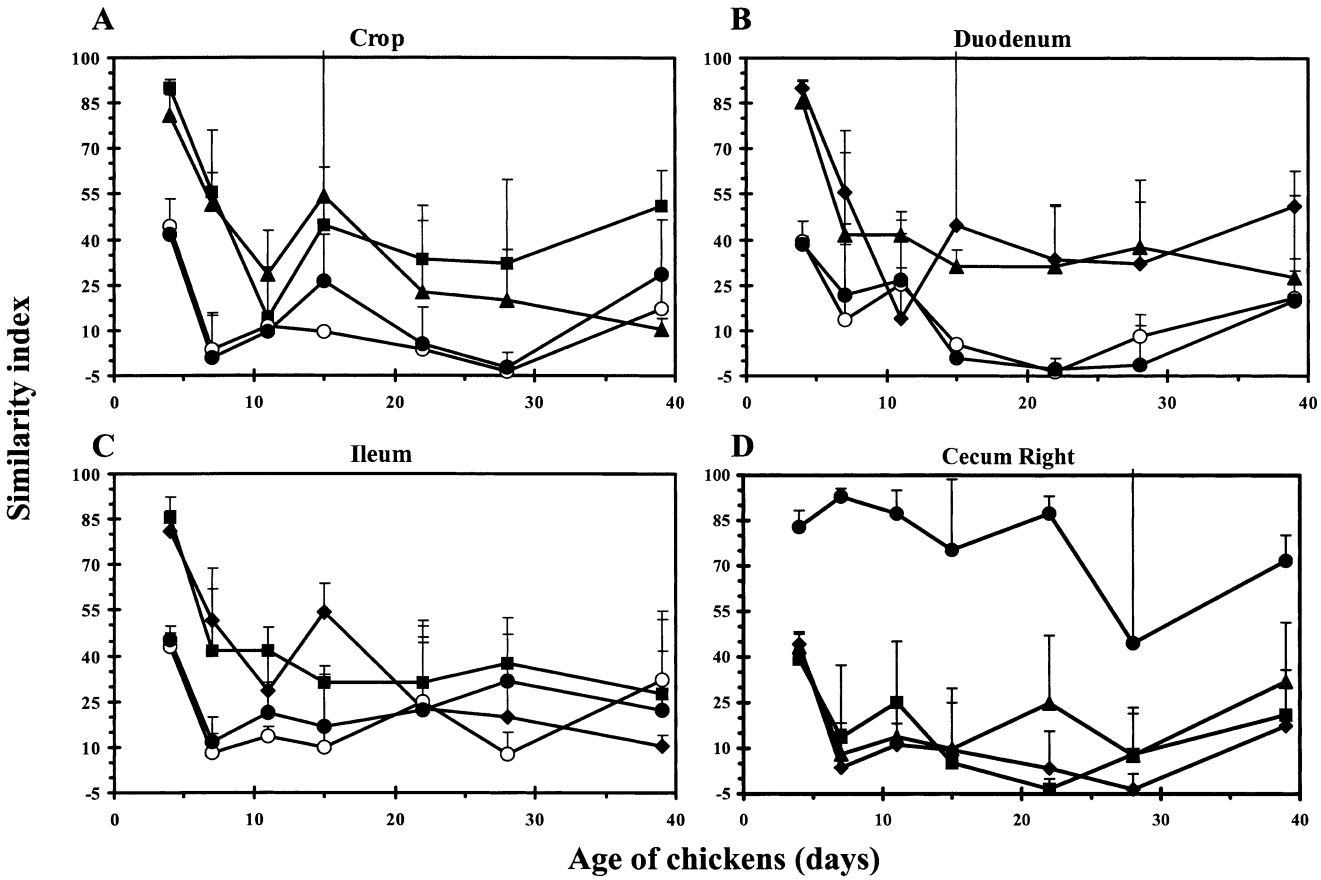

Fig. 4. Pearson product moment correlation coefficients of similarity between banding patterns of the dominant bacterial community of crop (A), duodenum (B), ileum (C), right cecum (D) and crop (◆), duodenum (■), ileum (△), left cecum (●), and right cecum (○). Results are given as mean of three chickens ± standard deviation.

16S rDNA sequences while this information cannot be obtained with bacterial %G + C-profiles.

At this moment we can only speculate about what type of host factors are important in the establishment of the bacterial community. Recently, it has been shown that the host genotype plays a role in the establishment of the host-specific microbiota [25, 31]. It has been hypothesized that immunological properties of the host, specific receptors for intestinal bacteria, or other communication systems with bacteria might be important factors in the establishment of a host-specific bacterial community [31]. Recent studies with mice have indicated that crosstalk between *Bacteroides thetaiotaomicron* and the host occurs on a molecular level [8, 11, 12] with *B. thetaiotaomicron* modulating expression of host genes important in several intestinal functions [12]. Yet, it remains uncertain whether this molecular mechanism between host and *B. thetaiotaomicron* is bacterium specific. Another study showed the importance of the major histocompatibility complex on the establishment of host-specific microbiota in mice [25]. However, both studies have been performed in mice under laboratory conditions and it remains uncertain whether these factors play a role in commercially raised broiler chickens as well.

Although crop, duodenum, and ileum were more similar to each other compared to the ceca, our results still showed that every part of the intestinal tract has its own specific bacterial community and can thus be seen as separate ecosystems. This result has also been observed when the cecal and ileal bacterial communities of adult chickens were compared with %G + C profiling [2] or traditional culturing methods [4, 6, 13, 19]. However, this study shows for the first time that crop, duodenum, and ileum have specific microbiota as well. This observation is in contrast with culturing techniques where the same bacterial species were isolated from the ileum and duodenum [21]. However, these traditional microbial methods have limitations. In this study, the dominant intestinal bacterial community

was compared using DGGE, a culture-independent approach. Therefore, better insight into the dominant bacterial community of the different parts of the intestinal tract was obtained. DGGE analysis of the dominant bacterial community of samples from different parts of the pig intestinal tract showed that cecal samples were not completely different from other parts of the intestinal tract [23]. This difference with our results is probably caused by differences in anatomy of the intestinal tracts of both animals.

Our results demonstrated that the dominant bacterial community in crop, duodenum, and ileum within one chicken was very similar in 4-day-old broilers. Even the similarity between the dominant bacterial community of the ceca with the other three parts of the intestinal tract was much higher in 4-day-old broilers. Immediately after hatching the intestinal tract of broiler chickens is germ-free and subsequently will be colonized by bacteria from the surrounding environment. The similarity between banding patterns of crop, duodenum, and ileum in 4-day-old broilers suggests that the environmental conditions along the intestinal tract are rather similar and do not allow niche differentiation. When broilers age, similarity between banding patterns of crop, duodenum, and ileum decreases considerably. This indicates that environmental factors in the intestine (e.g., pH, nutrition, bile salts, oxygen concentration) change specifically in each compartment. This study shows for the first time that these specific conditions in each compartments results in the development of a specific bacterial community for a given intestinal compartment.

Overall, we conclude from our study that every chicken as well as every compartment of the intestinal tract within one chicken has its own specific dominant bacterial community except for the left and right ceca, which are highly similar. Secondly, we conclude that crop, duodenum, and ileum in very young broilers have a similar dominant bacterial community but the bacterial community becomes intestinal compartment specific when broilers age. The results of this study can be important for studies related to the manipulation of the intestinal bacterial community in chickens such as competitive exclusion or probiotic research.

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