

Capillary electrophoresis single-strand conformation polymorphism for the monitoring of gastrointestinal microbiota of chicken flocks

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ABSTRACT The objective of the present study was to evaluate the capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) to characterize poultry gut microbiota and the ability of this molecular method to detect modifications related to rearing conditions to be used as an epidemiological tool. The V3 region of the 16S rRNA gene was selected as the PCR target. Our results showed that this method provides reproducible data. The microbiota analysis of individuals showed that variability between individual fingerprints was higher for ileum and cloaca than for ceca. However, pooling the samples decreased this variability. To estimate the variability within and between farms, we compared molecular gut patterns of animals from the same hatchery reared under similar conditions and fed the same diet in 2 separate farms. Total aerobic

bacteria, coliforms, and lactic acid bacteria were enumerated using conventional bacteriological methods. A significant difference was observed for coliforms present in the ceca and the cloaca depending on the farm. Ileal contents fingerprints were more closely related to those of cloacal contents than to those of ceca contents. When comparing samples from the 2 farms, a specific microbiota was highlighted for each farm. For each gut compartment, the microbiota fingerprints were joined in clusters according to the farm. Thus, this rapid and potentially high-throughput method to obtain gut flora fingerprints is sensitive enough to detect a “farm effect” on the balance of poultry gut microbiota despite the birds being fed the same regimens and reared under similar conditions.

Key words: gut microbiota, capillary electrophoresis single-strand conformation polymorphism

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INTRODUCTION

In poultry, as in the other vertebrates, the intestinal bacterial flora assists its host in the function of digestion and help to maintain the animal body in a state of health (Gabriel et al., 2006). Indeed, intestinal flora aids in the digestion of food by assisting in the absorption of vital nutrients required for energy and survival; they also destroy ingested toxins that can be harmful or fatal to its host. Vitamins such as vitamin K, niacin, B6, B12, and folic acid are synthesized in the digestive tract by intestinal flora (Gabriel et al., 2006). When the host becomes ill, helpful intestinal flora attacks harmful bacteria that disrupt the body's microbial balance. This vital function helps to restore the balance and to

ward off illness and disease. Finally, from birth, intestinal flora begins to develop in the digestive system and help to maintain the immunity by their ability to identify and destroy harmful bacteria without harming the helpful bacteria (Burel and Valat, 2009).

In poultry, cecal microflora is composed of 10^{11} bacteria/g of contents while 10^9 bacteria/g of contents are present in the ileum (Apajalahti et al., 2004). As is the case for other animals including humans, gram-positive bacteria are the most abundant (Franks et al., 1998; Leser et al., 2002; Gabriel et al., 2006). The microbiota appears to differ from one compartment of the intestinal tract to the other (Salanitro et al., 1978). In the jejunum, mostly facultative anaerobes, such as lactobacilli, streptococci, and enterococci, were isolated (Lu et al., 2003). In the ceca, the bacterial population is quite different, with the presence of strict anaerobes such as members of *Eubacterium*, *Bifidobacterium*, *Clostridium* genera and facultative anaerobes

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(Salanitro et al., 1978; Barnes, 1979; Gong et al., 2002). The composition of these microbiota varies with age (Knarreborg et al., 2002; Amit-Romach et al., 2004). The luminal flora appears to be different from the mucosal one (Gong et al., 2002; Lu et al., 2003). Gut microbiota bacteria interact with each other and directly with the lining of the gastrointestinal tract which may alter tract physiology and the immunological status of poultry (van Leeuwen et al., 2004). The balance of gut microbiota is conditioned by various factors such as diet, probiotics, antibiotics use, and stress (Netherwood et al., 1999; Apajalahti et al., 2001; Knarreborg et al., 2002). Antibiotic growth promotants were used to regulate microbial flora and mostly gram-positive bacteria to improve animal health and growth (Butaye et al., 2003). Among them, avilamycin was used to reduce clostridia populations (Butaye et al., 2003). Changes in European regulations, including the ban of antibiotic growth promotants, have led to modifications in feeding and rearing conditions of poultry, and it is important to understand their consequences on the balance of poultry gut microbiota.

The number of noncultivable bacteria in the gut microbiota is estimated to be as much as 90% (Lan et al., 2002). Molecular culture-independent methods have drastically improved the analysis of complex intestinal microflora in animals and especially in poultry (Apajalahti et al., 2001; van der Wielen et al., 2002; Hume et al., 2003; Lu et al., 2003; Apajalahti et al., 2004; Oviedo-Rondon, 2009). Consequences of feed composition or bacterial infection on the microbiota can be detected using these methods (Knarreborg et al., 2002; Johansen et al., 2006). For instance, whereas no change was detected by culturing, community hybridization of amplified 16S ribosomal DNA demonstrated that bacterial flora of the gastrointestinal tract changed significantly in response to probiotic treatments in poultry (Netherwood et al., 1999).

Among the molecular techniques, fingerprinting methods based on 16S rDNA analysis present several advantages: microbiota dynamics can be registered and profile modifications detected. One of the available fingerprinting methods is the single-strand conformation polymorphism (SSCP; Peters et al., 2000). This technique was first developed for the detection of gene polymorphisms in the human genome and later applied to the detection of mutations (Orita et al., 1989; Hayashi, 1991). In addition, it is currently being developed to study the composition and dynamics of different bacterial ecosystems, such as soil, anaerobic digesters, and food (Lee et al., 1996; Schwieger and Tebbe, 1998; Delbès et al., 2001; Dabert et al., 2005; Duthoit et al., 2005). Using the SSCP method, Ott et al. (2004) reported a reduction in the diversity of colonic mucosa-associated bacterial microbiota in patients with an active inflammatory bowel disease. And Hori and coworkers (2006) showed that SSCP was more discriminant than denaturing gradient gel electrophoresis (DGGE), another fingerprinting method. Recent studies have reported its

use for the analysis of bacterial communities in bovine, rabbit, and pig digestive tracts (Michelland et al., 2009; Waché et al., 2009; Combes et al., 2011).

The objective of this study was to determine if the CE-SSCP fingerprinting can be used as an epidemiological tool in poultry research. For that purpose, we aimed to establish if this method is able to point out a “farm effect” on the gut microbiota. To this end, 2 groups of broilers originating from the same hatchery were reared in 2 different experimental farms, in which the experimental rearing conditions, including feed, were maintained as closely related to the other as possible.

MATERIALS AND METHODS

Two experiments were performed: 1) the first experiment aimed to optimize the CE-SSCP method when applied on the intestinal contents of broilers, to evaluate the repeatability of this method when the same samples were analyzed several times, as well as to observe the extent of the individual variability in the intestinal microbiota profiles among a group of broilers reared together, 2) the second experiment aimed to compare the intestinal microbiota of broilers reared on 2 separate farms, but under similar conditions and fed the same diet.

Birds and Rearing Conditions

Animals were used in accordance with the “guidelines of the National Institutes of Health Guide and the French Ministry of Agriculture for the care and use of laboratory animals” and the experimental design had obtained the approval of the ethic committee of Anses. The same strain of broilers (male Ross PM3) provided by a French hatchery (Perot S.A., France) was used in the 2 experiments. Feed and water were provided ad libitum to the birds throughout the experiments. All the diets were manufactured at Anses Ploufragan-Plouzané (France).

In the first experiment, one-day-old broilers were reared in floor pens in the experimental farm of Anses Ploufragan-Plouzané (France). A starter feed was provided until the birds were 12 d old and then replaced by a grower feed until 24 d old. Their compositions are given in Table 1.

In the second experiment, one-day-old chicks coming from the same breeder flock were placed at the same time (d0) in 2 experimental farms, H1 (Anses Ploufragan-Plouzané, France) and H2 (INRA Nouzilly, France). The chicks were randomly distributed in 6 floor pens (64 birds per pen of 5 m²) in each farm and were reared under similar conditions until 25 d old, whatever the farm: the same lighting (23 h light/1 h dark between d0 and d4; 20 h light/4 h dark between d5 and d11; 18 h light/6 h dark between d12 and d25) and temperature (gradually decreased from 32°C at d0 to 28°C at d25) programs were followed. The

Table 1. Composition of the starter and the grower diets used in experiment 1

Item	Quantity (g/kg ¹)	
	Starter diet (0–12 d)	Grower diet (13–24 d)
Ingredient		
Wheat	300	250
Soybean meal 48	327	230
Corn	250	325
Peas	50	80
Soybean oil	27.5	35
Corn gluten 60	0	35
Bicalcic phosphate	13.5	11.5
Calcium carbonate	3.5	4
Sodium bicarbonate	0	2
Vitamin premix (NOV 998, NVV 934) ¹	20.8	20.8
NaCl	2.5	2
HCl lysine	1	2.5
DL-Methionine	2.5	2
Anticoccidian (Clinacox) ²	0.2	0.2
Calculated nutrient content		
ME (kcal/kg)	2,877	2,745
Proteins	215	200
Lysine	12.17	14.89
Methionine + cysteine	9.18	8.52
Tryptophan	2.53	2.09
Threonine	10.36	9.16
Calcium	6.55	6.27
Available phosphorus	4.70	4.05

¹Commercial vitamin premix (IDENA, Pontchâteau, France): composition not given.

²Clinacox (Janssen Santé Animale, Issy-les-Moulineaux, France).

common feeding program was composed of the same starter and grower diets (composition given in Table 2). Bird weights were determined at their arrival at the farms (d0) and then at d11 and d25 (individual BW). Feed intake ($n = 6$) was measured per pen at d11 and d25. Average weight gain ($n = 6$), feed intake ($n = 6$), and feed efficiency (live weight gain/feed intake; $n = 6$) were calculated for the periods 0 to 11 d, 12 to 25 d, and 0 to 25 d. Mortality was recorded daily.

Sample Collection

In the first experiment, fresh droppings ($n = 30$) were collected on the floor on d 24, and 30 birds were used. Cloacal contents were obtained by abdominal pressure on the broilers ($n = 11$). Then, the 30 birds were killed by intravenous injection in the wing with 1 mg/mL¹ of pentobarbital, and the ileal content (between Meckel's diverticulum and the ileocecal junction) as well as the content of the 2 ceca were collected. Samples were collected into sterile containers and kept on crushed ice. One gram of sample was taken for further CE-SSCP analysis.

In the second experiment, fresh droppings ($n = 6$ per floor) were collected on the floor in the 2 farms on d 25. Six chickens per floor pen, representative of the 64 birds of the group according to their BW were then used to obtain cloacal, ileal, and cecal contents as in the first experiment. The 6 intestinal samples were pooled by floor pen, given a total of 6 pools per farm per sample type. Pooled samples were divided into ali-

quots for molecular and conventional microbiota analysis: one gram of pooled sample was taken and preserved in 3 mL of 96% ethanol for further CE-SSCP analysis, while 3 g were subsampled and stored on ice for conventional bacteriological analysis.

Bacterial Counts

Bacterial counts were performed on fresh material within 48 h following sample collection. Total aerobic mesophilic bacteria and lactic bacteria were counted on Difco BHI agar (Dickinson and Company, France) and Difco MRS agar (Dickinson and Company), respectively, after incubation for 48 h at 37°C. Coliforms were numbered on Drigalski plates (Bio-Rad, France) incubated 24 h at 37°C. Results were expressed as log₁₀ cfu/g of sample.

DNA Extraction

The ethanol was removed from the samples after centrifugation ($9,000 \times g$ for 5 min at 20°C) and the pellet was rinsed 3 times with physiological saline by centrifugation. The DNA was extracted by using the QIAamp DNA Stool mini-kit (Qiagen, France). An additional treatment with 10 mg/mL of lysozyme was performed to improve the extraction yield of gram-positive bacterial DNA. Extracted DNA was loaded onto a 1% agarose gel and stained with 0.5 mg/mL of ethidium bromide to assess its quality and quantity. Images were captured with a Biocapt camera (Bioblock Scientific).

Table 2. Composition of the starter and the grower diets used in experiment 2

Ingredient	Quantity (g/kg ¹)	
	Starter Diet (0–12 d)	Grower diet (13–25 d)
Wheat	400	400
Soybean meal 48	369	281
Corn	134	217
Soybean oil	59	50
Corn gluten 60		17.4
Bicalcic phosphate	16.4	14.4
Calcium carbonate	12.9	9.7
Vitamin premix ¹	4.0	4.0
NaCl	3.0	3.0
HCl lysine	0.5	1.7
DL-Methionine	1.5	1.6
Anticoccidian (Clinacox) ²	0.2	0.2
Calculated nutrient content		
ME (kcal/kg)	3,000	3,050
Proteins	220	200
Lysine	12.0	11.0
Methionine + cysteine	8.5	8.2
Tryptophan	2.7	2.3
Threonine	8.2	7.3
Calcium	11.0	9.0
Available phosphorus	4.2	3.8

¹Premix composition (mg/kg of diet): Co, 0.6; Cu, 20; I, 2; Se, 0.2; Zn, 90; Fe, 50; Mn, 80 and CaCO₃, as support (carry 1 135 mg Ca); it supplied the following vitamins (per kg of diet): vitamin A (all trans-retinol) 15,000 IU, vitamin D₃ (cholecalciferol) 5,000 IU, vitamin E (DL- α -tocopheryl acetate) 100 mg, vitamin B₁ (thiamine mononitrate) 5 mg, vitamin K₃ (menadion) 5 mg, vitamin B₂ (riboflavin) 8 mg, vitamin B₆ (pyridoxine chlorhydrate) 7 mg, vitamin B₁₂ (cyanocobalamine) 0.02 mg, calcium pantothenate 25 mg, folic acid 3 mg, biotin 0.3 mg, choline chloride 550 mg, vitamin PP (niacin) 100 mg, butylated hydroxy toluene 125 mg.

²Clinacox (Janssen Santé Animale, Issy-les-Moulineaux, France).

PCR Reaction

The PCR amplifications of 3 different regions of the 16S rRNA gene (V2, V3, and V4-V5) were compared for purposes of total microbiota analysis. The V3 region amplification was performed according to Delbès et al. (2001) with W49 and W104 primers (Table 3). The V2 and V4-V5 regions were targeted by using ER10-ER111 and Com1-Com2 primers, respectively (C. Pissavin, unpublished data; Widjoatmodjo et al., 1994; Schwieger and Tebbe, 1998). These primers are specific to the eubacteria phylogenic domain except for the Com1-Com2 pair that enables the amplification of 16S eubacterial rDNA as well as the 18S rRNA gene of some eukaryotes (mold, yeast). Primers were labeled with 6-carboxyfluorescein (6-Fam) or 6-carboxy-1,4-dichloro-2',4',5',7'-tetra-chlorofluorescein (Hex) on the 5' end. The PCR reactions were performed with 1 μ L

of extracted DNA, that is, almost 10 to 50 ng. The V2 and V4-V5 regions amplifications were done in a reaction mix containing 1 μ M of ER10-ER111 or Com1-Com2 primers (Stratagene, France), 200 μ M of dNTPS, 1 \times enzyme buffer, and 2U pfu Turbo DNA polymerase (Stratagene). The V3 amplification was done in a reaction mix containing 130 nM of W49 and W104 primers, 200 μ M of dNTPS, 1 \times enzyme buffer, and 1.25 U pfu Turbo DNA polymerase.

After DNA denaturation for 10 min at 94°C, 25 cycles of 30 s at 94°C, 30 s at 61°C, and 30 s at 72°C were run with the primers W49-W104. The same steps were followed with the ER10-ER111, Com1-Com2 primers but with 30 cycles and an annealing temperature of 54°C. The PCR-amplified DNA was then loaded onto a 2% agarose gel and stained with 0.5 mg/mL of ethidium bromide. Images were captured with the Biocapt camera and DNA quantity evaluated.

Table 3. The PCR primers used for amplification of 16S rDNA

Primer	Sequence	<i>Escherichia coli</i> Position	Target	Region	Source of reference
W49 ¹	ACGGTCCAGACTCCTACGGG	330	Eubacteria	V3	(Delbès et al., 2001)
W104 ²	TTACCGCGGCTGCTGGCAC	500	Eubacteria	V3	(Delbès et al., 2001)
Com1 ²	CAGCAGCCGCGGTAATAC	519–536	Eub. + eukaryota	V4-V5	(Schwieger and Tebbe, 1998)
Com2 ¹	CCGTCAATTCCTTTGAGTTT	907–926	Eub. + eukaryota	V4-V5	(Schwieger and Tebbe, 1998)
ER10 ²	GGCGGACGGGTGAGTAA	103–119	Eubacteria	V2	(Widjoatmodjo et al., 1994)
ER111 ¹	CTGCCTCCCGTAGGAGT	341–357	Eubacteria	V2	(C. Pissavin, unpublished data)

¹The primer was labeled with Hex fluorescent dye.

²The primer was labeled with 6-Fam fluorescent dye.

CE-SSCP Electrophoresis

One microliter of PCR amplicons, diluted 2- to 50-fold after standardization on agarose gel, was mixed with formamide and Genescan 400 HD-Rox standard (Applied Biosystems, France) at a ratio of 1:18.5:0.5. After a denaturing step at 95°C for 10 min, the mix was quickly cooled on ice. A 96-well plate containing the samples was placed into an ABI Prism Genetic Analyzer 3100-Avent (Applied Biosystems). The nondenaturing polymer matrix used was 5.6% CAP polymer (Applied Biosystems) and 10% glycerol in 1× TBE. The electrophoresis was performed in 1× TBE buffer containing 10% glycerol. The samples were run at 15 kV at 32°C during 2,000 s. The data were collected with the Gene Mapper V4.0 software (Applied Biosystems), with a minimum peak height threshold of 50 relative units of fluorescence (RFU). Normalization was performed by using the internal standard 400 HD-Rox (Applied Biosystems).

CE-SSCP Standards

Amplified rDNA from *Clostridium* sp., *Enterococcus avium*, and *Lactobacillus paracasei* strains from the Anses collection (Anses, Laboratoire de Ploufragan-Plouzané, Unité Hygiène et Qualité des Produits Avicoles et Porcins, BP53, 22440 Ploufragan, France) were run individually as standards.

Statistical Analysis

Zootechnical performance and bacteriological count data were analyzed using Statview program version 5 (Abacus concepts, Berkeley, CA). Means were compared using Student's *t*-test ($P < 0.05$). Dendrograms from the CE-SSCP fingerprints were constructed using Bionumerics software (Applied Maths, Kortrijk, Belgium). Analysis was based on Pearson correlation coefficient obtained from the densitometric curves and unweighted pair group method using arithmetic averages.

RESULTS

CE-SSCP Optimization

Different parameters, such as temperature and the 16S rRNA gene target region of CE-SSCP, were optimized using samples taken from different gut compartments, that is, ileum, ceca, and cloaca obtained during the preliminary experiment. To verify the reliability of the target-variable region of the 16S rRNA gene, we compared the fingerprints of ileal, cecal, and cloacal samples after DNA amplification with primer pairs for the V2, V3, or V4-V5 regions (Figure 1). Complex fingerprints composed of 26, 33, and 27 peaks were obtained for the ceca contents by targeting the V2, V3, or V4-V5 regions, respectively (Figure 1). For the cloaca and the ileum contents, the most complex finger-

prints, composed of 25 and 27 peaks, respectively, were observed after amplification of the V3 region. Consequently, all the results presented below were obtained after DNA amplification with the V3-specific primers (W49 and W104).

Repeatability

To ensure reliability of the gut microbiota analysis using CE-SSCP, we studied the repeatability of the DNA extraction, PCR, and CE-SSCP electrophoresis steps. Three independent DNA extractions were performed with one cloaca and one cecal sample obtained during the preliminary experiment. They all led to the same fingerprint (data not shown). The PCR and CE-SSCP electrophoresis steps were tested on one ileum, one cecal, and one cloacal sample. The PCR product fingerprints obtained after 3 independent amplifications were identical, indicating a strict repeatability of the PCR reaction. After 4 independent runs performed on one sample, identical fingerprints were obtained. We concluded that there was a strict repeatability between capillaries and from one run to another. However, we noticed differences when several analyses of a given sample were separated by long time intervals even if the extracted DNA was stored at -20°C (Figure 2).

Individual Variability

To estimate inter-individual variability, we compared the fingerprints of samples from different birds sampled during the preliminary experiment. Thirty broilers have been sampled, but in many cases, the abdominal pressure was not successful and in one case, the cecal compartment was empty. We obtained 11 samples of cloacal content and 29 samples for the ceca. When the different gut compartments are taken into account, we observed a higher similarity percentage with cecal samples compared with those from the ileum and cloaca. The similarity between profiles of cecal, cloacal, and ileal contents from different birds reached 36.30%, 27.56%, and 2.65%, respectively. The droppings profiles exhibited only 1.03% similarity. To decrease the variability due to individuals, pools of 6 birds or droppings were used for the further studies presented herein.

Consequences of Husbandry on Broiler Performance

The broilers in the 2 farms during the main experiment were reared under conditions that were as similar as possible. Only slightly lower temperatures were noticed in husbandry H1 compared with husbandry H2. The differences were of 0.5, 1.5, 1.9, and 0.6°C at d 1, 7, 11, and 25, respectively. The mortality rate in H1 and H2 was 3.13% and 1.56%, respectively. Weight gain, feed intake, and feed conversion efficiency are indicated in Table 4. Zootechnical parameters observed during

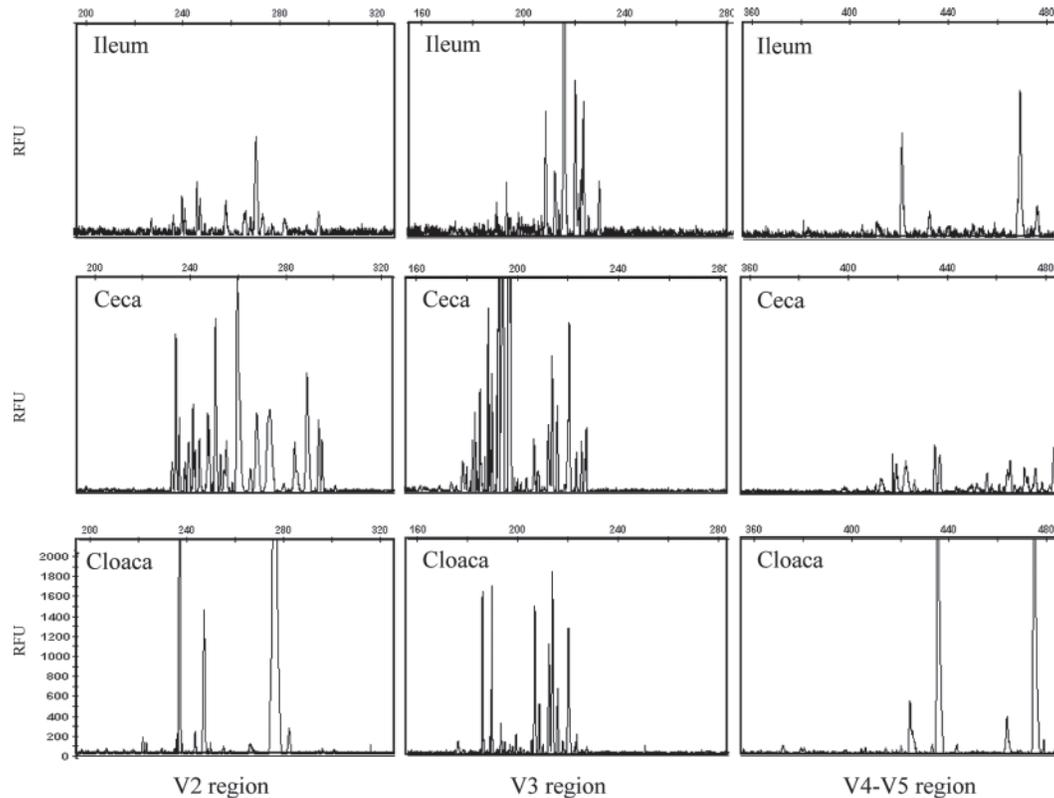


Figure 1. Capillary electrophoresis single-strand conformation polymorphism fingerprints of different regions of 16S rDNA. The tested regions are V2, V3, and V4-V5. The DNA was extracted from ileum, ceca, and cloaca of the same bird. The fluorochrome detected was 6-Fam. The relative fluorescence (RFU; y-axis) is plotted in function of the number of scans (x-axis).

the 2 rearing periods showed that the feed intake from 0 to 25 d was significantly higher in H1 than in H2 (10%), the feed efficiency was lower in H1 than in H2 (5%), but a higher weight gain was nevertheless observed in H1 compared with H2 (3.5%).

Consequences of Husbandry on Broiler Gut Microbiota

When comparing the bacterial counts in the different gut compartments, slight difference was noticed

between samples from H1 and H2. Coliforms were significantly more numerous in the ceca and cloaca of the birds from H2 than in those of the birds from H1 (Table 5). No difference in coliform counts for ileum and droppings appeared between the farms. No significant difference was observed concerning total aerobic mesophilic bacteria and lactic microbiota between the 2 farms (Table 5). Regardless of the farm, broiler ceca contained a significantly higher amount of total aerobic mesophilic bacteria, lactic flora, and coliforms than the ileum and cloaca (Table 5).

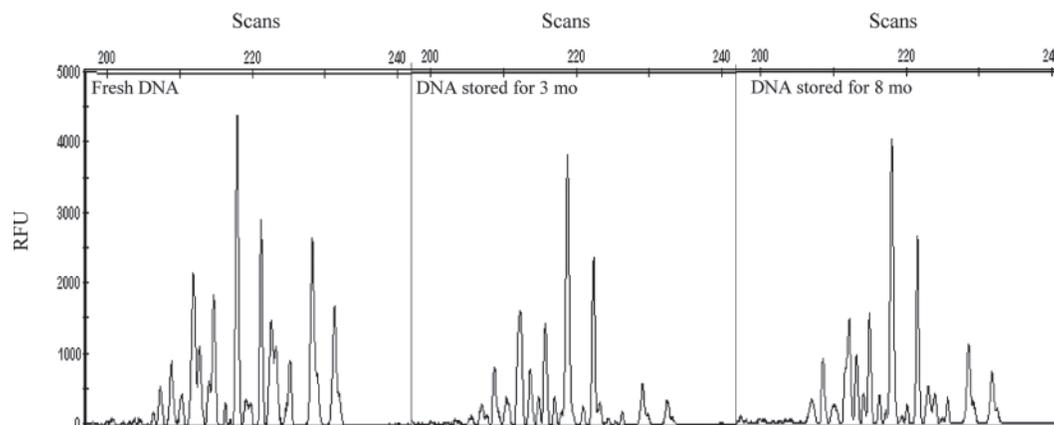


Figure 2. One ileal sample analyzed by capillary electrophoresis single-strand conformation polymorphism at 3 different times after sampling and extraction ($t = 0$, $t = 3$ mo, $t = 8$ mo). The fingerprints are obtained by 6-Fam detection. The relative fluorescence (RFU; y-axis) is plotted in function of the number of scans (x-axis).

Table 4. Average feed intake, weight gain, and feed efficiency of the broilers reared in the 2 farms (H1 and H2)¹

Parameter	Farm	Rearing period		
		0–11 d	12–25 d	0–25 d
Feed intake (g/animal)	H1	368 ± 2 ^a	1,569 ± 9 ^a	1,937 ± 9 ^a
	H2	326 ± 3 ^b	1,432 ± 11 ^b	1,758 ± 12 ^b
Weight gain (g/animal)	H1	308 ± 2 ^a	1,060 ± 7 ^a	1,367 ± 7 ^a
	H2	290 ± 2 ^b	1,030 ± 5 ^b	1,320 ± 6 ^b
Feed efficiency (g/g)	H1	0.84 ± 0.01 ^b	0.68 ± 0.01 ^b	0.71 ± 0.00 ^b
	H2	0.89 ± 0.01 ^a	0.72 ± 0.00 ^a	0.75 ± 0.00 ^a

^{a,b}For given time interval and parameters, the averages ± SE annotated with different superscripts are significantly different ($P < 0.05$).

¹The feed intake and weight gain were measured per pen ($n = 6$) but presented per bird.

The CE-SSCP was performed after PCR amplification of total microbiota DNA obtained from the samples. In parallel, the same experiment was performed with genomic DNA extracted from different bacterial strains used as a standard. Because sequence variations differently influence the folding of 2 DNA complementary strands, fingerprints obtained both with the Hex-labeled strand and with the Fam-labeled strand were analyzed.

The reference strains provided distinct peaks. Peaks co-migrating with the *Clostridium* standard peak were detected mostly in the ceca, whereas peaks co-migrating with *Lactobacillus* and *Enterococcus* appeared mainly in ileal and cloacal samples (Figure 3). These results were observed both in the 6-Fam and the Hex-labeled strands.

The dendrograms corresponding to pooled ileal, cecal, and cloacal contents obtained from the birds reared in H1 or H2 are presented in Figure 4. Each band presented in Figure 4 corresponds to one peak of the CE-SSCP fingerprint with respect to its intensity. For a given gut compartment, the samples were clustered according to each farm (Figure 4A). A higher similarity was observed within a farm than when the profiles were

compared between farms. The similarity between the H1 cluster and H2 cluster of ceca was 50.57%. For each farm, samples from ceca showed a higher similarity (H1: > 72.11%, H2: > 71.83%) compared with samples from ileum (H1: > 56.51%, H2: > 66.99%) and cloacal samples (H1: > 48.41%, H2: > 58.36%). Similarities between ileum and cloaca compartment contents were 48.41% and 47.88% for H1 and H2 birds, respectively. Comparable results were observed when the second Fam-labeled strand was considered because we noticed that cecal microflora showed higher similarity (H1: > 77.6%, H2: > 78.7%) between them than between those of the other gut compartments. In the ceca fingerprints of H1 birds, we noticed the presence of specific bands (indicated with black arrows) that were weaker or absent in the ceca of the H2 birds. On the contrary, one band is specifically present in the ceca patterns of H2 birds (indicated with a gray arrow).

The comparison of fresh droppings fingerprints (Figure 4B) revealed 2 clusters with 33.78% similarity corresponding to the 2 farms. Within a cluster, the similarity was higher than 63.05% and 55.33%, for H1 and H2, respectively.

Table 5. Bacteriological counts (\log_{10} cfu/g) of different gut parts ($n = 6$ pools of 6 individuals) and fresh droppings ($n = 6$ pools of 6) of chickens in the 2 farms (H1 and H2)

Microflora sample	Farm	Total aerobic mesophilic bacteria	Coliforms	Lactic bacteria
Ileum	H1	8.63 ± 0.15	5.51 ± 0.19	8.91 ± 0.16
	H2	8.45 ± 0.10	5.54 ± 0.22	8.64 ± 0.11
Ceca	H1	9.37 ± 0.21	7.94 ± 0.04 ^b	9.39 ± 0.12
	H2	9.28 ± 0.13	8.22 ± 0.11 ^a	9.53 ± 0.12
Cloaca	H1	8.75 ± 0.11	6.19 ± 0.15 ^b	8.95 ± 0.11
	H2	8.91 ± 0.12	6.69 ± 0.10 ^a	8.93 ± 0.06
Dropping	H1	9.25 ± 0.07	6.88 ± 0.23	9.12 ± 0.07
	H2	9.37 ± 0.09	7.16 ± 0.25	9.40 ± 0.11
Ileum ¹		8.54 ± 0.09 ^c	5.53 ± 0.14 ^d	8.77 ± 0.10 ^b
Ceca		9.32 ± 0.12 ^a	8.08 ± 0.07 ^a	9.46 ± 0.09 ^a
Cloaca		8.83 ± 0.08 ^b	6.44 ± 0.11 ^c	8.94 ± 0.06 ^b
Dropping		9.31 ± 0.06 ^a	7.02 ± 0.17 ^b	9.26 ± 0.07 ^a
P-value	Husbandry (H)	NS	*	NS
	Microflora sample (M)	***	***	***
	H × M	NS	NS	NS

^{a-d}Means in the same column with different superscripts for a given parameter differ significantly ($P \leq 0.05$).

¹For each gut compartment, the values are means of bacteriological counts of H1 and H2 farms.

NS refers to $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

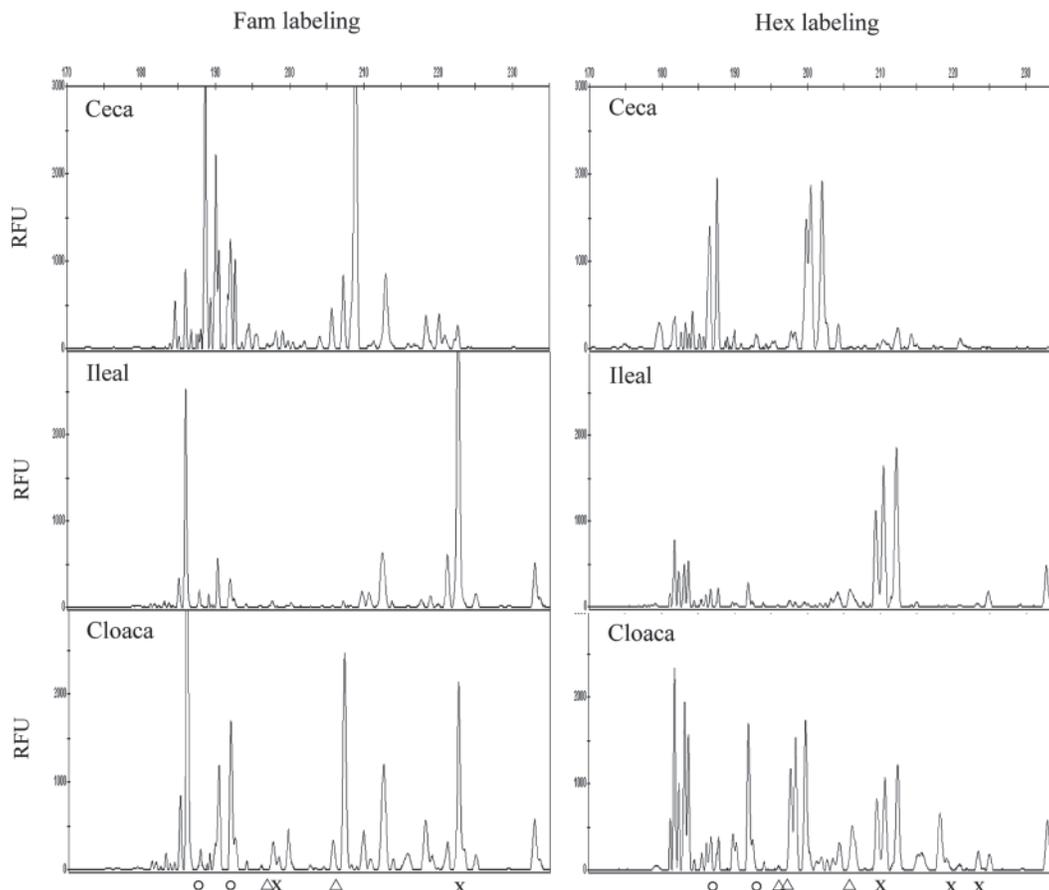


Figure 3. Representative fingerprints of pools (6 individuals per pool) of cecal, ileal, and cloacal contents. The fingerprints are obtained by 6-Fam or Hex detection. The position of peaks from reference bacteria is shown below the figures: *Clostridium sp.* (o), *Enterococcus avium* (x), and *Lactobacillus paracasei* (Δ). The relative fluorescence (RFU; y-axis) is plotted in function of the number of scans (x-axis).

DISCUSSION

Within the last decade, several molecular fingerprinting methods have been developed to analyze microbiota from animal gut or the environment (Dahllöf, 2002). Some of them, such as DGGE, temporal temperature gel electrophoresis (**TTGE**), terminal restriction fragment length polymorphism (**T-RFLP**), and restriction fragment melting curve analysis (**RFMCA**) have been used to explore poultry gut microbiota (Gong et al., 2002; van der Wielen et al., 2002; Zhu et al., 2002; Rudi et al., 2005; Torok et al., 2011). In contrast to DGGE and TTGE, T-RFLP and CE-SSCP do not require a gel gradient and silver staining, which can lead to problems with reproducibility. Moreover, these 2 capillary methods present the advantage that DNA amplification can be performed with one primer labeled with 6-Fam and the other with Hex or NED, providing 2 fingerprints from only one PCR reaction (Gong et al., 2002). The CE-SSCP method appears to be powerful for the study of complex microbiota (Baba et al., 2003; Duthoit et al., 2003; Peu et al., 2006; Hong et al., 2007). The biodiversity pattern depends on the region considered and is also a function of the method used (Dahllöf, 2002; Zhu et al., 2002). The choice of the region of the 16S rRNA gene is crucial to obtain representative finger-

prints of the microbiota and to draw pertinent conclusions. In our study, the V3 region provided the most complex fingerprints with several peaks in agreement with the generated numerical simulation fingerprinting patterns (Loisel et al., 2006).

We found specific fingerprints for individual chickens regardless of the gut compartment, as it was already demonstrated (van der Wielen et al., 2002). Fingerprint dissimilarity was not uniform; the patterns from cecal microbiota differed the least. It corroborates that each chicken reared under identical conditions showed quantitative and qualitative differences in microbiota, although some similarities were noticed for dominant microbiota (Zhu et al., 2002). Although, the number of combined samples may vary according to the age of the birds and the rearing conditions, we demonstrated that pooling gut samples from 6 birds appeared to be necessary and sufficient to decrease the variability and to highlight modifications of poultry gut microbiota in relation with the farm by the CE-SSCP described herein.

The highest number of total aerobic mesophilic bacteria, coliforms, and lactic bacteria were counted in the ceca, compared with the ileum and cloaca. This is in good agreement with the fact that the ileum contains 10^9 bacteria per gram whereas 10^{11} bacteria per gram are present in the ceca (Apajalahti et al., 2004). The

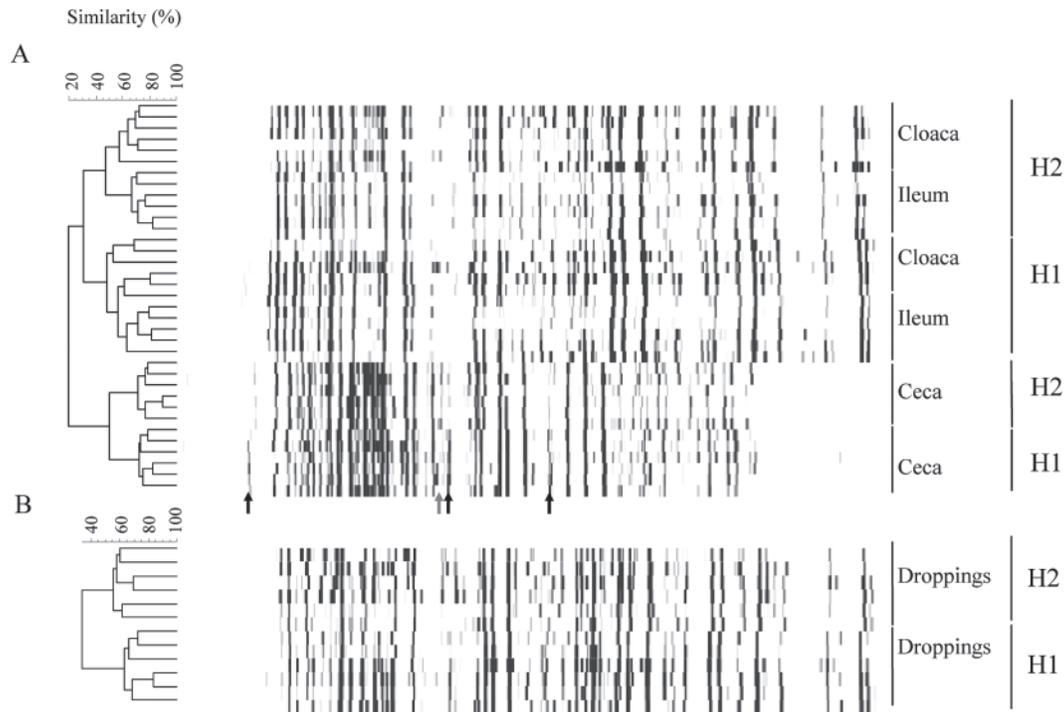


Figure 4. Similarity between pooled samples (6 individuals or droppings per pool) collected on the 2 farms (H1 and H2; $n = 6$ pools for each farm and for each sample type). A. Similarity between ileal, cecal, and cloacal contents. B. Similarity between fresh droppings. Hex-labeled V3 amplification products were analyzed. Similarity coefficients were calculated with Bionumerics software using Pearson correlation. Dendrograms were constructed based on the nonweighted pair-group method using arithmetic averages (UPGMA). The arrows indicate specific bacterial populations of H1 (black) or H2 (gray) husbandry.

highest number of bacteria in the cecum may be related to the slow turnover of contents (1 to 2 times/d; Gabriel et al., 2006).

The CE-SSCP fingerprints of the ileum were more similar to those of the cloaca than to those of the ceca, pointing out a specific flora in the ceca. The function of the ceca flora metabolism is improperly understood as of yet, but fermentative processes appear to be predominant with the production of organic acids (Mead, 1997). In the CE-SSCP fingerprint, peaks that co-migrated with *Clostridium* presented a higher intensity in the ceca than in the ileum and cloaca. Conversely, peaks that co-migrated with *Lactobacillus* and *Enterococcus* were higher in the ileum and the cloaca than in the ceca. These results are in good agreement with the conclusions of previous studies (Lu et al., 2003; Bjerum et al., 2006). It is noteworthy that peak intensity, related to the efficiency of polymerase processivity during PCR, may be different according to the sequence of bacterial species rDNA. But even if the method is semiquantitative, the presence of major peaks recovered in all samples from a given gut compartment may reveal the presence of predominant bacterial species or populations. Indeed, the CE-SSCP fingerprint provided a representative image of the gut microbiota biodiversity for the dominant bacterial genus. The detection of specific groups of bacteria can be improved by using group-specific primers (Peu et al., 2006). Moreover, it is probable that a more important functional analysis of the microbiota by CE-SSCP can be performed

because a precise mRNA quantification coupled with reverse transcription was reported by using this technique (Park et al., 2006).

Though the farms were run with as high degree of similar management as can be provided, the CE-SSCP approach allowed us to detect differences of poultry gut microbiota between the birds from the two farms. Differences of animal performance also have been measured, pointing out the relationship between intestinal flora and animal growth. We observed clusters of gut microbiota related to the farm, regardless of the gut compartment or the droppings analyzed. Our results suggest that certain major bacterial taxa were present in the broilers from different houses but at different relative abundances. Although the chicks came from the same hatchery and were fed with exactly the same diet, we could detect bacterial populations in the ceca that appeared to be specific to the husbandry. Some of these may be related to coliforms given that this bacterial population was recovered in greatest amounts in the ceca of the chickens from husbandry H2. The variability observed between pools of individuals was higher between farms than within each farm, suggesting that the environmental factors (litters, water, ventilation) can play a more important role than host factors. Although the host's diet is the strongest determinant (Oviedo-Rondon, 2009), it was already shown that litter type influences cecal microbiota, performance in broilers, and prevalence of pathogens such as *Campylobacter* (Line et al., 2002; Torok et al., 2009).

For epidemiological studies, samples of fresh droppings are often chosen because of their overall availability and easy access. However, Ott et al. (2004) showed that storage conditions of samples (that is, temperature, time of storage) may lead to a loss of diversity. Consequently, it should be taken into consideration that the fingerprints of the droppings collected on the floor evolved over time. Moreover, even if cloacal contents and floor droppings presented fingerprints with many common peaks, the presence of additional peaks in dropping profiles was suspected to arise from litter contamination. As a result, it does not appear that fresh droppings collected on the floor are the best indicators to associate an SSCP profile with the health status of a chicken flock. Ceca flora is of greater interest because of the presence of bacteria that may be responsible for food-borne diseases, for example, *Campylobacter* and *Salmonella*. Given the low variability of fingerprints from ceca pools, this digestive compartment appeared to be the most appropriate for using CE-SSCP as an epidemiological descriptor. However, some diseases may also be linked to microbiota modifications in another part of the gut, and if typical fingerprints can be associated with the symptoms or presymptomatic phase, the analysis of other gut compartments than ceca should be performed.

In conclusion, we demonstrated that CE-SSCP is a reproducible method to study the intestinal microbiota of poultry. The ability of this method to detect the influences of slight differences in rearing conditions in farms on poultry gut flora was demonstrated. It may be a powerful epidemiological tool with high throughput ability. Besides, to avoid methodological biases, it is recommended that samples be analyzed in a series of runs rather than separate ones. Its application in detecting gut microbiota modifications related to the use of alternatives to antibiotic growth promotants or related to infectious diseases should be helpful. For the purpose of epidemiological studies of infectious diseases, a dual approach combining conventional bacteriological counts and molecular fingerprinting is surely necessary because the detection of bacterial pathogens may be prevented by the presence of more important flora.

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