Effects of alternative to AGP on the digestive flora using fingerprint methods (RFLP, CE-SSCP and TTGE)

Irène Gabriel, Ignacio Badiola, Maria Francesh, Christine Pissavin, Henrik Christensen

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Report on the effects of alternative to in-feed antibiotic on the digestive flora of poultry, in comparison to AGP using fingerprint methods (RFLP, CE-SSCP and TTGE)

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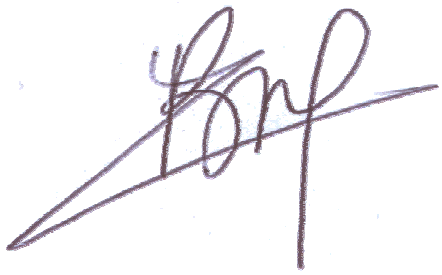
Dr. Christine BUREL - AFSSA Poultryflorgut Coordinator Phone: +33 (0)2 96 01 62 69 Email: c.burel@afssa.fr	Date: 03/04/2008 
Dr Henrik Christensen – KVL Work Package Leader Phone: +45 35 33 27 83 Email: hech@life.ku.dk	Date: In the paper format

Distribution list

Name	Organization	Email
BUREL Christine	AFSSA	c.burel@ploufragan.afssa.fr
CHEMALY Marianne	AFSSA	m.chemaly@ploufragan.afssa.fr
BOUVAREL Isabelle	ITAVI	bouvarel@itavi.asso.fr
CHRISTENSEN Henrik	KVL	hech@kvl.dk
FRANCESCH Maria	IRTA	maria.francesch@irta.es
GABRIEL Irène	INRA	gabriel@tours.inra.fr
HILBERT Friederike	VU	friederike.hilbert@vu-wien.ac.at
KLEIN Günter	TIHO	Guenter.Klein@tiho-hannover.de
MANFREDA Gerardo	UNIBO	gmanfreda@disa.unibo.it
PIESKUS Juozas	IIVU	jpieskus@imi.lt
STEINHAUSEROVA Iva	VFU	steinhauserovai@vfu.cz
VAN DER WAL Fimme	ASG-WUR	fimme.vanderwal@wur.nl
ADKIN Amie	VLA/DEFRA	a.adkin@vla.defra.gsi.gov.uk
MARTINO Gaetano	UNIPG	martinog@unipg.it
PEDERSEN Karl	DFVF	kpe@dfvf.dk
BADIOLA Ignacio	CReSA	ignacio.badiola@irta.es

Publishable executive summary

This report is on the effect of antibiotic growth promoters (AGP), avilamycin or salinomycin, and several alternatives to antibiotic growth promoters (AGP) (prebiotics, probiotics, botanicals, organic acids, diet structure) on animal performance and the digestive tract particularly on digestive microflora of broiler chickens. Digestive microflora was studied by conventional bacterial counts, and by molecular methods as Fluorescent In Situ Hybridization (FISH) and fingerprint methods. Among these latest molecular methods, there were two high-throughput fingerprint methods, Capillary Electrophoresis Single-Strand Conformation Polymorphism (CE-SSCP) and Restriction Fragment Length Polymorphism (RFLP), and another fingerprint method allowing identification of specific bacteria of dietary treatment, Temporal Temperature Gradient gel Electrophoresis (TTGE). Modification of digestive microflora was studied by comparing 5 or 6 pools of digestive contents of 6 birds (3 weeks of age) per dietary treatment. For this, four experiments were performed by three partners: IRTA Experiment 1 (Avilamycin and botanicals), IRTA Experiment 2 (Salinomycin and organic acids), AFSSA (Avilamycin and probiotics), and INRA (Avilamycin, prebiotics and diet structure).

For the first IRTA experiment (botanicals), the effects of avilamycin and some botanicals, (thymol, carvacrol, cinnamaldehyde, yucca extract), were studied. Analysis of ileal samples as well as by CE-SSCP (AFSSA) and by RFLP (CReSA) with universal primers revealed no significant differences between the dietary treatments. However analysis by RFLP (CReSA) showed a significant effect of block (cage of the same block were located nearest than cages of other blocks). These results could represent a cross-contamination between the microbiota of different treatments located in the proximity of other treatments

For the second IRTA experiment (organic acids), calcium formate, sodium butyrate, lactic acid, organic acids blend were studied. Results obtained by FISH showed higher number of bacteria belonging to Domain Bacteria (EUB338 probe) in ileum from birds fed sodium butyrate, compared with lactic acid, organic acids blend or negative control. Lower counts with LGC354C probes (*Enterococcus*, *Streptococcus*, *Lactococcus*) was observed with salinomycin compared with negative control or organic acids diets. Some organic acids affected members of *Enterobacteria*, *Bacteroides* and *Clostridium* significantly. Calcium formate decreased *Enterobacteria* compared with negative control and sodium butyrate diets. Lactic acid decreased Bacteroides compared with negative control, salinomycin and sodium butyrate diets. Calcium formate, lactic acid and organic acids blend diet decreased *Clostridium* compared with salinomycin diet. Analysis of ileal samples by RFLP (CReSA) with universal primers showed significant diet effect in ileum samples between treatments**.** Negative control as well feed supplemented with sodium butyrate are clearly different to the other treatments. RFLP profiles of feed supplemented with salinomycin and feed supplemented with calcium formate were not clearly different. RFLP profiles of feed supplemented with lactic acid and feed supplemented with formic and propionic acids and ammonium formate have some kind of association. Moreover, differences between treatments were detected in microbial biodiversity, as the number of electrophoretic bands in the RFLP profiles. Negative control biodiversity was significantly lower ($p<0.05$) than the biodiversity of feed supplemented with salinomycin, feed supplemented with sodium butyrate and feed supplemented with formic and propionic acids and ammonium formate. Analysis of ileal samples by CE-SSCP (AFSSA) with universal primers did not lead to cluster according to dietary treatments, but with salinomycin, it was observed a disappearance of some bands or bands with lower intensity compared to the other diets.

For the AFSSA experiment (probiotic), the effect of Bactocell® was studied. The fingerprint method CE-SSCP (AFSSA), did not lead to cluster according to dietary treatments (negative control, avilamycin, probiotic) neither with universal primers, nor with specific primers of Lactic Acid Bacteria. However, some specific bands of the dietary treatments were observed. Thus the absence or the weak intensity of two bands in the ileum and the cloaca of only the animals treated with the antibiotic may be related to this treatment. Other bands were absent in samples from birds fed avilamycin. Moreover bands not detected in samples of birds fed

negative control diet were observed with avilamycin. With the probiotic, the presence of some specific bands was detected. Thus the presence of one minor band in different gut compartment that did not correspond to lactic flora may be linked to this treatment. Another one was mostly detected in the fingerprints of the caecal lactic microflora of some animals treated with probiotic. On the contrary bands present in negative control diet, were absent with the probiotic. As with CE-SSCP, analysis of the digestive samples with universal primers by TTGE (INRA) did not lead to cluster according to dietary treatments whatever the digestive content (ileum, cloaca, caeca). However some bands characteristic of the dietary treatment were observed. In the ileal content, probiotic led to a band (a) with a high intensity corresponding to *Lactobacillus johnsonii* which was also detected in negative and positive control, but with lower intensity. In the cloacal content, avilamycin diet led to the appearance of one band (b) The probiotic diet led to the appearance of a band (c) migrating at the same level than the band (b) appearing with avilamycin diet. These bands (b, c) corresponded to a bacteria belonging to the order Clostridiales.
For the INRA experiment (prebiotics, diet structure), effect of FOS and whole wheat were studied. Analysis of ileal samples with universal primers by SSCP (KVL) showed no consistent patterns in relation to dietary treatment. Analysis of samples with universal primers by CE-SSCP (AFSSA) showed no cluster in relation with a specific treatment whatever the digestive content (ceca, ileum, cloaca contents). However, as for the other experiments, specific bands of dietary treatments were detected. Thus with avilamycin and whole wheat as well as appearance and disappearance of bands compare to control diet were observed. With FOS, specific bands were detected. Analysis of samples with universal primers by TTGE (INRA) also showed no consistent patterns in relation to dietary treatment. However some specific bands were observed in ileum and cloaca. In the ileal content, with the FOS diet, a band (a) corresponding to a segmented filamentous bacterium which was no present with control diet, was observed. With the whole wheat diet, a band (b) migrating at the same level than the band (a) of FOS diet was also observed. Moreover, another band (c) corresponding to a *Lactobacillus salivarius*, not detected in control diet, was also observed with whole wheat diet. In the cloacal content, with avilamycin, a band (d) corresponding to a long segmented filamentous micoroorganism, not present with control diet, was detected. Moreover another band (h), with a very low intensity was observed with avilamycin diet, but not with control diet. With FOS diet, a band (i) migrating at the same level than the band (h) of very low intensity, was observed. With whole wheat diet, a band (e) corresponding to *Escherichia coli*, present in control diet, was not detected. On the contrary a band (j) migrating at the same level than the bands (h) and (i) was observed with the whole wheat diet. The bands (h, i, j) corresponded to an uncultured bacteria belonging to the Clostridiales order.

In conclusion, when analysing microflora of ileal contents of birds fed a negative control diet, the AGP salinomycin or different organic acids as alternatives to AGP, among the two fingerprint techniques (RFLP, CE-SSCP) used in this study to detect difference between dietary treatments, only RFLP was able to conduct to clear cluster of microflora samples according to treatment. Salinomycin treatment and the 4 organic acid treatments were clearly different from the negative control diet. Analysis of these same ileal samples with CE-SSCP did not lead to cluster. However this technique was able to detect disappearance of some bands with salinomycin. No difference of organic acid treatments with negative control diet was observed.
When studying microflora of different digestive contents (ileum, caeca, cloaca and fresh dropping) of birds fed a negative control diet, the AGP avilamycin or alternatives to AGP (prebiotic, probiotic, diet structure) with two fingerprint techniques, CE-SSCP and TTGE, although no cluster were observed according to dietary treatment, specific bands to treatment were detected with the antibiotic and the alternatives to antibiotic.

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A. General introduction

Four experiments have been performed to validate fingerprint methods with nutritional compounds known to induce high (antibiotic growth promoter) or low (feed composition and structure) perturbations of digestive microflora.
The fingerprint methods used in this work were two high-throughput fingerprint methods, Capillary Electrophoresis Single-Strand Conformation Polymorphism (CE-SSCP) and Restriction Fragment Length Polymorphism (RFLP), and another fingerprint method allowing identification of specific bacteria of dietary treatment, Temporal Temperature Gradient gel Electrophoresis (TTGE).
The modifications of diet composition were additives as prebiotics (FOS by INRA), probiotics (Bactocell ® by AFSSA), botanicals (essential oils and saponin by IRTA) and organic acids (calcium formate, sodium butyrate, lactic acid, organic acids blend by IRTA). Diet structure was modified by the use of wheat given separately as whole grain with a protein concentrate instead of ground in the complete diet (INRA). For a better comparison of the results, a negative and a positive control treatment with an antibiotic growth promoter (avilamycin or salinomycin) was included in all experiments.
The effect of the different alternatives to in-feed antibiotics was studied on animal performance and the digestive tract particularly on digestive microflora of broiler chickens.
Microflora was studied by classical bacterial counts (INRA: *E. coli*, *Lactobacillus spp.* and aerobic mesophil for INRA and AFSSA experiments; CReSA: *E. coli*, *Clostridium perfringens*, and *Lactobacillus spp.* for IRTA experiments) and molecular methods as Fluorescent In Situ Hybridization (FISH by KVL) and fingerprint methods (CE-SSCP by AFSSA, SSCP by KVL, RFLP by CReSA and TTGE by INRA).
Moreover the effect of the prebiotic and whole wheat on functionality of the digestive tract was assessed by measuring the gut morphology (villus and crypt) and the intestinal enzymatic activities (INRA). The effect of the probiotic on the morphology of intestinal tract was also studied. The effect of botanicals and organic acids on physical characteristics of the digestive content was assessed by viscosity measurement (IRTA).

B. Effects of botanicals on intestinal microbiota of broilers chickens by IRTA

Summary

One experiment was conducted to evaluate the feasibility of the PCR-SSCP, FISH and RFLP methods to demonstrate perturbations (of high or low extent) in the chicken intestinal flora, induced by the use of botanicals (essential oils and saponin (yucca extract)) as alternative to in-feed antibiotics. The study involved to test the efficacy of some botanicals on performance of broilers chickens and to provide intestinal content samples to other partners of the project, to elucidate the effect of these compounds in the intestinal microbiota composition by molecular techniques. Six hundred and forty eight male broiler chickens of the Ross 380 strain were used, distributed into 36 cages at eighteen chickens per cage. Birds were fed with a mash diets based on wheat, barley and soybean meal. Six dietary treatments were tested as follow: T-1) a negative control; T-2) positive control 10 ppm Avilamycin; T-3) thymol essential oil at 225 g/ton, T-4) carvacrol essential oil at 75 g/ton; T-5) cinnamaldehyde essential oil at 500 g/ton and T-6) yucca extract at 750 g/ton. Dietary treatments were replicated six times each and allocated at random by blocks. Body weight, average daily gain, average feed consumption, feed to gain ratio and mortality were determined at 10, 24 and 36 days. At day 22, six chickens per cage were sacrificed and samples from the distal part of the small intestine were taken, pooled and sent for microbiota analysis.

From 0 to 24 days, dietary treatments affected significantly ($P<0.05$) body weight, weight gain and feed to gain ratio. Birds fed yucca extract grew faster than birds given cinnamaldehyde, thymol or control diets and chickens fed carvacrol and avilamycin also grew faster than control birds ($P<0.05$). Birds fed yucca extract showed better feed to gain ratio than those birds fed avilamycin or control diets. The use of carvacrol or cinnamaldehyde also improved feed to gain ratio compared to control ($P<0.05$). In overall, no differences in feed intake were observed. From 24 to 36 days and in overall experiment (from 0 to 36 days) dietary treatments did not affect significantly performance parameters. However, birds from yucca extract and carvacrol groups were numerically heavier than the control and avilamycin groups. Numerically better feed to gain ratio was obtained with carvacrol, cinnamaldehyde or yucca extract compared with avilamycin. The mortality rate in overall experiment was 2.2% and no significant effect of treatments was detected. Supernatant jejunum digesta viscosities ranged from 7.66 cps for the control to 8.71 cps for yucca extract group, and no effect of dietary treatment was observed.

Analysis of microflora of ileal samples by conventional bacterial counts showed no effect of botanicals. Analysis by the two fingerprint techniques, CE-SSCP (AFSSA) and RFLP (CReSA) with universal primers, revealed no significant differences between the dietary treatments. With CE-SSCP, only Yucca extract may provoke a very slight specific modification of the microflora. Analysis by RFLP (CReSA) showed a significant effect of block (cage of the same block were located nearest than cages of other blocks). These results could represent a cross-contamination between the microbiota of different treatments located in the proximity of other treatments

1. Introduction

The main objective of the study was to evaluate the feasibility of the PCR-SSCP, FISH and RFLP methods to demonstrate perturbations (of high or low extent) in the chicken intestinal flora, induced by the use of botanicals as alternative to in-feed antibiotics.

The study involved to test the efficacy of some botanicals on performance of broilers chickens and to provide intestinal content samples to other partners of the project, to

elucidate the effect of these compounds in the intestinal microbiota composition by molecular techniques.

2. Materials and methods

Botanical test products

Pure essential oils (thymol, carvacrol and cinnamaldehyde) were provided by LUCTA SA (Ctra. Masnou a Granollers, Km. 12.4, 08170 Montornés del Valles, Barcelona, Spain) and yucca extract as a source of saponins was provided by ITPSA (Avda. Roma 157, 7è, 08011 Barcelona, Spain).

Feeding program

The feeding program consisted of two steps: starter diet from 0 to 24 days (3000 kcal/kg, 21.5% of crude protein) and grower diet from 24 to 36 days (3100 kcal/kg, 19.5% of crude protein).

Experimental diets were based on wheat-barley and soybean meal and their composition and calculated nutrient content are presented in Table 1.

The premix and experimental feeds were manufactured at Feed Mill of IRTA. All feed ingredients, except fat, salt, dicalcium phosphate, calcium carbonate, the vitamin and mineral premix and test products were ground through a 25 CV hammermill until the particles pass through a 3 mm sieve. The mixer was a 1000 L capacity horizontal mixer, and the mixing time was 5 min. Amino acids, mineral and vitamin premix and test products were mixed with and aliquot of 10 kg of feed ration in a small mixer, and then added immediately to the mixer.

Feed samples were taken for analysis of crude protein by the Dumas procedure by means of a Nitrogen/protein FP-528 determinator (LECO Corp. St Joseph, MO, USA), moisture (AOAC, 2000), ether extract by means of a Buchi Extraction System B-811 (Buchi Labortechnik AG, Flewil, Switzerland) and chloride by AOAC (2000), to test the homogeneity of mixing.

Feed and water were provided *ad-libitum* during the experiment and feed was presented in mash form.

The feed used in this trial did not contain any anticoccidial drug, antibiotic growth promoter or any other probiotic feed additive.

Housing and management

The animal experiment was performed in Experimental farm of IRTA in Centre de Mas Bové, Ctra. Reus a El Morell, km. 4.5, 43120 Constantí (Spain) during thirty six days.

Chickens were housed in room provided with 36 cages of 1 m2 each. The house was provided with forced ventilation, artificial light and gas heating. On arrival, chickens were distributed at random at 18 chicks per cage. Feed and water will be provided ad-libitum throughout the experiment and feeders and waterers were of plastic. Temperature inside the house on arrival was programmed as follows: 31-33°C (d0-d3), 31-32°C (d4 - d7), 29-31°C (d8 - d14), 28-29°C (d15 - d21), 25-27°C (d22 - d24), 22-24°C (d25 - d27), 20-22° C (d28 - d36). The lighting programme was scheduled as follows: 24h of light (d0 – d4), 20h of light (d5 - d11) and 18h of light (d12 – d36).

Typical prophylactic cleaning, disinfecting and vaccination were carried out according to the routine practice. Twice daily observations were recorded for general flock condition, temperature, lighting, water, feed, litter condition and mortality.

Animals

Six hundred and forty eight male broiler chickens of the Ross 380 strain were used, and distributed into 36 cages, at eighteen chickens per cage. Only animals free of any clinical signs, e.g. no leg problems, eyes opened, active behaviour, and no other problems, were included in the trial.

Treatments and experimental design

The experiment was designed as a randomised complete block design, with six blocks, six dietary treatments and six replicates of 18 birds per treatment.
The arrangement of treatments was: T1) negative control; T2) positive control- avilamycin (10 g/ton); T3) thymol (225 g/ton); T4) carvacrol (75 g/ton); T5) cinnamaldehyde (500 g/ton); T6) yucca extract (750 g/ton).
The dose used for each additive was selected according to their in-vitro antimicrobial activity, flavour impact and toxicity.

Zootechnical performance
Chicks were weighed in bulk on arrival, and per cage at 10, 24 and 36 days. Feed consumption per cage was recorded at days 10, 24 and 36. Average daily gain, average daily feed consumption and feed to gain ratio were calculated for the periods 0 to 10 days, 10 to 24 days, 24 to 36 days and for the overall experiment. Mortality was checked and recorded daily, including the cause of the death.

Sampling of the intestinal content for microbial measurements
At day 22, six chickens per cage, representative of the cage according to their apparent weight, were sacrificed by intravenous injection of Tiobarbital (150 mg / kg body weight) according to the experimental procedure num. 689, approved by the Ethical Commission of IRTA. Samples from the distal part of the small intestine (from Meckel's diverticulum to ileo-caecal junction) were taken by gentle squeezing. Samples from the six birds per cage were collected into sterile containers for pooling. Samples were kept at 4ºC (crushed ice).during sampling and before pooling / splitting.
From the pooled samples the following sub-sampling was done:
- 1 g of sample preserved in 3 ml ethanol 96% were stored at 4ºC and sent for RFLP measurements.
- 1 g of sample preserved in 3 ml ethanol 96% were stored and maintained at 4°C during transport and sent for PCR-SSCP measurements
- 1 (±0.1) g of sample preserved in 3 ml in ethanol:PBS (1:1) were stored and maintained at 4°C during transport and sent for FISH measurements. The exactly weight of sample were recorded by weighting the tube+preserver before and after adding the sample.
- 3 g of samples freezed and stored at -70ºC were sent with dry ice (-80°C) for *E. coli*, *Clostridium perfringens* and *Lactobacillus sp*. counts.
The PBS used for sample for FISH measurements was prepared as follows: 8 g of NaCl per liter, 0.2 g of KCl per liter, 1.44 g of Na2HPO4 per liter, 0.24 g of KH2PO4 per liter, pH 7.2. The chemicals were of analytical quality and water double distilled. Before use, the solution was filtered to exclude bacteria sized particles. The ethanol was of 96%.

Intestinal digesta viscosity
At the same day of sampling from microbial measurements and from two birds from each cage, jejunum digesta samples (from the distal part of duodenum to Meckel's diverticulum) were collected and kept on ice, to measure supernatant digesta viscosity. Samples were centrifuged at 10000 rpm for 15 min. at 15ºC and supernatant viscosity was measured by using a Brookfield digital viscometer (model LVTDVCP-II, Brookfield Engineering Laboratories, Stouhton, MA), maintained at 30ºC and reading after 1 min.

Conventional bacterial counts
The samples for bacterial analysis were successively diluted at 1/10 in 9.5 g/l Maximum Recovery Diluent (Merck) and analysed for *Escherichia coli*, *Lactobacillus spp* and *Clostridium perfringens*. *E. coli* were counted by plating serial dilution on McConkey Agar (Difco) and incubated aerobically for 24 hours at 37ºC. The lactic acid bacteria were counted after being plated onto MRS agar (Lactobacilli MRS Broth, Difco, supplemented with 15g/l of Bacto Agar, Difco) and incubated anaerobically for 48 hours at 37ºC, and *Clostridium perfringens* were counted by plating on Perfringens Agar (Oxoid) supplemented with

Perfringens Supplement A and B (Oxoid) and incubated anaerobically for 24 hours at 37ºC.. The results were expressed as $\log_{10}$ colony forming units (CFU)/g of digestive contents.

Bacterial numbers according to 16S rRNA group specific FISH oligonucleotide probes
Several probes were used for *Lactobacillus, Bacillus, Enterococcus and Streptococcus, Streptococcus, Enterobacteria, Salmonella* (Olsen et al. 2006).

Fingerprint techniques

DNA extraction
The ethanol was removed from samples (pools of ileal content of 6 birds) after centrifugation (9 000 g) and the pellet was rinsed three times with physiological water. DNA was extracted from 200 mg samples using the QIAamp DNA Stool Mini Kit (Qiagen) as described by the manufacturer. An additional treatment with lysozyme was performed in order to improve the extraction of Gram positive bacteria DNA. After the step of incubation of samples with ASL buffer during 5 minutes at 95°C, and before the use of InhibitEX tablets, 140µl of a 10 mg/mL of lysozyme (Sigma L-7651) in Tris-EDTA pH 8 (Tris 10 mM, EDTA 1 mM) was added to each extraction tube. Samples with lysozyme were incubated at 37°C during 30 min. At the end of the procedure, the purified DNA was stabilised with the addition of 4µL of 40 mg/ml BSA (Bovine Serum Albumin, Sigma B-4287) plus 2 µl of Ribonuclease–A (Sigma R-4642) and maintained at -20°C until used. The concentration and integrity of nucleic acids were determined by electrophoresis on 1% agarose gel containing ethidium bromide. The DNA extracted from each of the 6 pools of digestive content of each dietary treatment was used to compare the 6 replicates per dietary treatment by CE-SSCP and RFLP.

CE-SSCP
PCR reaction: For total microflora analysis, PCR was performed according to Delbes *et al*., (2001) by amplification of the V3 region with the primers W49 (ACG GTC CAG ACT CCT ACG GG) and W104 (TTA CCG CGG CTG CTG GCA C). These primers are specific for the Eubacteria phylogenic domain. Primers W49 and W104 were labelled on the 5' end with hexachloro derivative of fluorescein (Hex) and 5'-fluorescein-CE phosphoramidite (6-Fam) respectively. The amplification of the V3 region were performed by using the *pfu* Turbo enzyme (Stratagene, La Jolla, CA). After a step of DNA denaturing 10 minutes at 94°C, 25 cycles composed of 30 sec. at 94°C, 30 sec. at 61°C (W49-W104) and 30 sec. at 72°C were run. After PCR, amplified DNA was loaded onto a 2% agarose gel and stained with 0.5 mg/ml ethidium bromide. Images were captured with a Biocapt camera (Bioblock Scientific).
CE-SSCP electrophoresis: DNA was mixed with formamide and Genescan 400 HD-Rox standard (Applied Biosystems, France) according to the ratios 1:18.5:0.5. After a denaturing step at 95°C during 10 minutes, the mix was quickly cooled on ice. The 96-well plate containing the samples was placed into an ABI Prism Genetic Analyzer 3100-*Avent* (Applied Biosystems, France). The non-denaturing polymer matrix used was 5.6% CAP polymer (Applied Biosystems, France) - 10% Glycerol - 1x TBE. The electrophoresis was performed in 1x TBE buffer - 10% Glycerol. The samples were run at 15 kV at 32°C. The data were collected with the Gene Mapper V4.0 software. A normalisation was performed by using the internal standard 400 HD-Rox.

RFLP
PCR reaction: Two primers 5'-CTACGGGAGGCAGCAGT-3' and 5'-CCGTCWATTCMTTTGAGTTT-3' (Sigma-Genosys) designed for regions of the 16S rRNA gene highly conserved among a wide range of microorganisms were used for PCR amplification.
PCR reaction included: PCR-Master Mix (Applied Biosystems), with 1.25 IU of Taq polymerase, DNA template, the preceding primers, and distilled water in a total volume of 50 µl.

PCR mixtures were heated to 94°C for 5 minutes once, followed by 35 cycles of denaturation at 94°C for 1 minute, primer annealing at 45°C for 1 minute, and DNA extension at 72° for 1:15 minutes. The last extension cycle was continued for 5 minutes. The PCR amplification reaction was conducted in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems).
In general, PCR amplification products were processed just after amplification, but they will be maintained at -20ºC until use in other case.
RFLP analysis : The DNA fragments amplified by PCR were digested with *Alu* I, *Rsa* I, *Hpa* II, *Sau* 3A I or *Cfo* I restriction endonucleasas (Sigma-Aldrich) in accordance with manufacturer specifications, but with SA buffer as common buffer to avoid the effect of the pH differences of samples on electrophoresis separation of DNA fragments.
The restriction endonuclease fragments were analysed using a 2% wide range agarose electrophoresis, supplemented with ethidium bromide. For the electrophoresis separation 150 V during 60 minutes were applied. The bands of DNA were visualized in an UV Chemigenious Image System (SynGene) using the GeneSnap software (SynGene). Pictures with 4.63 seconds exposure were stored.
The electrophoretic profiles obtained, known as Restriction Fragment Length Polymorphism (RFLP), are highly characteristic of the microbial genera, and in some cases, of the microbial species.
Two molecular weight controls were used: Step Ladder, 50 bp (Sigma-Aldrich) and our own control prepared by digestion of amplified fragments of 16S r-DNA of well characterized animal bacteria.

Statistical analysis
The experiment was set up us a randomised block design and data (performance, viscosity, microbial counts by conventional and FISH method) were subject to a two way analysis of variance (block and treatment) by using the General Linear Models (GLM) procedures of SAS. The level of statistical significance was pre-set at $P<0.05$. Treatments means were compared by the multiple range test of Duncan.
The CE-SSCP profiles were compared using Bionumerics software (Applied Maths, Belgium). The comparisons of profiles were based on the Pearson similarity coefficient which tooks into account the number of bands, their position, and their intensity. Similarity coefficient, calculated for each pair of profiles, yielded a similarity matrix. A dendrogram was constructed from this matrix by using the unweighted pair group method using arithmetic averages (UPGMA).
For RFLP profiles, the sizes of all the bands obtained were calculated with the aid of the GeneTools software (SynGene). Dendrograms considering the size and the intensity of the bands were constructed. Biodiversity degree was calculated as the number of bands of each sample.

3. Results

Performances
From 0 to 10 days (Table 2), dietary treatments affected significantly ($P<0.05$) body weight, weight gain and feed to gain ratio. Weight gain of birds fed carvacrol or yucca extract were higher compared with birds from the negative control ($P<0.05$). Carvacrol group shown better feed to gain ratio in comparison with avilamycin or control groups ($P<0.05$). No differences in feed intake were observed between treatments.
From 10 to 24 days (Table 3), birds fed the yucca extract grew faster than broilers fed cinnamaldehyde, thymol or control ($P<0.05$) and at the same time, birds fed carvacrol or avilamycin grew faster than the control group ($P<0.05$). The better feed to gain ratio was obtained with yucca extract, significantly different ($P<0.05$) from feed to gain ratio of birds fed thymol or control diets and not different from that obtained with cinnamaldehyde, carvacrol or avilamycin. Dietary treatment did not affected significantly daily feed intake.

In the accumulate period (from 0 to 24 days), results shown similar trends (Table 4). Birds fed yucca extract grew faster than birds given cinnamaldehyde, thymol or control diets and chickens fed carvacrol and avilamycin also grew faster that control birds ($P<0.05$). Birds fed yucca extract sowed better feed to gain ratio than those birds fed avilamycin or control diets. The use of carvacrol or cinnamaldehyde also improved feed to gain ratio compared with the control ($P<0.05$). In overall, no differences in feed intake were observed.
From 24 to 36 days (Table 5) and in overall experiment (from 0 to 36 days, Table 6) dietary treatments did not affect significantly performance parameters. Although differences did not reach significance ($P>0.05$), numerically differences between treatments were observed in final body weight, weight gain and feed to gain ratio in overall. The heavier birds were from yucca extract and carvacrol groups (103 and 89 g more than the control, respectively), the medium birds from avilamycin group (54 g) and the lighters birds from cinnamaldehyde, thymol and control groups. Numerically better feed to gain ratio was obtained with carvacrol, cinnamaldehyde or yucca extract compared with thymol, avilamycin or control diets.
The mortality rate from 0 to 10 days was 0.8% on average, from 10 to 24 days 0.9%, from 24 to 36 days 1.0% and 2.2% in overall experiment (Table 7). There was no significant effect of treatments over this parameter in any period and in overall experiment.

Digesta viscosity
Results from the supernatant jejunum digesta viscosity measurements at day 22 are presented in Table 8. Digesta viscosities ranged from 7.66 cps for the control to 8.71 cps for yucca extract group, but dietary treatment did not affect this parameter ($P<0.05$).

Conventional microbial counts
No effect of botanicals was observed on bacterial counts (data not shown).

Bacterial numbers according to 16S rRNA group specific FISH oligonucleotide probes
The study of the effects of avilamycin and some botanicals on different bacteria groups (*Lactobacillus, Bacillus, Enterococcus and Streptococcus, Streptococcus, Enterobacteria, Salmonella*) by FISH counts in ileal contents showed a high variability between samples (Table 9). With avilamycin, numerically lower counts of each bacteria group were observed (except similar value for *Streptococcus*) compared with negative control. The use of thymol and carvacrol also tended to reduce the number of cells of each bacteria group, compared with the negative control, and the effect was similar to that of avilamycin. With cinnamaldehyde numerically lower counts of each bacteria group were observed compared with all the other treatments including avilamycin. Yucca extract resulted in higher counts of Bacteroides, compared with botanicals and avilamycin, and also compared with negative control. The higher *Salmonella* counts were found in the negative control group and the lower counts in avilamycin and carvacrol groups**.**

Microbiota profiles
CE-SSCP
No detectable flora modification was observed for the animals treated with Avilamycin.
No high modification of the microflora was observed whatever the botanical used. In consequence, the samples were not joined together in clusters in function of the treatment. No significant band was detected when the fingerprint with 6-Fam labelling was observed (Figure 1). On the contrary, one band (Figure 2, position 214.8) was present in two samples from animals treated with Yucca extract (n°24 and 38) when the fingerprint with hex labelling was studied. This band was absent, in all the other samples.
In conclusion, only Yucca extract may provoke a very slight specific modification of the microflora detectable by CE-SSCP with universal primers.

RFLP

Analysis of samples by RFLP showed no significant differences between treatments but a significant effect of block (cage of the same block were located nearest than cages of other blocks) (data not shown). These results could represent a cross-contamination between the microbiota of different treatments located in the proximity of other treatments.

4. Discussion

The results of the present experiment suggested that some botanical compounds tested, added to a wheat and barley-based diet, may improve performance of birds in terms of growth and feed to gain ratio. The improvement was significant during the first twenty-four days of the growing period for weight gain with yucca extract at 750 g/ton and carvacrol at 75 g/ton, and this improvement was numerically higher than those obtained with avilamycin at 10 g/ton. Birds fed yucca extract, carvacrol and cinnamaldehyde showed better feed to gain ratio than those birds fed control diets. No significant effects of botanicals compounds were observed from 24 to 36 days of age, however birds from yucca extract and carvacrol groups were numerically heavier than the control and avilamycin group. Numerically better feed to gain ratio was obtained with carvacrol, cinnamaldehyde or yucca extract compared with avilamycin. The growth enhancing effect of essential oils might be attributed to their antimicrobial activity, antioxidative properties or the stimulating effect on animal digestive system (Lee et al., 2004). Yucca plant extract contains a glyco-component fraction which binds ammonia and a steroidal saponin fraction which has surface active properties (Cheeke, 2000) and it is believe that yucca can inhibit the production of ammonia by bacteria in the gut, and possible, to produce some change in the intestinal microbiota profile.

5. References

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS (2000) Official methods of analysis. 17th ed., Association of Official Analytical Chemist. Washington D. C

Cheeke, P.R. 2000. Actual and potential applications of *Yucca schidigera* and *Quaillaja saponaria* saponins in human and animal nutrition. Proc. American Society of Animal Science.

Lee, K.W., Everts, H. and Beynen, A.C. 2004. Essential oils in broiler nutrition. International Journal of Poultry Science, 3: 738-752.

Olsen, K. N., Francesch, M. and Christensen, H. 2006. Fluoroscence in situ hybridization of chicken intestinal samples with bacterial rRNA targeted oligonucleotide probes. EPCXII Verona.

6. List of tables

Table 1. Composition and calculated nutrient composition of experimental feeds

	Starter diet (0-24 days)	Grower diet (24-36 days)
Ingredient (%)		
Wheat	44.1	48.8
Barley	15.0	15.0
Soybean oil	4.0	6.0
Full fat extruded soybeans	12.3	5.8
Soybean meal 48	20.6	20.9
DL-Methionine	0.245	0.218
Lysine HCl	0.067	-
Threonine	0.048	-
Tryptophan	0.001	
Calcium carbonate	1.275	1.229
Dicalcium phosphate	1.591	1.301
Salt	0.324	0.336
Minerals and vitamins[1]	0.400	0.400
Choline chloride (50%)	0.037	0.011
Calculated nutrient content[3]		
Metabolisable energy (kcal/kg)	3000	3100
Crude protein (%)	21.5	19.5
Crude fibre (%)	3.35	3.10
Crude fat (%)	7.82	8.48
Ash (%)	5.99	5.46
Lysine (%)	1.100	0.930
Methionine (%)	0.550	0.500
Met + Cys (%)	0.908	0.835
Threonine	0.794	0.677
Tryptophan	0.259	0.239
Calcium (%)	1.000	0.900
Available phosphorus (%)	0.429	0.370

[1]One kg of feed contains: Vitamin A: 12000 IU; Vitamin D_3: 5000 IU; Vitamin E: 30 mg; Vitamin K_3: 3 mg; Vitamin B_1: 2,2 mg; Vitamin B_2: 8 mg; Vitamin B_6: 5 mg; Vitamin B_{12}: 11 μg; Folic acid: 1,5 mg; Biotin: 150 μg; Calcium pantothenate: 25 mg; nicotinic acid: 65 mg; Mn: 60 mg; Zn: 40 mg; I: 0,33 mg; Fe: 80 mg; Cu: 8 mg; Se: 0,15 mg; Etoxiquín: 150 mg.

Table 2. Effects of botanicals on growth performance of broilers chickens from 0 to 10 days

		Body weight at day 10 (g)	Daily weight gain (g)	Daily feed intake (g)	Feed to gain ratio
T-1	Negative control	187 ^{c}	14.3 ^{c}	20.7	1.445 ^{a}
T-2	Avilamycin	194 ^{abc}	15.1 ^{abc}	21.8	1.455 ^{a}
T-3	Thymol	190 ^{bc}	14.7 ^{bc}	20.3	1.388 ^{ab}
T-4	Carvacrol	205 ^{a}	16.1 ^{a}	21.3	1.321 ^{b}
T-5	Cinnamaldehyde	194 ^{abc}	15.0 ^{abc}	20.7	1.381 ^{ab}
T-6	Yucca extract	202 ^{ab}	15.8^{ab}	21.7	1.377 ^{ab}
	Standard error	3.76	0.38	0.49	0.0293
	Anova (Pr>F)	*0.05*	*0.05*	0.21	*0.05*

Values are means of 6 replicates of 18 chickens per treatment. Means within a column with different superscript differ significantly (P<0.05).

Table 3. Effects of botanicals on growth performance of broilers chickens from 10 to 24 days

		Body weight at day 24 (g)	Daily weight gain (g)	Daily feed intake (g)	Feed to gain ratio
T-1	Negative control	828 ^{c}	45.8 ^{c}	67.2	1.465 ^{a}
T-2	Avilamycin	885 ^{ab}	49.3 ^{ab}	70.7	1.434 ^{bc}
T-3	Thymol	843 ^{bc}	46.6 ^{bc}	67.4	1.458 ^{ab}
T-4	Carvacrol	888 ^{ab}	48.9 ^{abc}	69.9	1.433 ^{bc}
T-5	Cinnamaldehyde	866 ^{bc}	48.0 ^{bc}	68.6	1.429 ^{bc}
T-6	Yucca extract	927 ^{a}	51.8 ^{a}	73.2	1.413 ^{c}
	Standard error	17.0	1.02	1.59	0.0101
	Anova (Pr>F)	*0.01*	*0.01*	0.11	*0.05*

Values are means of 6 replicates of 18 chickens per treatment. Means within a column with different superscript differ significantly (P<0.05).

Table 4. Effects of botanicals on growth performance of broilers chickens from 0 to 24 days

		Body weight at day 24 (g)	Daily weight gain (g)	Daily feed intake (g)	Feed to gain ratio
T-1	Negative control	828 [c]	32.7 [c]	47.8	1.461 [a]
T-2	Avilamycin	885 [ab]	35.0 [ab]	50.3	1.438 [ab]
T-3	Thymol	843 [bc]	33.3 [bc]	47.8	1.435 [abc]
T-4	Carvacrol	889 [ab]	35.2 [ab]	49.6	1.410 [bc]
T-5	Cinnamaldehyde	866 [bc]	34.3 [bc]	48.6	1.420 [bc]
T-6	Yucca extract	927 [a]	36.8 [a]	51.7	1.405 [c]
	Standard error	17.0	0.71	1.05	0.0097
	Anova (Pr>F)	*0.01*	*0.01*	*0.09*	*0.01*

Values are means of 6 replicates of 18 chickens per treatment. Means within a column with different superscript differ significantly (P<0.05).

Table 5. Effects of botanicals on growth performance of broilers chickens from 24 to 36 days

		Body weight at day 36 (g)	Daily weight gain (g)	Daily feed intake (g)	Feed to gain ratio
T-1	Negative control	1797	80.7	138.1	1.712
T-2	Avilamycin	1851	80.6	139.6	1.736
T-3	Thymol	1789	78.8	136.1	1.727
T-4	Carvacrol	1886	83.2	142.7	1.717
T-5	Cinnamaldehyde	1808	78.5	134.9	1.721
T-6	Yucca extract	1900	81.1	142.1	1.752
	Standard error	33.1	1.94	2.79	0.0203
	Anova (Pr>F)	0.10	0.59	0.31	0.76

Values are means of 6 replicates of 12 chickens per treatment.

Table 6. Effects of botanicals on growth performance of broilers chickens from 0 to 36 days

		Body weight at day 36 (g)	Daily weight gain (g)	Daily feed intake (g)	Feed to gain ratio
T-1	Negative control	1797	48.7	76.6	1.573
T-2	Avilamycin	1851	50.2	78.6	1.567
T-3	Thymol	1789	48.5	75.8	1.563
T-4	Carvacrol	1886	51.8	79.0	1.544
T-5	Cinnamaldehyde	1808	49.0	75.9	1.548
T-6	Yucca extract	1900	51.6	79.9	1.549
	Standard error	33.1	0.92	1.46	0.0109
	Anova (Pr>F)	0.10	0.10	0.24	0.35

Values are means of 6 replicates of 18 chickens per treatment from 0 to 24 days and 12 chickens from 24 to 36 days.

Table 7. Effects of botanicals on mortality rate

		0-10 days	**10-24 days**	**24-36 days**	**0-36 days**
T-1	Negative control	0.83	0.00	1.52	1.67
T-2	Avilamycin	0.83	0.00	0.00	0.83
T-3	Thymol	0.00	0.00	0.00	0.00
T-4	Carvacrol	0.00	0.00	2.78	1.76
T-5	Cinnamaldehyde	1.68	1.80	1.38	4.03
T-6	Yuca extract	1.76	3.70	0.00	5.09
	Standard error	0.798	1.105	1.089	1.487
	Anova (Pr>F)	*0.48*	*0.14*	*0.37*	0.14

Values are means of 6 replicates of 18 chickens per treatment from 0 to 24 days and 12 chickens from 24 to 36 days.

Table 8. Effects of botanicals on supernatant jejunum digesta viscosity ad day 22

		Viscosity (cps)
T-1	Negative control	7.66
T-2	Avilamycin	8.00
T-3	Thymol	8.28
T-4	Carvacrol	8.02
T-5	Cinnamaldehyde	8.14
T-6	Yuca extract	8.71
	Standard error	0.752
	Anova (Pr>F)	*0.92*

Values are means of 6 replicates per treatment.

Table 9. Effects of botanicals on bacterial numbers in ileum according to 16S rRNA group specific FISH oligonucleotide probes (10^7 cells / g (mean (SD))

	Oligonucleotide probe				
	EUB338	LGC354A	LGC354B	LGC354C	ProbeD
Control	19.7 (14.5)	10.7 (13.0)	11.9 (16.0)	8.10 (6.54)	9.49 (11.2)
Avilamycin	15.0 (16.2)	4.99 (7.30)	6.43 (5.78)	6.90 (9.50)	4.99 (7.50)
Thymol	12.3 (9.64)	3.06 (3.37)	3.76 (2.25)	5.03 (7.09)	4.69 (6.79)
Carvacrol	14.7 (6.35)	5.49 (5.90)	6.27 (4.96)	5.95 (2.72)	5.06 (4.90)
Cinnamaldehyde	8.89 (5.19)	1.88 (1.49)	3.21 (2.37)	3.64 (2.56)	2.37 (3.50)
Yucca	20.8 (15.1)	12.2 (11.6)	6.78 (6.94)	7.78 (4.37)	8.81 (10.8)

	Oligonucleotide probe			
	Strc493	Bacto1080	Chis150	Sal3
Control	5.87 (8.50)	10.2 (9.21)	8.19 (6.92)	10.4 (11.8)
Avilamycin	5.06 (6.86)	2.30 (1.29)	4.70 (4.14)	3.52 (4.44)
Thymol	3.38 (4.04)	3.62 (6.45)	3.57 (5.28)	4.48 (8.22)
Carvacrol	4.26 (4.95)	5.65 (4.09)	4.16 (3.89)	4.15 (5.36)
Cinnamaldehyde	2.07 (2.46)	3.28 (4.02)	2.13 (1.90)	2.57 (1.93)
Yucca	5.15 (4.10)	18.4 (30.0)	5.44 (4.25)	9.21 (10.6)

7. List of figures

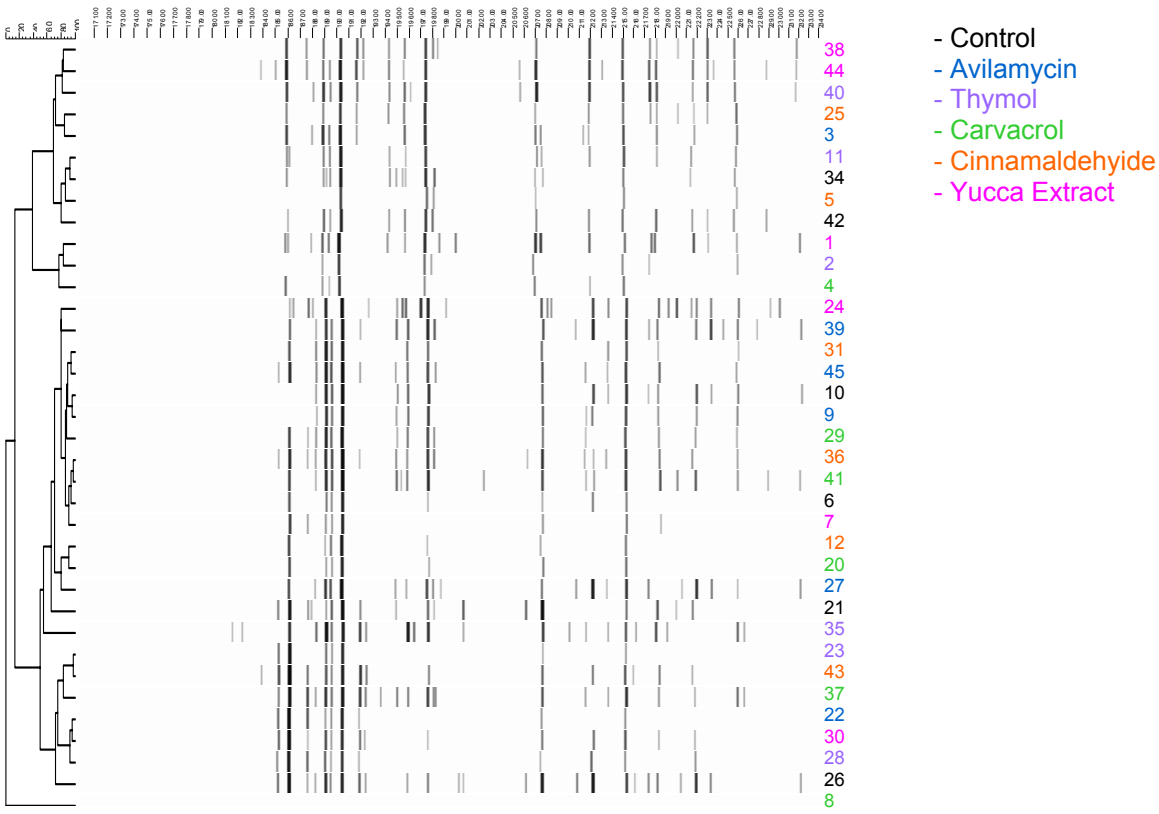

Figure 1. Similarity of the fingerprints obtained by CE-SSCP (6-Fam labelling) from ileum pools after different treatments with botanicals. Dendrograms were realised by using Pearson correlation coefficient and UPGMA method.

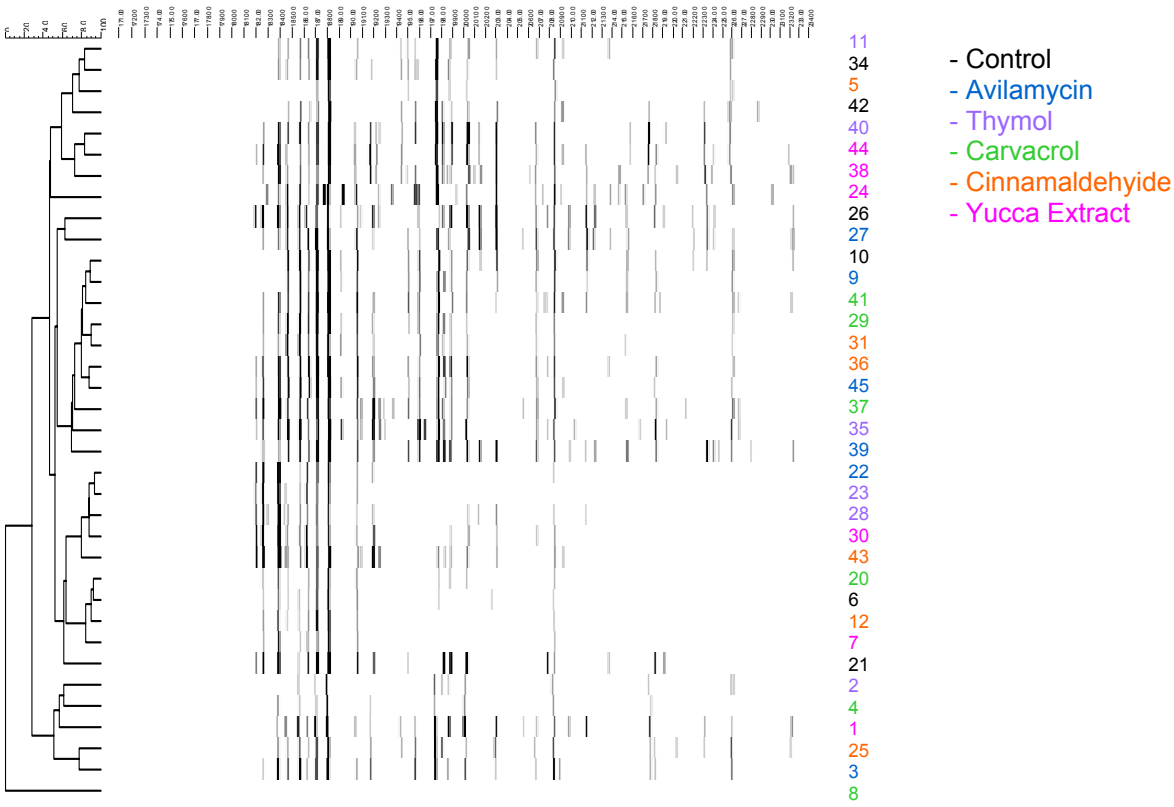

Figure 2. Similarity of the fingerprints obtained by CE-SSCP (Hex labelling) from ileum pools after different treatments with botanicals. Dendrograms were realised by using Pearson correlation coefficient and UPGMA method.

C. Effects of organic acids on intestinal microbiota of broilers chickens by IRTA

Summary

One experiment was conducted to evaluate the feasibility of molecular methods as Fluorescent In Situ Hybridization (FISH) and fingerprint methods (Capillary Electrophoresis Single-Strand Conformation Polymorphism (CE-SSCP) and Restriction Fragment Length Polymorphism (RFLP)), to demonstrate perturbations (of high or low extent) in the chicken intestinal flora, induced by the use of organic acids as alternative to in-feed antibiotics. The study involved to test the efficacy of some organic acids on performance of broilers chickens and to provide intestinal content samples to other partners of the project, to elucidate the effect of these compounds in the intestinal microbiota composition by molecular techniques. Six hundred and forty eight male broiler chickens of the Ross 380 strain were used, distributed into 36 cages at eighteen chickens per cage. Birds were fed with mash diets based on wheat, barley and soybean meal. Six dietary treatments replicated six times were tested as follows: T-1) NC (negative control); T-2) positive control SAL (Salinomycin, 75 mg/kg feed); T-3) CF (calcium formate, 68% formic acid, at 7.4 kg/ton), T-4) SBC (sodium butyrate coated, 30% butyric acid, at 1 kg/ton from 0-24 d and 0.5 kg/ ton from 24-35 d); T-5) LA (lactic acid 63% at 5 kg/ton) and T-6) OAB (blend of formic and propionic acids and ammonium formate, 43% expressed as formic acid and ammonium formate, at 4 kg/ton). Body weight, average daily gain, average feed consumption, feed to gain ratio and mortality were determined at 10, 24 and 35 days. At day 21, six chickens per cage were sacrificed and samples from the small intestine content were taken and pooled for ileum microbiota analysis and supernatant jejunum digesta viscosities measurements.

During the first 24 days, the blend of formic and propionic acids with ammonium formate, as well as calcium formate, improved substantially weight gain and increased feed intake, without affecting FCR. The improvement was similar to those obtained with salinomycine. In overall experiment (from 0 to 35 days), the organic acids blend tended to increase weight gain and feed consumption compared to the negative and lactic acid groups. The use of calcium formate, sodium butyrate and the organic acids blend impaired feed to gain ratio compared to negative control and salinomycine groups. In overall growing period, positive effects of the use of organic acids in terms of weight gain could be detected. The lowest digesta viscosity was observed with calcium formate and the highest with sodium butyrate.

Analysis of ileal microflora by conventional bacterial count showed higher *Lactobacillus spp*. counts with salinomycin and organic acids compared with negative control. No effect was observed on *Escherichia coli* and *Clostridium perfringens* intestinal counts. Results obtained by FISH showed higher number of bacteria belonging to Domain Bacteria (EUB338 probe) in ileum from birds fed sodium butyrate, compared with lactic acid, organic acids blend or negative control. Lower counts with LGC354C probes (*Enterococcus*, *Streptococcus*, *Lactococcus*) was observed with salinomycin compared with negative control or organic acids diets. Some organic acids affected members of *Enterobacteria*, *Bacteroides* and *Clostridium* significantly. Calcium formate decreased *Enterobacteria* compared with negative control and sodium butyrate diets. Lactic acid decreased Bacteroides compared with negative control, salinomycin and sodium butyrate diets. Calcium formate, lactic acid and organic acids blend diet decreased *Clostridium* compared with salinomycin diet. Analysis of ileal samples by RFLP (CReSA) with universal primers showed significant diet effect in ileum samples between treatments**.** Negative control as well feed supplemented with sodium butyrate are clearly different to the other treatments. RFLP profiles of feed supplemented with salinomycin and feed supplemented with calcium formate were not clearly different. RFLP profiles of feed supplemented with lactic acid and feed supplemented with formic and

propionic acids and ammonium formate have some kind of association. Moreover, differences between treatments were detected in microbial biodiversity, as the number of electrophoretic bands in the RFLP profiles. Negative control biodiversity was significantly lower ($p<0.05$) than the biodiversity of feed supplemented with salinomycin, feed supplemented with sodium butyrate and feed supplemented with formic and propionic acids and ammonium formate. Analysis of ileal samples by CE-SSCP (AFSSA) with universal primers did not lead to cluster according to dietary treatments, but with salinomycin, it was observed a disappearance of some bands or bands with lower intensity compared to the other diets.

1. Introduction

The objective of the study was to evaluate the feasibility of the PCR-SSCP, FISH and RFLP methods to demonstrate perturbations (of high or low extent) in the chicken intestinal flora, affected by organic acids, as alternative to in-feed antibiotics.

2. Materials and methods

Test products

The organic acids studied were calcium formate, sodium butyrate, lactic acid and organic acids blend.

Feeding program

The feeding program consisted of two diets: starter diet from 0 to 24 days (3000 kcal/kg, 21.5% of crude protein) and grower diet from 24 to 35 days (3100 kcal/kg, 19.5% of crude protein).

Experimental diets were based on wheat-barley and soybean meal and their tentative composition and calculated nutrient content are presented in Table 1.

The premix and experimental feeds was manufactured at Feed Mill of IRTA. All feed ingredients, except fat, salt, dicalcium phosphate, calcium carbonate, the vitamin and mineral premix and test product were ground through a 25 CV hammermill until the particles pass through a 3 mm sieve. The mixer is a 1000 L capacity horizontal mixer, and the mixing time will be 5 min. Amino acids, mineral and vitamin premix and test products were mixed with and aliquot of 10 kg of feed ration in a small mixer, and then added immediately to the mixer.

Feed samples were taken for analysis of crude protein by the Dumas procedure by means of a Nitrogen/protein FP-528 determinator (LECO Corp. St Joseph, MO, USA), moisture (AOAC, 2000), ether extract by means of a Buchi Extraction System B-811 (Buchi Labortechnik AG, Flewil, Switzerland) in the laboratory of the Department of Animal Nutrition of IRTA. Also, samples were analysed for chloride by AOAC (2000) to test the homogeneity of mixing.

The feed used in this trial did not contain any anticoccidial drug, antibiotic growth promoter or any other probiotic feed additive. Feed were presented in mash form.

Location, housing and management

The animal experiment was conducted in the experimental farm of the Department of animal Nutrition of IRTA (Mas de Bover, Ctra. Reus a El Morell, km. 3.8, 43120 Constantí, Spain) during five weeks. Chickens were housed in room provided with 48 cages of 1 m2 each, but only 36 cages were used. The floor, walls and ceiling of cages were on wire and they were adapted to use litter. Moreover, every group of six cages was separately physically by cardboard walls, to allocate each dietary treatment separately from the others treatments. The house was provided with forced ventilation, artificial light and gas heating. On arrival, chickens were distributed at random at 18 chicks per cage. Feed and water were provided *ad-libitum* throughout the experiment and feeders and waterers were plastic made. Temperature inside the houses were programmed as follows: 31-33°C from day 0 to 3, 31-32°C from day 4 to 7, 29-31°C from day 8 to 14, 28-29°C from day 15 to 21, 25-27°C from

day 22 to 24, 22-24°C from day 25 to 27 , 20-22° C from day 28 to 34, and 19°C at day 35. From day 0 to 4 the lighting programme consisted of 24 hours light, from day 5 to 11, it consisted of 20 hours light and 4 hours dark (22pm-2 am) and from day 12 to the end of the experiment 18 hours light and 6 hours dark (21pm-3 am).
Typical prophylactic cleaning, disinfecting and vaccination (infectious bronchitis) were carried out according to the routine practice. Twice daily observations were recorded for general flock condition, temperature, lighting, water, feed, litter condition and mortality.

Animals
Six hundred and forty eight male broiler chickens of the Ross 380 strain were used, and distributed into 36 cages, at eighteen chickens per cage. Only animals free of any clinical signs, e.g. no leg problems, eyes opened, active behaviour, and no other problems, were included in the trial.

Treatments and experimental design
As in the previous experiment there was a stronger effect of the block location than the dietary treatment in the intestinal microbiota composition, birds fed with the same experimental diet were placed in contiguous cages, separately physically from the other dietary treatments. Six dietary treatments replicate six times each with 18 birds per replicate, were tested.
The arrangements of treatments were: T-1) negative control, T-2) positive control (Salinomycin, 75 mg/kg feed), T-3) calcium formate, T-4) sodium butyrate, T-5) lactic acid, T-6) Combination of formic and propionic acids and ammonium formate.
From 30 to 35 days, birds from T-2 were fed with the negative control diet. For T-3 treatment, the corresponding Ca supply by the calcium formate was removed from de calcium carbonate.
The organic acids were added on top of the diet and the following organic acids products were studied. The product containing calcium formate (68% formic acid) was added at 7.4 kg/ton. As the product contains 30% of calcium, 0.55% of calcium carbonate of the formula was reduced. The product containing sodium butyrate coated (30% butyric acid) was added at 1 kg/ton from 0-24 d and 0.5 kg/ ton from 24-35 d. The product containing lactic acid (63%) was added at 5 kg/ton. The product containing blend of formic and propionic acids and ammonium formate (43% expressed as formic acid and ammonium formate) was added at 4 kg/ton.

Zootechnical performance
Chicks were weighed in bulk on arrival, and per cage at 10, 24 and 35 days. Feed consumption per cage was recorded at days 10, 24 and 35. Average daily gain, average daily feed consumption and feed to gain ratio were calculated for the periods 0 to 10 days, 10 to 24 days, 24 to 35 days and for the overall experiment.
Mortality was checked and recorded daily, including the cause of the death.

Sampling of the intestinal content for microbial measurements
At day 21-22, six chickens per cage, representative of the cage according to their apparent weight, were sacrificed by intravenous injection of Tiobarbital (150 mg / kg body weight) according to the experimental procedure num. 689, approved by the Ethical Commission of IRTA, and samples from the distal part of the small intestine (from Meckel's diverticulum to ileo-caecal junction) were taken by gentle squeezing. Samples from the six birds per cage were collected into sterile containers for pooling. Samples during sampling and before pooling / splitting were kept at 4ºC (crushed ice).
From the pooled samples the following sub-sampling were done:
- 1 g of sample preserved in 3 ml ethanol 96% were stored at 4ºC and sent at ambient temperature for RFLP measurements.
- 1 g of sample preserved in 3 ml ethanol 96% were stored and maintained at 4°C during transport for PCR-SSCP measurements.

- 1 (±0.1) g of sample preserved in 3 ml in ethanol:PBS (1:1) were stored and maintained at 4°C during transport and sent for FISH measurements. The exactly weight of sample will be recorded by weighting the tube+preserver before and after adding the sample.
- 3 g of samples freezed and stored at -70ºC were sent with dry ice (-80°C) for *E. coli*, *Clostridium perfringens* and *Lactobacillus sp*. counts.

The PBS used for sample for FISH measurements was prepared as follows: 8 g of NaCl per liter, 0.2 g of KCl per liter, 1.44 g of Na2HPO4 per liter, 0.24 g of KH2PO4 per liter, pH 7.2. The chemicals were of analytical quality and water double distilled. Before use, the solution was filtered to exclude bacteria sized particles. The ethanol was of 96%.

Intestinal digesta viscosity
At the same day of sampling from microbial measurements and from two birds from each cage, jejunum digesta samples (from the distal part of duodenum to Meckel's diverticulum) were collected and kept on ice, to measure supernatant digesta viscosity. Samples were centrifuged at 10000 rpm for 15 min. at 15ºC and supernatant viscosity was measured by using a Brookfield digital viscometer (model LVTDVCP-II, Brookfield Engineering Laboratories, Stouhton, MA), maintained at 30ºC and reading after 1 min.

Conventional bacterial counts
The samples for bacterial analysis were successively diluted at 1/10 in 9.5 g/l Maximum Recovery Diluent (Merck) and analysed for *Escherichia coli*, *Lactobacillus spp* and *Clostridium perfringens*. *E. coli* were counted by plating serial dilution on McConkey Agar (Difco) and incubated aerobically for 24 hours at 37ºC. The lactic acid bacteria were counted after being plated onto MRS agar (Lactobacilli MRS Broth, Difco, supplemented with 15g/l of Bacto Agar, Difco) and incubated anaerobically for 48 hours at 37ºC, and *Clostridium perfringens* were counted by plating on Perfringens Agar (Oxoid) supplemented with Perfringens Supplement A and B (Oxoid) and incubated anaerobically for 24 hours at 37ºC.. The results were expressed as $\log_{10}$ colony forming units (CFU)/g of digestive contents.

Bacterial numbers according to 16S rRNA group specific FISH oligonucleotide probes
The following probes were used: EUB338 (domain bacteria), LGC354A (Lactobacillus), LGC354B (Bacillus), LGC354C (Enterococcus, Streptococcus, Lactococcus), Probe D (Enterobact.), Bacto1080 (Bacteroides) and Chis150 (Clostridium) (Olsen et al, 2006).

Fingerprint techniques

DNA extraction
The ethanol was removed from samples (pools of ileal content of 6 birds) after centrifugation (9 000 g) and the pellet was rinsed three times with physiological water. DNA was extracted from 200 mg samples using the QIAamp DNA Stool Mini Kit (Qiagen) as described by the manufacturer. An additional treatment with lysozyme was performed in order to improve the extraction of Gram positive bacteria DNA. After the step of incubation of samples with ASL buffer during 5 minutes at 95°C, and before the use of InhibitEX tablets, 140µl of a 10 mg/mL of lysozyme (Sigma L-7651) in Tris-EDTA pH 8 (Tris 10 mM, EDTA 1 mM) was added to each extraction tube. Samples with lysozyme were incubated at 37°C during 30 min. At the end of the procedure, the purified DNA was stabilised with the addition of 4µL of 40 mg/ml BSA (Bovine Serum Albumin, Sigma B-4287) plus 2 µl of Ribonuclease–A (Sigma R-4642) and maintained at -20°C until used. The concentration and integrity of nucleic acids were determined by electrophoresis on 1% agarose gel containing ethidium bromide. The DNA extracted from each of the 6 pools of digestive content of each dietary treatment was used to compare the 6 replicates per dietary treatment by CE-SSCP and RFLP.

CE-SSCP

PCR reaction: For total microflora analysis, PCR was performed according to Delbes *et al*., (2001) by amplification of the V3 region with the primers W49 (ACG GTC CAG ACT CCT ACG GG) and W104 (TTA CCG CGG CTG CTG GCA C). These primers are specific for the Eubacteria phylogenic domain. Primers W49 and W104 were labelled on the 5' end with hexachloro derivative of fluorescein (Hex) and 5'-fluorescein-CE phosphoramidite (6-Fam) respectively. The amplification of the V3 region was performed by using the *pfu* Turbo enzyme (Stratagene, La Jolla, CA). After a step of DNA denaturing 10 minutes at 94°C, 25 cycles composed of 30 sec. at 94°C, 30 sec. at 61°C (W49-W104) and 30 sec. at 72°C were run. After PCR, amplified DNA was loaded onto a 2% agarose gel and stained with 0.5 mg/ml ethidium bromide. Images were captured with a Biocapt camera (Bioblock Scientific).

CE-SSCP electrophoresis: DNA was mixed with formamide and Genescan 400 HD-Rox standard (Applied Biosystems, France) according to the ratios 1:18.5:0.5. After a denaturing step at 95°C during 10 minutes, the mix was quickly cooled on ice. The 96-well plate containing the samples was placed into an ABI Prism Genetic Analyzer 3100-*Avent* (Applied Biosystems, France). The non-denaturing polymer matrix used was 5.6% CAP polymer (Applied Biosystems, France) - 10% Glycerol - 1x TBE. The electrophoresis was performed in 1x TBE buffer - 10% Glycerol. The samples were run at 15 kV at 32°C. The data were collected with the Gene Mapper V4.0 software. A normalisation was performed by using the internal standard 400 HD-Rox.

RFLP

PCR reaction: Two primers 5'-CTACGGGAGGCAGCAGT-3' and 5'-CCGTCWATTCMTTTGAGTTT-3' (Sigma-Genosys) designed for regions of the 16S rRNA gene highly conserved among a wide range of microorganisms were used for PCR amplification.

PCR reaction included: PCR-Master Mix (Applied Biosystems), with 1.25 IU of Taq polymerase, DNA template, the preceding primers, and distilled water in a total volume of 50 µl.

PCR mixtures were heated to 94°C for 5 minutes once, followed by 35 cycles of denaturation at 94°C for 1 minute, primer annealing at 45°C for 1 minute, and DNA extension at 72° for 1:15 minutes. The last extension cycle was continued for 5 minutes. The PCR amplification reaction was conducted in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems).

In general, PCR amplification products were processed just after amplification, but they will be maintained at -20ºC until use in other case.

RFLP analysis:

The DNA fragments amplified by PCR were digested with *Alu* I, *Rsa* I, *Hpa* II, *Sau* 3A I or *Cfo* I restriction endonucleasas (Sigma-Aldrich) in accordance with manufacturer specifications, but with SA buffer as common buffer to avoid the effect of the pH differences of samples on electrophoresis separation of DNA fragments.

The restriction endonuclease fragments were analysed using a 2% wide range agarose electrophoresis, supplemented with ethidium bromide. For the electrophoresis separation 150 V during 60 minutes were applied. The bands of DNA were visualized in an UV Chemigenious Image System (SynGene) using the GeneSnap software (SynGene). Pictures with 4.63 seconds exposure were stored.

The electrophoretic profiles obtained, known as Restriction Fragment Length Polymorphism (RFLP), are highly characteristic of the microbial genera, and in some cases, of the microbial species.

Two molecular weight controls were used: Step Ladder, 50 bp (Sigma-Aldrich) and our own control prepared by digestion of amplified fragments of 16S r-DNA of well characterized animal bacteria.

Statistical analysis
Data (performance, viscosity, microbial counts by conventional and FISH method) were subject to a one way analysis of variance by using the General Linear Models (GLM) procedures of SAS. Significance were based on a 5% probability level.
The CE-SSCP profiles were compared using Bionumerics software (Applied Maths, Belgium). The comparisons of profiles were based on the Pearson similarity coefficient which took into account the number of bands, their position, and their intensity. Similarity coefficient, calculated for each pair of profiles, yielded a similarity matrix. A dendrogram was constructed from this matrix by using the unweighted pair group method using arithmetic averages (UPGMA).
For RFLP profiles, the sizes of all the bands obtained were calculated with the aid of the GeneTools software (SynGene). Dendrograms considering the size and the intensity of the bands were constructed. Biodiversity degree was calculated as the number of bands of each sample.

3. Results

Performances
During the first 24 days (Table 2), birds fed the blend of formic and propionic acids with ammonium formate grew faster than birds given sodium butyrate, lactic acid or control diets and chickens fed salimomicyn or calcium formate grew faster that control birds ($P<0.05$). Feed consumption was numerically higher in birds fed organic acids or salinomycin compared to control group ($P<0.06$), In overall, no differences in FCR were observed., In overall experiment (from 0 to 35 days, Table 3), organic acids blend, sodium butyrate or calcium formate impaired FCR compared to salinomycin or negative control, whereas lactic acid impaired FCR compared to salinomycin ($P<0.05$). Dietary treatments did not affect significantly growth and feed intake. Although differences did not reach significance ($P>0.05$), the heavier birds were from organic acids blend and salinomycin groups (130 and 93 g more than the control), followed by calcium formate and sodium butyrate (60 and 25 g more than the control, respectively). Weight of birds fed lactic acid was similar to that of control group. Feed intake was numerically increased by the supplementation of organic acids.

Digesta viscosity
The lowest digesta viscosity was observed with calcium formate and the highest with sodium butyrate (Table 4).

Conventional microbial counts
Higher *Lactobacillus spp*. counts were observed with salinomycin and organic acids compared with negative control (Figure 1). No effect was observed on *Escherichia coli* (Figure 2) and *Clostridium perfringens* intestinal counts (data not shown).

Bacterial numbers according to 16S rRNA group specific FISH oligonucleotide probes
Higher number of bacteria belonging to Domain Bacteria (EUB338 probe) was detected in ileum from birds fed sodium butyrate, compared with lactic acid, organic acids blend or negative control. Lower counts with LGC354C probes (*Enterococcus*, *Streptococcus*, *Lactococcus*) was observed with salinomycin compared with negative control or organic acids diets. Some organic acids affected members of *Enterobacteria*, *Bacteroides* and *Clostridium* significantly. Calcium formate decreased *Enterobacteria* compared with negative control and sodium butyrate diets. Lactic acid decreased *Bacteroides* compared with negative control, salinomycin and sodium butyrate diets. Calcium formate, lactic acid and organic acids blend diet decreased *Clostridium* compared with salinomycin diet.

Microbiota profiles

CE-SSCP

The CE-SSCP results are presented in the Figures 3 and 4.

No cluster could be related to a treatment. No detectable high modification of the gut microflora was noticed by CE-SSCP. However, we could detect some minors modifications related to the use of salinomycin. With 6-Fam labelling, one band (Figure 3, position 186), presented a lower intensity in the 6 ileum fingerprint of the animals treated with salinomycin. With Hex-labelling, two areas (182-184 and 189-193) with less bands in the ileum fingerprint of the animals treated with salinomycin on the contrary to the other dietary treatments were observed.

In conclusion, some modifications related to the use of salinomycin were highlighted by using CE-SSCP.

RFLP

Analysis of samples by RFLP showed significant differences between treatments (Figure 5)**.** RFLP profiles corresponding to negative control as well feed supplemented with sodium butyrate were clearly different to the other treatments. RFLP profiles of feed supplemented with salinomycin and feed supplemented with calcium formate were not clearly different. RFLP profiles of feed supplemented with lactic acid and the blend of formic and propionic acids and ammonium formate have some kind of association. Moreover, differences between treatments were detected in microbial biodiversity, as the number of electrophoretic bands in the RFLP profiles (Figure 6). Negative control resulted in a significant lower biodiversity ($p<0.05$), compared with the supplementation of salinomycin, sodium butyrate or organic acids blend. Furthermore, but without statistical significance, feed supplemented with calcium formate or lactic acid tended to increase the biodiversity in reference to negative control.

4. Discussion

Organic acids, as well as salinomycin, improved performance of chickens from 0 to 24 days. Organic acids increased *Lactobacillus* spp. intestinal counts. No differences between treatments on *Escherichia coli* and *Clostridium perfringens* intestinal counts.

By FISH, some organic acids showed weak variations on some bacterial groups and affected members of *Enterobacteria*, *Bacteroides* and *Clostridium* significantly.

Thus whereas only one difference was observed with conventional microbial counts, several difference were observed with FISH count.

Among the two fingerprint methods used in this study, the difference highlighted between the dietary treatments were not the same. CE-SSCP did not allow clustering profiles according to dietary treatment, but allowed to show some differences between salinomycin and the other dietary treatments. RFLP allowed clustering some dietary treatments. Thus salinomycin was differentiated from negative control and 3 of the 4 dietary treatments with organic acid, but not from calcium formate diet. All the dietary treatments with organic acids were differentiated from the negative control. Among the 4 dietary treatments with organic acid, 2 were not differentiated, lactic acid and blend of organic acids, whereas the two other were significantly differentiated, calcium formate and sodium butyrate. Moreover, with RFLP, microbial biodiversity, as the number of electrophoretic bands, was showed to be higher with salinomycin, and two organic acid treatments, sodium butyrate and organic acids blend, compared to negative control diet.

5. References

Olsen, K. N., Francesch, M. and Christensen, H. 2006. Fluoroscence in situ hybridization of chicken intestinal samples with bacterial rRNA targeted oligonucleotide probes. EPCXII Verona.

6. List of table

Table 1. Composition and calculated nutrient composition of experimental feeds

	Starter diet (0-24 days)	Grower diet (24-35 days)
Ingredient (%)		
Wheat	46.8	52.1
Barley	15.0	15.0
Soybean oil	3.5	5.5
Full fat extruded soybeans	12.2	5.6
Soybean meal 48	18.5	18.3
DL-Methionine	0.240	0.214
Lysine HCl	0.097	0.043
Threonine	0.004	-
Tryptophan	0.002	-
Calcium carbonate	1.281	1.237
Dicalcium phosphate	1.605	1.320
Salt	0.325	0.338
Minerals and vitamins[1]	0.400	0.400
Choline chloride (50%)	0.044	0.020
Calculated nutrient content[3]		
Metabolisable energy (kcal/kg)	3000	3100
Crude protein (%)	21.5	19.5
Crude fibre (%)	3.20	3.08
Crude fat (%)	7.23	7.92
Ash (%)	5.95	5.40
Lysine (%)	1.100	0.930
Methionine (%)	0.550	0.500
Met + Cys (%)	0.932	0.862
Threonine	0.794	0.681
Tryptophan	0.259	0.235
Calcium (%)	1.000	0.900
Available phosphorus (%)	0.429	0.370

[1]One kg of feed contains: Vitamin A: 12000 IU; Vitamin D_3: 5000 IU; Vitamin E: 30 mg; Vitamin K_3: 3 mg; Vitamin B_1: 2,2 mg; Vitamin B_2: 8 mg; Vitamin B_6: 5 mg; Vitamin B_{12}: 11 μg; Folic acid: 1,5 mg; Biotin: 150 μg; Calcium pantothenate: 25 mg; nicotinic acid: 65 mg; Mn: 60 mg; Zn: 40 mg; I: 0,33 mg; Fe: 80 mg; Cu: 8 mg; Se: 0,15 mg; Etoxiquín: 150 mg.

Table 2. Effects of organic acids on growth performance of broilers chickens from 0 to 24 days

		Body weight at day 24 (g)	Daily weight gain (g)	Daily feed intake (g)	Feed conversion ratio
T-1	Negative control	778 [c]	30.4 [c]	44.1 [b]	1.449
T-2	Salinomycin [(1)]	857 [ab]	33.7 [ab]	48.1 [ab]	1.428
T-3	Calcium formate [(2)]	858 [ab]	33.7 [ab]	48.3 [ab]	1.432
T-4	Sodium butyrate [(3)]	817 [bc]	32.0 [bc]	46.6 [ab]	1.455
T-5	Lactic acid [(4)]	825 [bc]	32.3 [bc]	46.7 [ab]	1.443
T-6	Blend [(5)]	906 [a]	35.7 [a]	51.3 [a]	1.437
	Standard error	22.4	0.93	1.55	0.0147
	P	**	**	(*)	NS

Values are means of 6 replicates per treatment of 18 chickens from 0 to 21 days and 12 chickens from 21 to 24 days. Means within a column with different superscript differ significantly (P<0.05).
NS (P>0.1), (*) P<0.1, ** P<0.01.
[(1)] Salinomycin at 75 mg/kg. [(2)] Calcium formate (68% formic acid) at 7.4 kg/ton. [(3)] Sodium butyrate coated (30% butyric acid) at 1 kg/ton. [(4)] Lactic acid (63%) at 5 kg/ton. [(5)] Blend of formic and propionic acids and ammonium formate (43% expressed as formic acid and ammonium formate) at 4 kg/ton.

Table 3. Effects of organic acids on growth performance of broilers chickens from 0 to 35 days

		Body weight at day 35 (g)	Daily weight gain (g)	Daily feed intake (g)	Feed conversion ratio
T-1	Negative control	1664	46.1	73.3	1.586 [bc]
T-2	Salinomycin [(1)]	1757	48.8	76.3	1.563 [c]
T-3	Calcium formate [(2)]	1724	47.9	77.9	1.629 [a]
T-4	Sodium butyrate [(3)]	1689	46.9	76.8	1.637 [a]
T-5	Lactic acid [(4)]	1659	46.0	74.2	1.613 [ab]
T-6	Blend [(5)]	1794	49.9	81.6	1.637 [a]
	Standard error	42.3	1.21	2.23	0.0129
	P	NS	NS	NS	**

Values are means of 6 replicates of 18 chickens per treatment from 0 to 21 days and 12 chickens from 21 to 35 days. Means within a column with different superscript differ significantly (P<0.05).
NS (P>0.1), ** P<0.01.
[(1)] Salinomycin at 75 mg/kg. [(2)] Calcium formate (68% formic acid) at 7.4 kg/ton. [(3)] Sodium butyrate coated (30% butyric acid) at 1 kg/ton from 0 to 24 days and 0.5 kg/ton from 24 to 35 days. [(4)] Lactic acid (63%) at 5 kg/ton. [(5)] Blend of formic and propionic acids and ammonium formate (43% expressed as formic acid and ammonium formate) at 4 kg/ton.

Table 4. Effects of organic acids on supernatant jejunum digesta viscosity ad day 21

		Viscosity (cps)
T-1	Negative control	4.73
T-2	Salinomycin [(1)]	4.63
T-3	Calcium formate [(2)]	3.96
T-4	Sodium butyrate [(3)]	5.43
T-5	Lactic acid [(4)]	4.46
T-6	Blend [(5)]	4.35
	Standard error	0.514
	P	NS

Values are means of 6 replicates per treatment.
[(1)] Salinomycin at 75 mg/kg. [(2)] Calcium formate (68% formic acid) at 7.4 kg/ton. [(3)] Sodium butyrate coated (30% butyric acid) at 1 kg/ton. [(4)] Lactic acid (63%) at 5 kg/ton. [(5)] Blend of formic and propionic acids and ammonium formate (43% expressed as formic acid and ammonium formate) at 4 kg/ton.

Table 5. Effects of organic acids on bacterial numbers in ileum according to 16S rRNA group specific FISH oligonucleotide probes (Log cfu/g)

		EUB338 Domain bacteria	LGC354A Lactobacillus	LGC354B Bacillus	LGC354C Enterococcus Streptococcus Lactococcus
T-1	Negative control	8.00 b	7.79	7.57	7.59 a
T-2	Salinomycin (1)	ND	7.80	8.18	6.71 b
T-3	Calcium formate (2)	8.04 ab	7.69	7.79	7.52 a
T-4	Sodium butyrate (3)	8.33 a	7.80	7.82	7.20 a
T-5	Lactic acid (4)	7.97 b	7.70	7.61	7.57 a
T-6	Blend (5)	7.97 b	7.60	7.59	7.22 a
P		<0.09	NS	NS	<0.001

		ProbeD Enterobact.	Bacto1080 Bacteroides	Chis150 Clostridium
T-1	Negative control	7.64 a	7.83 a	6.90 abc
T-2	Salinomycin (1)	7.57 ab	7.87 a	7.37 a
T-3	Calcium formate (2)	7.20 b	7.50 ab	6.25 c
T-4	Sodium butyrate (3)	7.83 a	7.85 a	7.21 ab
T-5	Lactic acid (4)	7.45 ab	7.35 b	6.17 c
T-6	Blend (5)	7.59 ab	7.67 ab	6.45 bc
P		<0.05	<0.05	<0.01

Values are means of 6 replicates per treatment.
(1) Salinomycin at 75 mg/kg. (2) Calcium formate (68% formic acid) at 7.4 kg/ton. (3) Sodium butyrate coated (30% butyric acid) at 1 kg/ton. (4) Lactic acid (63%) at 5 kg/ton. (5) Blend of formic and propionic acids and ammonium formate (43% expressed as formic acid and ammonium formate) at 4 kg/ton.

7. List of figures

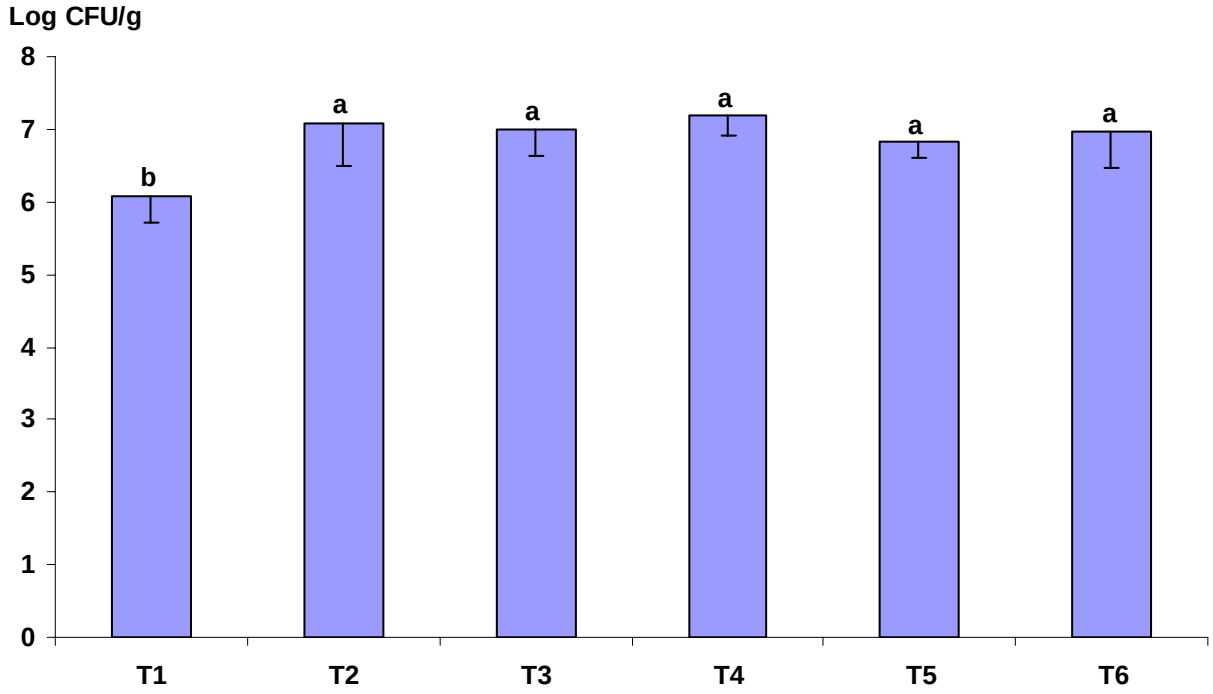

Figure 1. Bacterial plate count of intestinal contents of *Lactobacillus* spp.. T1 : Negative control T2 : Salinomycin at 75 mg/kg. T3 : Calcium formate (68% formic acid) at 7.4 kg/ton. T4 : Sodium butyrate coated (30% butyric acid) at 1 kg/ton. T5 : Lactic acid (63%) at 5 kg/ton. T6 : Blend of formic and propionic acids and ammonium formate (43% expressed as formic acid and ammonium formate) at 4 kg/ton. (Treatment with no common letter differed significantly)

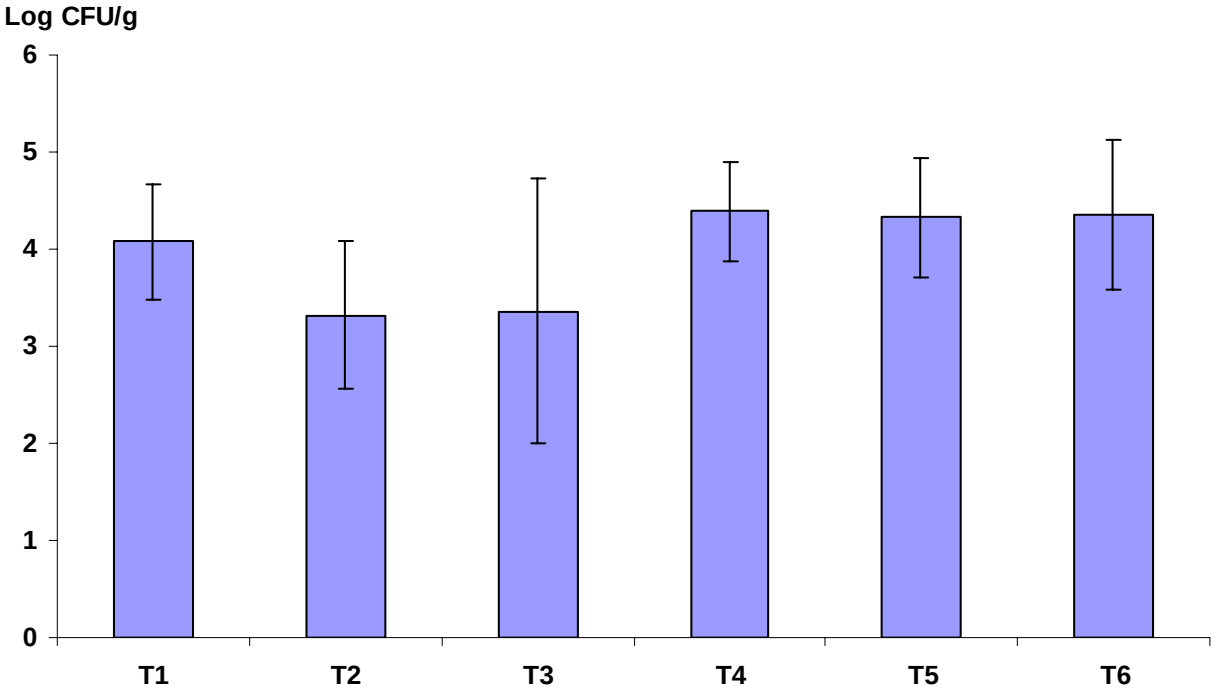

Figure 2. Bacterial plate count of intestinal contents of *Escherichia coli*. T1 : Negative control T2 : Salinomycin at 75 mg/kg. T3 : Calcium formate (68% formic acid) at 7.4 kg/ton. T4 : Sodium butyrate coated (30% butyric acid) at 1 kg/ton. T5 : Lactic acid (63%) at 5 kg/ton. T6 : Blend of formic and propionic acids and ammonium formate (43% expressed as formic acid and ammonium formate) at 4 kg/ton.

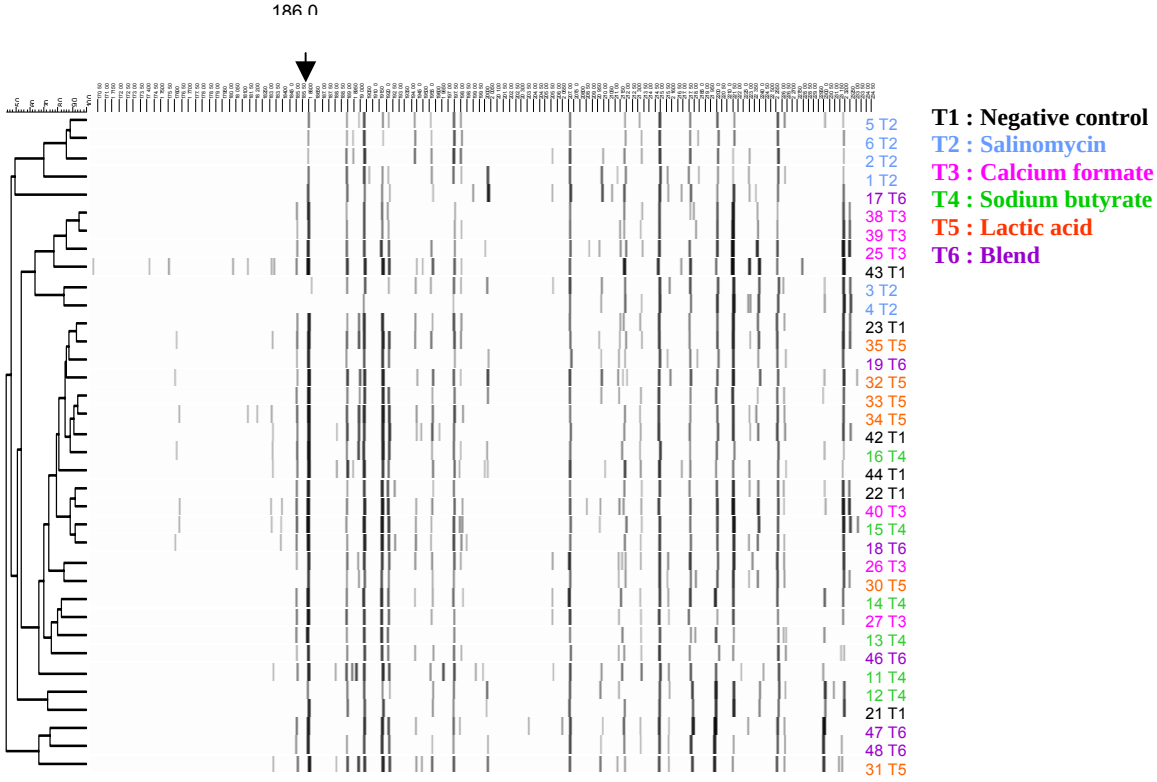

Figure 3. CE-SSCP fingerprint (V3 region of the total 16S rDNA from the bacterial flora targeted by using universal primers; 6-Fam labelling) obtained from ileum pools after treatments with organic acids.

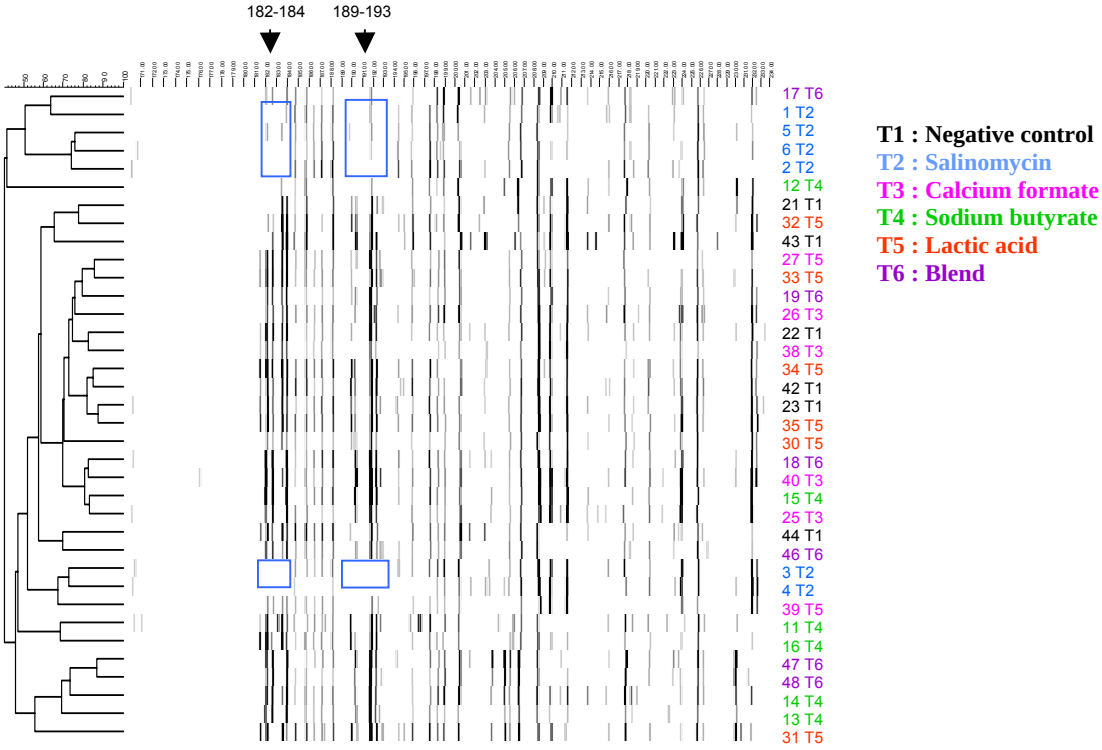

Figure 4. CE-SSCP fingerprint (V3 region of the total 16S rDNA from the bacterial flora targeted by using universal primers; Hex labelling) obtained from ileum pools after treatments with organic acids. Groups of specific bands are surrounded.

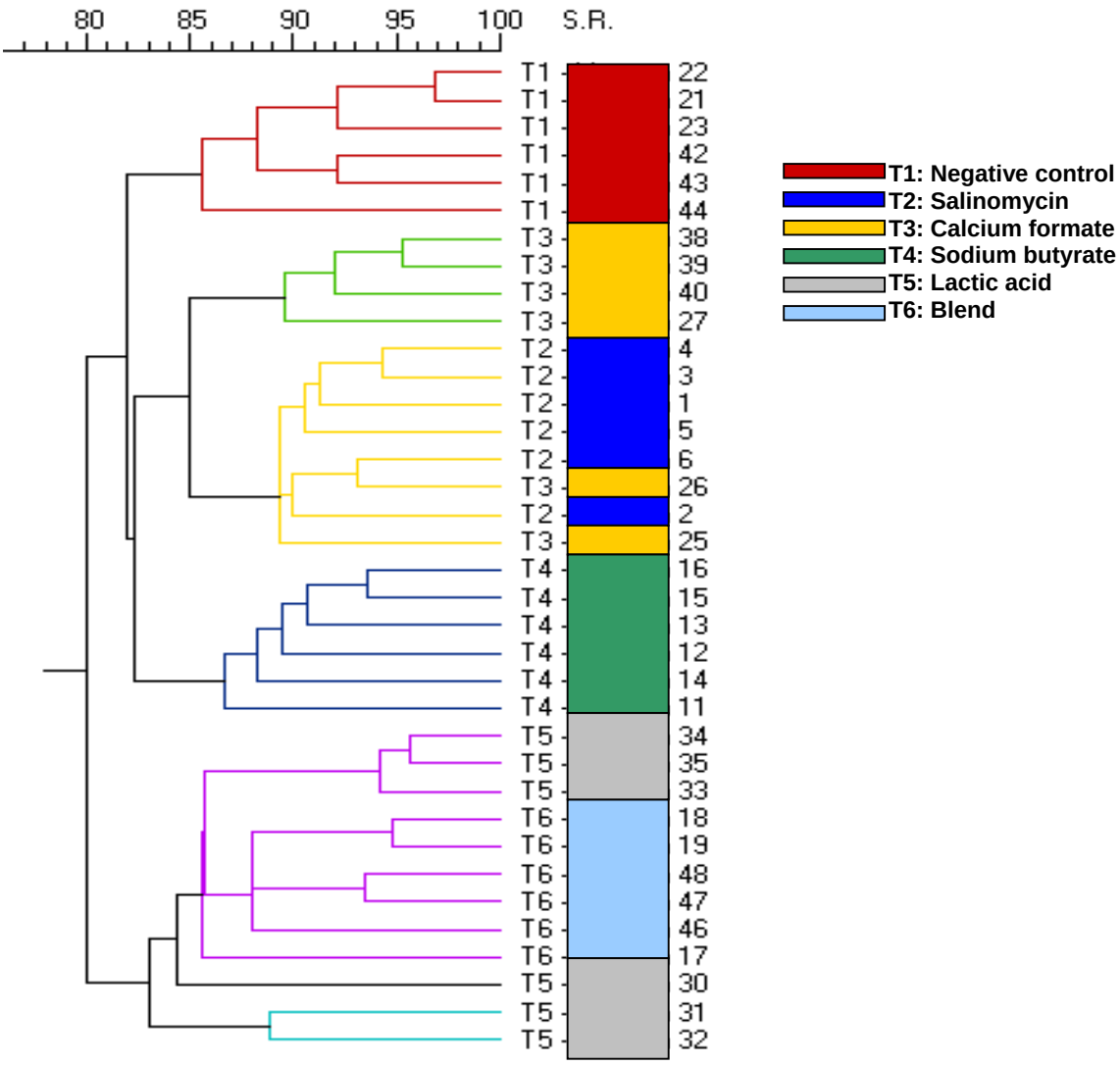

Figure 5. Dendrogram of similarity of RFLP profiles obtained from ileum pools after treatments with organic acids

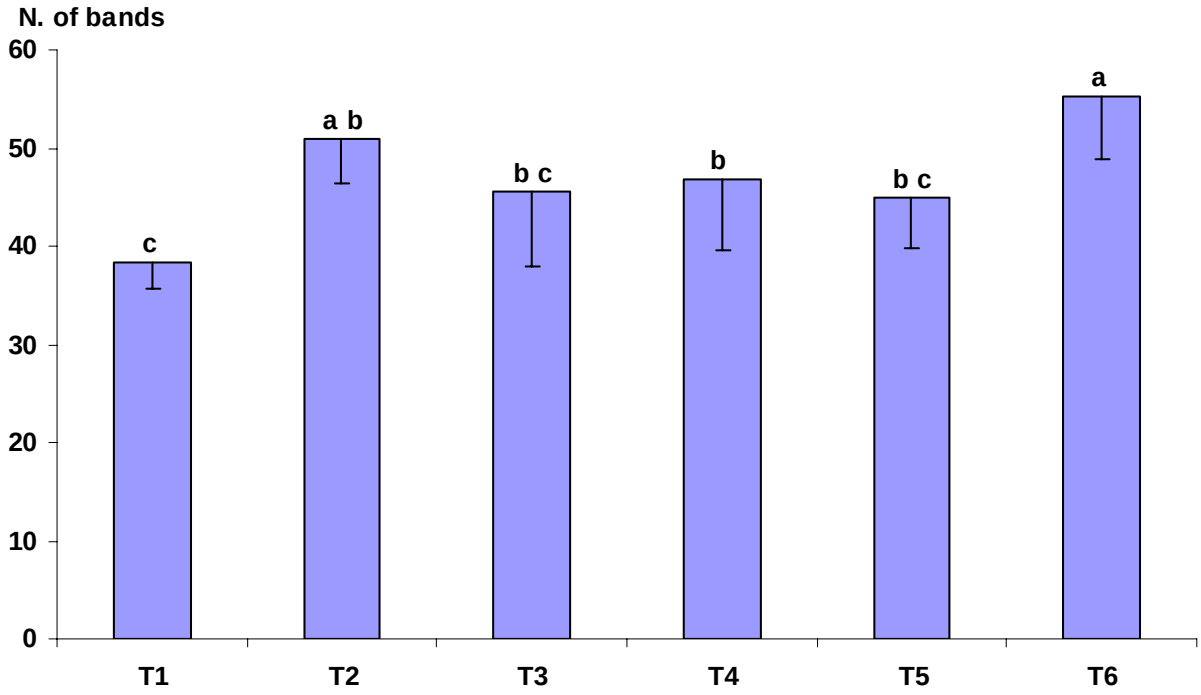

Figure 6. Biodiversity degree of RFLP profiles as number of electrophoresis bands. T1 : Negative control T2 : Salinomycin at 75 mg/kg. T3 : Calcium formate (68% formic acid) at 7.4 kg/ton. T4 : Sodium butyrate coated (30% butyric acid) at 1 kg/ton. T5 : Lactic acid (63%) at 5 kg/ton. T6 : Blend of formic and propionic acids and ammonium formate (43% expressed as formic acid and ammonium formate) at 4 kg/ton. (Treatment with no common letter differed significantly)

D. Effect of probiotics on digestive microflora of chicken compared to an antibiotic growth promoter by AFSSA

Summary

The effect of a probiotic (Bactocell®) was studied on animal performance, intestinal structure and digestive microflora. The animal performance results showed an increase in feed intake and a negative effect on feed conversion ratio (FCR) with antibiotics (avilamycin) during the whole period (1-42 d) compared to the negative control. With the probiotic a positive effect was observed on FCR during the whole period (1-42 d) although a negative effect was observed from 14 to 25 d. Analysis of intestinal structure showed no difference in surface of villi of ileum between dietary treatments, but a lower crypt area was observed with antibiotic compared to control diet. Moreover a lower heterogeneity of surface of the villi was observed with antibiotic compared to the control diet. Analyses of digestive microflora by classical bacterial counts did not show any effect of dietary treatments in ileal, cloacal and caecal content. However, in the fresh droppings, avilamycin as well as the probiotic increased the number of coliform. Analyses of digestive microflora by the fingerprint method CE-SSCP, did not lead to cluster according to dietary treatments (negative control, avilamycin, probiotic) neither with universal primers, nor with specific primers of Lactic Acid Bacteria. However, some specific bands of the dietary treatments were observed. Thus the absence or the weak intensity of two bands in the ileum and the cloaca of only the animals treated with the antibiotic may be related to this treatment. Other bands were absent in samples from birds fed avilamycin. Moreover bands not detected in samples of birds fed negative control diet were observed with avilamycin. With the probiotic, the presence of some specific bands was detected. Thus the presence of one minor band in different gut compartment that did not correspond to lactic flora may be linked to this treatment. Another one was mostly detected in the fingerprints of the caecal lactic microflora of some animals treated with probiotic. On the contrary some bands present in negative control diet, were absent with the probiotic. As with CE-SSCP, analysis of the digestive samples with universal primers by TTGE did not lead to cluster according to dietary treatments whatever the digestive content (ileum, cloaca, caeca). However some bands characteristic of the dietary treatment were observed. In the ileal content, probiotic led to a band (a) with a high intensity corresponding to *Lactobacillus johnsonii* which was also detected in negative and positive control, but with lower intensity. In the cloacal content, avilamycin diet led to the appearance of one band (b). The probiotic diet led to the appearance of a band (c) migrating at the same level than the band (b) appearing with avilamycin diet. These bands (b,c) corresponded to a bacteria belonging to the order Clostridiales.

1. Introduction

The mean objective of this study was to use two fingerprint methods to study the effect of a probiotic (Bactocell®) on digestive microflora, as well as the effect of an antibiotic growth promoter (avilamycin). This AGP was used as a positive control to study if fingerprint technic were able to detect difference in digestive microflora.
Bactocell contained *Pediococcus acidilactici*, a lactic acid bacterium that produces bacteriocin (Bhunia et al, 1988).

2. Material and Methods

2.1. Dietary treatments

The birds were allocated to three dietary treatments: 1) a negative control with no additives (Nc), 2) a positive control containing 0.01 g/kg avilamycin (Av), 3) a treatment containing 1 g/kg (10^{10} UFC / kg) of probiotic Bactocell® (P).
The feeding program consisted of three different diets for each treatment: a starter diet (from 1 to 28 days of age), a grower diet (from 28 to 35 days of age), a finisher diet (from 35 to 47 days of age). The composition of the basal diets (the negative control diets) is shown in table 1. The diets were steam pelleted (2.5 mm in diameter). For the treatments avilamycin and probiotic, the the avilamycin and Bactocell® were incorporated at the expense of the same amount of maize.
The feed and the water were supplied *ad libitum*.

2.2. Birds and housing

A total of 900 Ross PM3 male broiler chickens vaccinated against infectious bronchitis were obtained from a commercial hatchery (Perrot, Pommerit jaudy, France). The chickens were raised in 5 m^2 floor pens with a stocking density of 12 birds/m^2 with 5 replicates per treatment. From day 1 to 4 the lighting programme consisted of 23 hours light and 1 hour dark (0-1 am), from day 5 to 11, it consisted of 20 hours light and 4 hours dark (10 pm-2 am) and from day 12 to the end of the experiment 18 hours light and 6 hours dark (9 pm-3 am). The temperature was 32°C from day 1 to 6 , it was reduced to 30°C at day 8, 29°C at day 15, 26°C at day 22, 23°C at day 25, 21°C at day 28 and 20°C at day 35.

2.3. Experimental protocol and sample collection

The experiment was carried out in accordance to the specific guidelines for experiments on animals (Decree, 2001).
At arrival, the birds were randomly distributed with 60 birds in each pen with a similar weight per pen. The birds were individually weighed on day 14, 25 and 42. The feed intake in each pen was measured at the same age and the FCR calculated. The mortality was checked daily. Feed intake was expressed as animal present each day (i.e. dead birds were not included). To calculate Daily Live Weight Gain (DLWG) any females and dead birds were taken out of the calculation, but they were included in the FCR calculation.
At day 25, fresh droppings were collected on the floor. At day 26, 6 chickens representative of their pens were selected (according to their weight) from each pen. The cloacal contents were obtained by abdominal pressure on the birds, and were pooled from the 6 animals per pen. Fresh dropping and cloacal contents were collected into sterile containers and kept on crushed ice. The birds were killed by intravenous injection of sodium pentobarbital (82 mg/kg). Their digestive tract was removed from the Meckel's diverticulum to the end of the intestine. For histological analysis, the middle part (1.5 cm long) of the ileum was taken, from 3 of the 5 replication pens per dietary treatment (6 birds per pen). The samples were opened longitudinally, rinsed with cold saline (NaCl 9 g/l) and fixed in a buffered formaline solution overnight. They were then rinsed and stored in ethanol/water (70/30, v/v) and stored at 4°C until further analysis. The contents from the ileum and the two caeca were collected by gentle pressure. These digestive content samples were pooled from the 6 animals per pen into sterile containers and kept on crushed ice. Fresh dropping, cloacal, ileal and caecal contents were divided into aliquots for molecular and conventional methods for microflora analysis. 200 mg of pooled sample was taken and stored at -70°C until further CE-SSCP analysis. One gram was taken and preserved in 3 ml 96% ethanol, send with dry ice (-80°C), and stored at -20°C until further TTGE analysis. Three grams were sub-sampled, also send with dry ice (-80°C), and stored at -70°C until further conventional bacteriological count.

2.4. Histological analysis of intestinal wall (ileum)

The ileal samples were analysed as described by Goodlad *et al.* (1991). A 0.5 cm sample was cut off and kept in ethanol/acetic acid (75/25, v/v) for 24 hours, followed by a rehydration in ethanol/water (50/50, v/v) and then in distilled water. Thereafter, the samples were stained by the Feulgen reaction: first a hydrolysis in hydrochloric acid 1 N at 60ºC for 6 minutes, then rinsed with distilled water and thereafter stained with Schiff reagent for 30 minutes. Finally, the samples were rinsed in distilled water and stored in acetic acid/water (45/55 v/v) at 4ºC until analysis.
For histological measurements, villi with their attached crypts of Lieberkühn were individually dissected under a dissecting microscope then mounted between a slide and a cover slip in an aqueous mounting agent (Aquatex, Merck). They were measured under the magnification of 40 for crypts and 10 for villi, using an optical microscope (Leitz, Laborux), a camera (Scion corporation, CFW 1308C) and an image analysis software (Visilog 6.3, Noesis). The length and width of 10 villi and the depth and width of 20 crypts were measured from each segment of each bird. The surface area was calculated for each villi and crypt. An average value was calculated for each bird ileal segment.

2.7. Conventional bacterial counts

The samples for bacterial analysis were successively diluted at 1/10 in 9 g/l NaCl and analysed for coliform, lactic acid bacteria and aerobic mesophilic bacteria. The lactic acid bacteria were counted after being plated onto MRS agar (Man, Rogosa, Sharpe) and incubated for 48 hours, the coliforms were plated onto Drigalski agar and incubated for 24 hours and the aerobic mesophilic bacteria on brain heart infusion agar and incubated for 48 hours. All the plates were incubated aerobically at 37°C. The results were expressed as $\log_{10}$ colony forming units (CFU)/g of digestive contents.

2.6. Search for Bactocell®

Pediococcus acidilactici was numerated in feed (meal before pelleting, pellet at the beginning and the end of the experiment) onto MRS-NaCl-TTC-vancomycine. It was also quantified by PCR. *Pediococcus acidilactici* was also numerated in dropping, at the beginning (5 day) and at the end (35 day) of the experiment
The detection of the strain *Pediococcus acidilactici* present in the probiotic Bactocell®, was performed by PCR on pools of samples from negative control, avilamycin and probiotic (5 pools / treatment). Three digestive segments have been studied (caeca, ileum, cloaca) and droppings on the litter. PCR was performed with the primers *pu* and *ppe* specific of the strain *Pediococcus acidilactici* as described by Mora et al. (1997).

2.7. Analysis of digestive microflora by molecular methods (fingerprint techniques)

DNA extraction:

DNA was extracted by using the QiaAmp DNA Stool mini-kit (Qiagen, France). An additional treatment with 10 mg/mL lysozyme was performed in order to improve the extraction of Gram positive bacteria DNA. Extracted DNA was loaded onto a 1% agarose gel and stained with 0.5 mg/ml ethidium bromide in order to assess its quality and quantity. Images were captured with a Biocapt camera (Bioblock Scientific).

CE-SSCP analysis

PCR reaction: For total microflora analysis, PCR was performed according to Delbes *et al*., (2001) by amplification of the V3 region with the primers W49 (ACG GTC CAG ACT CCT ACG GG) and W104 (TTA CCG CGG CTG CTG GCA C). These primers are specific for the

Eubacteria phylogenic domain. Primer W49 was labelled with hexachloro derivative of fluorescein (Hex) and primer W104 with 5'-fluorescein-CE phosphoramidite (6-Fam) on the 5' end. The amplification of the V3 region was performed by using the *pfu* Turbo enzyme (Stratagene, La Jolla, CA). After a step of DNA denaturing 10 minutes at 94°C, 25 or 30 cycles composed of 30 sec. at 94°C, 30 sec. at 61°C and 30 sec. at 72°C were run. After PCR, amplified DNA was loaded onto a 2% agarose gel and stained with 0.5 mg/ml ethidium bromide. Images were captured with a Biocapt camera (Bioblock Scientific), and DNA quantity was evaluated.
Nested PCR was performed with the primers W18 and W108 to specifically target for lactic bacteria (Walter *et al.*, 2001).

CE-SSCP electrophoresis: DNA, diluted 2 to 50 fold to standardise the quantity, was mixed with formamide and Genescan 400 HD-Rox standard (Applied Biosystems, France) according to the ratios 1:18.5:0.5. After a denaturing step at 95°C during 10 minutes, the mix was quickly cooled on ice. A 96-well plate containing the samples was placed into a ABI Prism Genetic Analyzer 3100-*Avent* (Applied Biosystems, France). The non-denaturing polymer matrix used was 5.6% CAP polymer (Applied Biosystems, France) - 10% Glycerol - 1x TBE. The electrophoresis was performed in 1x TBE buffer - 10% Glycerol. The samples were run at 15 kV at 32°C. The data were collected with a Gene Mapper V4.0 software. A normalisation was performed by using the internal standard 400 HD-Rox.

TTGE

PCR reaction: Primers Bact 968-GC-f (5' GCclamp- AAC GCG AAG AAC CTT AC) and Bact 1401-r (5' CGG TGT GTA CAA GAC CC) were used to amplify the V6-V8 region of bacterial 16S rRNA genes (Nubel et al., 1996). The following program was used : 95° C for 15 min, 30 cycles of 97°C for 1 min, 58°C for 1 min, 72°C for 1 min 30 s and finally 72°C for 15 min. PCR products were analyzed by electrophoresis on 1% agarose gels containing ethidium bromide to check their size (473 bp) and estimate their concentration.

TTGE: PCR products were separated by Temporal Temperature Gradient gel Electrophoresis (TTGE) using the Dcode Universal Mutation Detection System (Biorad, Paris, France). Electrophoresis was run for 17 hours at a fixed voltage corresponding to 63 mA, an initial temperature of 66°C, and a ramp rate of 0.2°C/h. For better resolution, the voltage was fixed at 20 V for 15 min at the beginning of the electrophoresis. On each gel, the 5 replicates of control diet were compared to the 5 replicates of one of the other dietary treatments (avilamycin or probiotic). A TTGE ladder consisting of a PCR amplicon mix of 8 cloned rDNAs from different bacterial species was used to normalize the profiles (Suau et al., 1999; Johansen et al, 2006). Gels were stained in the dark by immersion for 30 min in a solution of SYBR green I (nucleic acid gel stain, Roche Diagnostics, GmbH, Mannheim, Germany) and the fluorescence was read with a UV camera (Gel DOC XR, Biorad).

Identification of bacteria by sequencing TTGE fragment: DNA fragments of interest were excised aseptically from the polyacrylamide gel and placed in 200µl of water. The samples were heated 10 mn 50°C to allow elution of the DNA. After centrifugation (8 000 rpm, 4 min), the supernatant was purified using the QIAquick PCR Purification Kit (Qiagen Ref 28104). Prior to sequencing, the samples were amplified by PCR with Primers Bact 968-GC-f and Bact 1401-r, and were checked by TTGE with the original sample from which the band was excised. Only products that migrated as single band and to the same position with respect to the original sample were used for sequencing. When products resulting from excision were not composed of single band, the band of interest was excised and processed as previously described. For sequencing, PCR amplification was performed using primers without the GC clamp. Sequencing was carried out by Genome Express (Grenoble, France). The sequences retrieved were compared with the GenBank database using BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). Positive identifications of unknown sequences were

considered significantly similar when sequences were more than 98% identical to BLAST database sequences. The RDP II database was used to obtain classification with the classification algorithm of Wang et al (2007) for unidentified bacteria with the GenBank database (http://rdp.cme.msu.edu/classifier/classifier.jsp).

2.8. Statistical analysis

Data of the zootechnical performances, histological analysis and bacteriological counts were computed using Statview ® programm version 5 (Abacus concepts, Berkeley, CA, USA). The data were analysed by one-way analysis of variance (ANOVA), and significant differences between treatments were determined by Student Newman-Keuls test ($P < 0.05$).
From the CE-SSCP fingerprints, dendrograms were constructed by using Bionumerics software (Applied Maths, Kortrijk, Belgium). The TTGE profiles were compared using the GelCompar II software (Applied Maths, Belgium). Only samples run on the same gel were compared. The profiles were normalized by means of TTGE ladder. The comparisons were based on the Pearson similarity coefficient (SCp) which took into account the number of bands, their position on the gel, and their intensity. Similarity coefficient, calculated for each pair of profiles, yielded a similarity matrix. A dendrogram was constructed from this matrix by using the unweighted pair group method using arithmetic averages (UPGMA).

3. Results

3.1. Performances

The animal performance showed an increase in feed intake and a negative effect on feed conversion ratio (FCR) with antibiotic (avilamycin) from 14 to 25 d and during the whole period (1-42d) compared to the negative control (Table 2). With the probiotic, an increase in feed intake was observed from 14 to 25 d, and a negative effect on FCR. However, during the whole period (1-42d), a positive effect was observed on FCR.

3.2. Gut morphology

No difference in surface of villi of ileum was observed between dietary treatments, but a lower crypt area was observed with antibiotic compared to control diet (Table 3). Moreover a lower heterogeneity of surface of villi was observed with antibiotic compared to control diet.

3.3. Bacterial counts

Classical bacterial counts (Table 4) showed no effect of dietary treatment in ileal, cloacal and caecal content. However, in the fresh droppings, avilamycin as well as the probiotic increased the number of coliform.

3.4. Detection of the probiotic

A band of identical size to the control, corresponding to the strain *Pediococcus acidilactici* used in this study, was detected in pools of ileum of birds of 3 pens (P3, 4, 5). It was detected neither in pools of caeca and cloaca of birds from these pens, nor in pools of fresh dropping. No band was detected in samples of birds from control or avilamycin diet, whatever the digestive content or the fresh dropping.

3.5. CE-SSCP Profil

With this fingerprint method (with universal primers or with specific primers of Lactic Acid Bacteria), whatever the gut compartment, dendrograms performed after comparison of the

profiles with the Pearson similarity coefficient and by using the UPGMA method did not lead to clear cluster of all pools of each diet. However this fingerprint method revealed that some bands seemed to be specific of studied dietary treatments (Table 5; Figures 1 to 16).

Total Microflora :
Analysis of total microflora was performed with the primers W49 and W104 (Delbes et *al*., 2001) labelled with fluorochromes Hex and 6-Fam respectively. No bands migrating at the same level that positive control *Pediococcus acidilactici* was detected in digestive samples of birds fed the probiotic or the other diets.
Dendrograms obtained by statistical analysis did not reveal the presence of any cluster. However, some minor differences were observed.
Some bands different according to samples were observed in profiles obtained with fluorochrome Hex (Figures 1-4). A band (position 206) was observed in 3 pools of ileal samples of birds fed probiotic, and where probiotic was detected by PCR (P3, 4, 5) (Figure 1). This band was absent in ileum samples of birds fed negative control and avilamycin diets. It was detected in 3 pools of samples of cloaca (P2, P4, P5) of birds fed probiotic (Figure 2). Among these samples, 2 pools were performed from pens where the probiotic was detected in the pools of ileum samples of birds. At the same position, a band was detected in all the pools of caecal samples of birds fed probiotic, in two pools of birds fed negative control diet (Nc1, Nc5) and one pool of birds fed avilamycin (Av1) (Figure 3). A PCR and CE-SSCP performed on the pure *Pediococcus* strain used as probiotic showed that this band at position 206 did not correspond to the *Pediococcus* strain. Analysis of profiles of fresh dropping samples showed a band (position 195) in 4 pools of birds fed probiotic (P1, P2, P4, P5) (Figure 4). This band was not detected in fresh dropping samples of other birds.
Comparison of profiles obtained with florochrome 6-Fam revealed that in ileum (Figure 5), one band (position 211,8) present in all the pools of birds fed negative control or probiotic, was absent in 3 pools of birds fed avilamycin (Av2, Av4, Av5) and with a very long intensity in the 2 other pools of birds with this dietary treatments. Another band (position 213) was also present in all the pools of birds fed negative control or probiotic, but was absent in 3 pools of birds fed avilamycin (Av 3, Av4, Av5). Moreover, a band (224.0) which was present in 3 pools of negative control (Nc1, Nc2, Nc3), was absent in all pools of avilamycin fed birds. In cloaca samples (Figure 6), as in ileum, the band (position 211,8) which was present in all the pools of negative control fed birds and in 4 of the 5 pools of probiotic fed birds (P1, P2, P4, P5) was absent in 4 pools of birds fed avilamycin (Av2, Av3, Av4, Av5). The band (position 213) also present in all the pools of negative control fed birds and probiotic fed birds, was absent in 3 pools of birds fed antibiotic (Av2, Av3, Av5). Moreover, a band (220.0) which was absent in negative control fed birds, was present in 3 pools of birds fed avilamycin (Av 1, Av4, Av5), and 2 pools of birds fed probiotic (P2, P4). A band (221.0) present in 3 pools of negative control fed birds (Nc1, Nc4, Nc5) was absent in all the pools of birds fed probiotic. In caecal samples (Figure 7), a band (185.0) present in 3 pools of negative control fed birds (Nc1, Nc3, Nc4), was absent in all the pools of birds fed antibiotic or probiotic. Another band (221.0) was present in 4 pools of negative control fed birds (Nc1, Nc3, Nc4, Nc5) but was absent in pools of avilamycin fed birds and present in only one pool of probiotic fed birds (P5). In samples of fresh droppings (Figure 8), a band (184.5) was present in 3 pools of avilamycin fed birds (Av2, Av4, Av5) but absent in samples of birds fed negative control diet or probiotic. Another band (204.0) was present in 3 pools of avilamycin fed birds (Av3, Av4, Av5) but was absent in samples of birds fed negative control diet and present in only one pool of birds fed probiotic (P5). Another band (position 211.5) which was present in 3 pools of negative control diet fed birds (Nc2, Nc3, Nc4), and 4 pools of probiotic fed birds (P1, P3, P4, P5) was absent in all the pools of pens of birds fed antibiotic. This characteristic of birds fed antibiotic was not observed after the nested PCR specific for Lactic Acid Bacteria (Figure 16).

Lactic Flora
With specific primers of Lactic Acid Bacteria, some clusters of some of the pools were observed (data not shown). A cluster for some control animals was observed in the ceca

(3/5). Moreover, some samples from animals treated with Avilamycin were joined together in clusters in the cloaca (4/5) and the fresh droppings (3/5).
With the Hex labelling (Figures 9-12), profiles of 3 caecal samples (P1, P2, P5) of birds fed probiotic showed 2 bands (207-208) that were not observed in samples of birds fed avilamycin, and present only with very low intensity in 2 samples of birds fed control diet (Figure 11). These two bands corresponding to DNA of lactic bacteria, were not detected in profiles of total microflora. It does not correspond to the band of the probiotic strain.
With the 6 Fam labelling (Figure 13-16), one band (position 210.5) was specifically observed in 4 pools of caecal samples of birds fed avilamycin (Av2, 3, 4, 5) (Figure 15). This band was also detected in 3 pools of ileal samples of birds fed avilamycin (Av2, 4, 5), and one pools of birds fed probiotic diet (P2) (Figure 13), as well as in cloacal samples of 3 pools of birds fed avilamycin (Av2, 4, 5) and 1 pool fed probiotic (P3) (Figure 14). One band (position 197) was detected in 3 pools of cloacal samples of birds fed probiotic (P1, P2, P4) (Figure 14) but only one of these pools come from a sample in which the probiotic was detected. In fresh droppings, one band (position 214.5) migrating with one of the band of the control *Pediococcus acidilactici*, was specifically detected in pools of birds fed probiotic (P3, 5) (Figure 16).

3.6. TTGE

The TTGE profiles of bacterial community of digestive content of chickens are shown in figures 17 to 21. Whatever the gut compartment, no clear cluster of all pools of each diet was observed (data not shown). Only cluster of some of the pools were observed. With the pools of 6 birds of ileal samples, when comparing control and avilamycin diets, clusters were observed for control diet (3/5) and avilamycin diet (4/5). When comparing control and probiotic diet, no cluster was observed. With the pools of 6 birds of cloaca samples, no cluster were observed, whatever the diets compared. With the pools of caeca samples, clusters were observed for control diet (3/5) and avilamycin diet (4/5). When comparing control and probiotic diet, clusters were also observed for control diet (3/5) and Pro diet (3/5). However a higher similarity between microbiota of ileal content of birds fed avilamycin compared to control diet was observed (Table 6).
As for SSCP and CE-SSCP, in order to know if the dietary treatments (antibiotic, probiotic) led to characteristic bands compared to control diet, we compared profiles obtained for each intestinal segment. We observed some differences for some dietary treatment. In the ileal content, no difference was observed between control diet and avilamycin (Figure 17). With the probiotic diet, a band (a) which was present in all the samples, had a low intensity with all the pools of control diet, but had a high intensity in 3 of the 5 pools of probiotic (P2, P4, P5) (Figure 18). In the cloacal content, a band (b) which had a very low intensity in all the pools of control diet, was also present in all the pools of avilamycin diet, but showed a moderate intensity for 4 of the 5 pools of this diet (Av2, Av3, Av4, Av5) (Figure 19). With the probiotic diet, a band (c) which was present in all the samples, had a very low intensity with all the pools of control diet, but had a moderate intensity for 4 of the 5 pools of this diet (P2, P3, P4, P5) (Figure 20). In the caecal content, no difference between dietary treatments was observed (data not showed).
In order to characterize the specific band of dietary treatment observed with TTGE, for each digestive segment, the samples of all birds were pooled for each dietary treatment (30 birds). As observed previously with pools of six birds, we observed specific bands of dietary treatments (Figure 21). In the ileal content, probiotic led to a high intensity for the band (a) which was also detected in negative and positive control but with a moderate intensity. In the cloacal content, avilamycin diet lead to the appearance of the band (b) compared to control diet. The probiotic diet lead to the appearance of the band (c) migrating at the same level than the band (b) appearing with avilamycin diet.
Identification of the bands by sequencing showed that the band observed in the ileum with higher intensity with probiotic diet compared to control and avilamycin diet corresponded to the species *Lactobacillus johnsonii* (Table 7). The band present with avilamycin and probiotic

diets (b,c), but not with control diet, corresponded to an uncultured bacterium according to the GenBank database, and was classified as a bacteria belonging to the order Clostridiales with the RDP II database.

4. Discussion

4.1. Comparison of fingerprint techniques

Whatever the two fingerprint techniques, it was not possible to join together in cluster samples of the same dietary treatments. However, several differences between dietary treatments were observed.
As CE-SSCP has higher resolution than TTGE as show by the detection of more bands, and due to its double labelling CE-SSCP allowed to detect band with one label or the other, this fingerprint technique could be expected to shown more difference than TTGE, which was the case (Table 5). Thus in the caeca, whereas TTGE was not able to show difference between dietary treatments, CE-SSCP shown difference as well as for the antibiotic and for the probiotic compare to control diet. However, TTGE was able to detect differences that CE-SSCP did not show. Thus, in ileum, the band (a) of high intensity detected with the probiotic and present with low intensity with the control diet was not detected by CE-SSCP. In the same manner, the bands (b) and (c) of moderate intensity observed by TTGE in 4 pools of cloacal contents of avilamycin and probiotic fed birds and only with low intensity in pools of negative control diet fed birds, were not observed by CE-SSCP.
Thus these two fingerprint techniques used in this study allowed detecting differences between dietary treatment, and these differences were not the same. Moreover CE-SSCP is a high throughput method allowing comparing numerous samples, contrary to the limitation of the method on gel as TTGE where only samples on the same gel can be compared. However, TTGE allows to easily identifying bands on the gel. Thus these techniques appear to be complementary. The difference of detection of characteristic of dietary treatment is not only due to the resolution of the technique as shown by the detection of difference between diets by TTGE that were not detected by CE-SSCP. The choice of primers for PCR and mode of separation of DNA fragment are probably implied. Indeed in this study, we do not use the same universal primers for the two techniques. For CE-SSCP, PCR was performed with the primers W49 and W104 to amplify the V3 region according to Delbes *et al*., (2001), and for TTGE primers Bact 968-GC-f and Bact 1401-r were used to amplify the V6-V8 region of bacterial 16S rRNA genes (Nubel et al., 1996). Whereas during CE-SSCP, DNA fragments are denatured before being separated on a capillary, during TTGE, DNA fragments are denatured during their migration on an acrylamide gel. Thus the use of these two complementary techniques allows detecting difference in the digestive microflora due to the AGP avilamycin and one alternative to AGP, a probiotic, whereas conventional methods with selective medium of culture have difficulties to show difference.

4.2. Effect of avilamycin

With this AGP, a negative effect on FCR was observed from 14 to 25 d and during the whole period (1-42d) due to a higher feed intake without change in weight gain. This negative effect of AGP is unusual.
The lower crypt area in the ileum compared to control diet observed in this study was also previously reported by Sarica et al (2005) with avilamycin. The reduction in crypt area may be related to a lower secretory activity such as a lower mucus production, goblet cells being particularly concentrated in the crypts (Langhout et al., 1999). This reduction may also be related to a decrease in cell turn-over as shown by relationship between crypt depth and cell proliferation (Brunsgaard and Eggum, 1995; Hedemann et al., 2003). In the two cases, it decreases nutrient use by intestinal tissue, and it is thus considered as positive for animal. The lower mucus production and / or decrease in cell turn-over may be due to the

disappearance of some bacterial species in the ileum as showed by the results of CE-SSCP (Gabriel et al, 2006).
The lower heterogeneity of surface of villi compared to control diet observed in this study, can be considered as a positive effect of the AGP on intestinal morphology. It may also be explained by the the disappearance of some bacterial species (Gabriel et al, 2006).
Whereas no effect on microflora was observed with bacterial counts of ileal, cloacal and caecal samples, fingerprint methods showed several effects.
The effect of avilamycin on digestive micriflora was showed by the higher similarity between microbiota TTGE profiles of ileal content of birds fed avilamycin compared to control diet.
Samples of birds fed avilamycin showed the disappearance of several bands with CE-SSCP. Ileal and cloacal samples of birds fed avilamycin were characterized by the absence or presence with low intensity of 2 bands (211.8 and 213.0; universal primer, 6-Fam) contrarily to negative control diet. Moreover, ileal samples of birds fed avilamycin did not present the band 224.0 (universal primer, 6-Fam) contrarily to 3 of the 5 pools of negative control fed birds. In caecal samples, 2 bands (universal primer, 6-Fam), position 185 and 221, present in 3 and 4 pools respectively, with negative control diet, were absent with avilamycin. In fresh dropping, band (211.5, universal primer, 6-Fam) present in 3 pools of negative control diet samples, was absent in avilamycin samples.
However, with avilamycin, some supplementary bands compared to negative control diet were observed. Thus with TTGE, in the cloaca, a band corresponding to a bacteria belonging to the order Clostridiales was observed with a moderate intensity for 4 of the 5 pools of this diet whereas it was present only with a very low intensity in pools of birds fed control diet. With CE-SSCP, in the cloacal samples, 3 of the 5 avilamycin samples showed a band (220; universal primer, 6-Fam) that were absent in negative control diet samples. One band (position 210.5, LAB primers, 6-Fam) was observed in ileal and cloacal samples of 3 pools of birds fed avilamycin and in 4 pools of caecal samples of birds fed avilamycin, and not in corresponding samples of negative control fed birds.
In fresh dropping samples, 2 bands (184.5 and 204.0; universal primer, 6-Fam) absent with negative control diet, were present in 3 pools of avilamycin diet.

4.3. Effect of probiotic

The specific detection of *Pediococcus acidilactici* only in samples of birds fed this probiotic showed that there was no cross-contamination between these birds and those fed control diet or avilamycin. As only 3 pools were positive, it can be suggested that in the other 2 pools, the probiotic was eliminated or its level was under the detection limit. It was not possible to detect a band corresponding to the DNA of this bacteria in samples of digestive content. This is in accordance with the low number of *Pediococcus acidilactici* that can be detected in digestive samples, 10^4 CFU / g samples (ileum, fresh dropping), compared to the total microflora in digestive tract, 10^{11} and 10^9 CFU/g in caeca and ileum respectively (Apajalahti et al, 2004).
With the probiotic a negative effect on FCR was observed from 14 to 25 d due to a higher feed intake without change in weight gain. However, during the whole period (1-42d), a positive effect was observed on FCR. No effect of probiotic was observed on intestine morphometry.
Whereas no effect on microflora was observed on bacterial counts of ileal, cloacal and caecal samples, fingerprint methods showed several effects.
Samples of birds fed probiotic showed the appearance of several bands. With CE-SSCP, a band (position 206 universal primer, Hex), that did not correspond to *Pediococcus acidilactici*, was observed in ileum samples of birds fed probiotic but absent in ileum samples of birds fed negative control diets. This band was also observed in cloacal samples of 3 pools of birds fed probiotic, but not in samples of negative control fed birds. Another band (position 195 universal primer, Hex), was observed in 4 of the 5 pools of fresh dropping samples of birds fed probiotic but not with the other diets. In caecal contents, 3 samples of birds fed probiotic showed 2 bands (207-208, LAB primer, Hex) that were present only with

very low intensity in 2 samples of birds fed control diet. These two bands corresponding to DNA of lactic bacteria, were not detected in profiles of total microflora, and did not correspond to the band of the probiotic strain. One band (position 197, LAB primers, 6-Fam) was detected in 3 pools of cloacal samples of birds fed probiotic but only one of these pools come from a sample in which the probiotic was detected. Another band (position 214.5, LAB primers, 6-Fam) was detected in 2 pools of fresh dropping samples of birds fed probiotic but not with the other diets. With TTGE, in the ileum, a band of higher intensity than with control diet was observed for probiotic treatment. It was identified as *Lactobacillus johnsonii*. This bacteria was detected by Zhu et al (2002) in caecal content of broilers. Moreover, another band corresponding to a bacteria belonging to the order Clostridiales was observed with moderate intensity in the cloaca, whereas it was present only with a very low intensity with control diet.

On the contrary, some bands disappeared with probiotic. A band (221.0; universal primer, 6-Fam) present in 3 cloacal pools of negative control fed birds, was absent in all the cloacal pools of probiotic fed birds. In caecal samples, another band (185; universal primer, 6-Fam) present in 3 pools of negative control fed birds, was absent in all the pools of probiotic fed birds.

5. References

Apajalahti, J., Kettunen, A., Graham, H., 2004. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. World's Poultry Science Journal 60, 223-232.

Brunsgaard, G., Eggum, B.O., 1995. Small intestinal tissue structure and proliferation as influenced by adaptation period and indigestible polysaccharides. Comp. Biochem. Physiol. A Physiol. 112A, 365-377.

Delbes, C., M. Leclerc, E. Zumstein, J. J. Godon, and R. Moletta. 2001. A molecular method to study population and activity dynamics in anaerobic digestors. Water Sci Technol **43:**51-7

Gabriel, I., Lessire, M., Mallet, S., Guillot, J.F., 2006. Microflora of the digestive tract: critical factors and consequences for poultry. World's Poultry Science Journal 62, 499-511.

Hedemann, M.S., Hojsgaard, S., Jensen, B.B., 2003. Small intestinal morphology and activity of intestinal peptidases in piglets around weaning. J. Anim. Physiol. Anim. Nutr. 87, 32-41.

Langhout, D.J., Schutte, J.B., Van Leeuwen, P., Wiebenga, J., Tamminga, S., 1999. Effect of dietary high- and low-methylated citrus pectin on the activity of the ileal microflora and morphology of the small intestinal wall of broiler chicks. Br. Poult. Sci. 40, 340-347.

Mora, D., Fortina, M.G., Parini, C., Manachini, P.L., 1997. Identification of *Pediococcus acidilactici* and *Pediococcus pentosaceus* based on 16S rRNA and ldhD gene-targeted multiplex PCR analysis. FEMS Microbiol Lett 151, 231-236.

Sarica, S., Erdogan, S., Koc, A., Erdogan, Z., 2005. Addition of avilamycin, mannanoligosaccharide and organic acids mixture to corn-soyabean meal based broiler diets. Indian J. Anim. Sci. 75, 961-964.

Wang, Q., Garrity, G.M., Tiedje, J.M., R., C.J., 2007. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl Environ Microbiol 73, 5261-5267.

Zhu, X.Y., Zhong, T., Pandya, Y., Joerger, R.D., 2002. 16S rRNA-based analysis of microbiota from the caecum of broiler chickens. Applied Environmental Microbiology 68, 124-137.

6. List of tables

Table 1. Composition of basal diets (g/kg)

Period (days)	Starter 1-28	Grower 28-35	Finisher 35-47
Ingredients			
Maize	250.00	325.00	440.00
Wheat	300.00	25.00	200.00
Maize gluten meal	-	35.00	45.00
Soyabean meal	327.00	230.00	188.00
Pea	50.00	80.00	50.00
Calcium carbonate	3.50	4.00	3.00
Mono-Dicalcium phosphate	13.50	11.50	11.00
Sodium chloride	2.50	2.00	2.50
Soda bicarbonate	1.50	2.00	1.00
Soyabean oil	27.50	35.00	35.00
Methionine	2.50	2.00	1.50
Lysine	1.00	2.50	2.50
Choline	3.00	3.00	3.00
Mineral premix	10.00	10.00	10.00
Vitamin premix	8.00	8.00	7.50
Anticoccidian (Clinacox ^{TM})	0.20	0.20	-
Calculated nutrient analysis			
Metabolisable energy (kcal/kg)	2848.96	2997.37	3075.66
Crude protein	21.56	20.04	18.46
Lysine	1.25	1.2	1.06
Methionine + cystine	0.99	0.92	0.84
Tryptophane	0.27	0.22	0.19
Threonine	0.79	0.73	0.67
Calcium	1.04	1.00	0.93
Available phosphorus	0.43	0.37	0.34

Table 2. Performance of broiler chickens fed the experimental diets from 1 to 42 days

	Control	Antibiotic (Avilamycin)	Probiotic (Bactocell)	*p* value
Daily feed intake (g/animal/day) [1]				
Days 14-25	96.9 ± 5.5 a	119.7 ± 4.6 b	125.0 ± 1.8 b	<0.001
Days 1-42	132.5 ± 2.8 a	138.2 ± 1.4 b	131.1 ± 2.8 a	0.001
Daily live weight gain (g/animal/day) [2]				
Days 14-25	80.6 ± 2.5	81.2 ±1.4	81.2 ± 1.1	NS
Days 1-42	74.7 ± 2.1	75.2 ±1.2	75.3 ± 2.0	NS
Feed conversion ratio [1]				
Days 14-25	1.20 ± 0.05 a	1.47 ± 0.05 b	1.54 ± 0.03 c	<0.001
Days 1-42	1.63 ± 0.01b	1.70 ± 0.02 c	1.59 ± 0.02 a	<0.001

(a-c) Mean (± standard error) with different letters for a given parameter differ significantly ($p \leq 0.05$) ;
NS : not significant.
[1] Data represent the mean value of 5 replication pens.
[2] Data represent the mean value of 5 replication pens with 60 birds in each from the beginning of the experiment until the slaughtering of birds (26d), and with 54 birds in each pen after slaughtering of birds.

Table 3. Histological measurements of the intestinal wall (ileum) of broiler chickens (26 d) fed the experimental diets[1]

Parameter		Control	Antibiotic (Avilamycin)	Probiotic (Bactocell)	*P*
Measure	Villi surface (μm^2)	310 763 ± 13 850	312 334 ± 19 455	312 853 ± 12 775	NS
	Crypt surface (μm^2)	10 682 ± 628 a	8 946 ± 284 b	9 499 ± 323 ab	0.021
Variation of measure	Villi surface (%)	22.8 ± 1.51 a	18.1 ± 1.09 b	21.7 ± 1.42 ab	0.049
(SE/mean x 100)	Crypt surface (%)	24.6 ± 1.04	23.4 ± 1.46	23.0 1.20	NS

(a-b) Mean (± standard error) with different letters for a given parameter differ significantly ($p \leq 0.05$);
NS : not significant.
[1] Data represent the mean value of 18 birds (3 of the 5 replication pens x 6 birds/pen).

Table 4. Digestive flora (log $_{10}$ CFU/g intestinal content) of broiler chickens (26 d) fed the experimental diets[1]

	Control	Antibiotic (Avilamycin)	Probiotic (Bactocell)	P
Ileum				
Aerobic mesophilic	8.49 ± 0.19	8.28 ± 0.28	8.69 ± 0.15	NS
Lactic acid bacteria	8.54 ± 0.16	8.21 ± 0.26	8.62 ± 0.18	NS
Coliform	7.56 ± 0.20	7.53 ± 0.25	7.49 ± 0.20	NS
Caeca				
Aerobic mesophilic	9.17 ± 0.19	9.04 ± 0.22	9.12 ± 0.07	NS
Lactic acid bacteria	9.26 ± 0.23	9.05 ± 0.30	9.23 ± 0.12	NS
Coliform	7.70 ± 0.18	7.88 ± 0.21	7.96 ± 0.10	NS
Cloaca				
Aerobic mesophilic	8.71 ± 0.14	8.90 ± 0.11	8.88 ± 0.11	NS
Lactic acid bacteria	8.77 ± 0.14	8.92 ± 0.10	8.91 ± 0.10	NS
Coliform	7.32 ± 0.18	7.74 ± 0.29	7.40 ± 0.22	NS
Fresh dropping				
Aerobic mesophilic	9.11 ± 0.15	9.48 ± 0.10	9.50 ± 0.13	NS
Lactic acid bacteria	8.86 ± 0.12	8.93 ± 0.13	9.28 ± 0.12	NS
Coliform	6.80 ± 0.09 b	7.33 ± 0.21 a	7.51 ± 0.14 a	0.018

(a, b) : Means (± standard error) in the same row with no common letter differ significantly ($P < 0.05$).
NS : not significant
[1] Data represent the mean value of 5 replication pens with pools of 6 birds in each.

Table 5. Specific bands of dietary treatments observed with CE-SSCP and TTGE [1]

Digestive segment	Dietary treatment	CE-SSCP [2]	TTGE (Univ) [2]
Ileum	Avilamycin	- Presence band 210.5 (LAB, 6-Fam) (Av 2, 4, 5) - Absence band 211.8 (Univ, 6-Fam) (Av 2, 4, 5; Very low intensity Av 1, 3) - Absence band 213 (Univ, 6-Fam) (Av 3, 4, 5 ; Very low intensity Av 1, 2) - Absence band 224 (Univ, 6-Fam)	
	Probiotic	- Presence band 206 (Univ, Hex) (P3, 4, 5)	- Presence band a (high intensity P2,4,5)
Cloaca	Avilamycin	- Presence band 220 (Univ, 6-Fam) (Av 1, 4, 5) - Presence band 210.5 (LAB, 6-Fam) (Av 2, 4, 5) - Absence band 211.8 (Univ, 6-Fam) (Av 2, 3, 4, 5) - Absence band 213 (Univ, 6-Fam) (Av 2, 3, 5; Very low intensity Av 1, 4)	- Presence band b (moderate intensity Av 2,3,4,5)
	Probiotic	- Presence band 206 (Univ Hex) (P2, 4, 5) - Presence band 197 (LAB, 6-Fam) (P1, 2, 4) - Absence band 221 (Univ, 6-Fam)	- Presence band c (moderate intensity P 2,3,4,5)
Caeca	Avilamycin	- Presence band 210.5 (LAB, 6-Fam) (Av 2, 3, 4, 5) - Absence band 185 (Univ, 6-Fam) - Absence band 221 (Univ, 6-Fam)	
	Probiotic	- Presence band 207 (LAB, Hex) (P1, 2, 5) - Presence band 208 (LAB, Hex) (P1, 2, 5) - Absence band 185 (Univ, 6-Fam)	
Fresh dropping	Avilamycin	- Presence band 184.5 (Univ, 6-Fam) (Av 2, 4, 5) - Presence band 204 (Univ, 6-Fam) (Av 3, 4, 5) - Absence band 211.5 (Univ, 6-Fam)	ND
	Probiotic	- Presence band 195 (Univ, Hex) (P1, 2, 4, 5) - Presence band 214.5 (LAB, 6-Fam) (P3, 5)	ND

ND : not determined

[1] Specific bands: absent or present bands with the dietary treatments (Avilamycin, probiotic) compared to negative control diet.

[2] Univ : universal primers ; LAB : Lactic Acid Bacteria primers ; Hex : Hex labelling ; 6-Fam : 6-Fam labelling

Table 6. Similarity coefficient (Pearson correlations, %) obtained with TTGE for each alternative to antibiotic and each digestive segment [1]

Dietary treatments	Intestinal segment	Dietary treatments		SEM	Proba
Control/Avilamycin		Control	Avilamycin		
	Ileum	58 b	89 a	4,95	<0,001
	Cloaca	75	82	3,1	NS
	Caeca	69	77	3,6	NS
Control/Probiotic		Control	Probiotic		
	Ileum	76	74	4,33	NS
	Cloaca	76	76	2,87	NS
	Caeca	74	71	3,46	NS

(a, b) : Means in the same row with no common letter differ significantly (P < 0.05). NS : not significant
[1] Five profiles of microflora of pools of 6 birds were compared per dietary treatment

Table 7. Characterization of bands observed by TTGE affected by dietary treatments

Digestive segment	Dietary treatment	Band	Bacteria identified	Accession number	Similarity (%)
Ileum	Probiotic	a	*Lactobacillus johnsonii*	AB295648.1	100%
Cloaca	Avilamycin	b	Order Clostridiales	AF376252.1	100%
	Probiotic	c	Order Clostridiales	AF376252.1	100%

7. List of figures

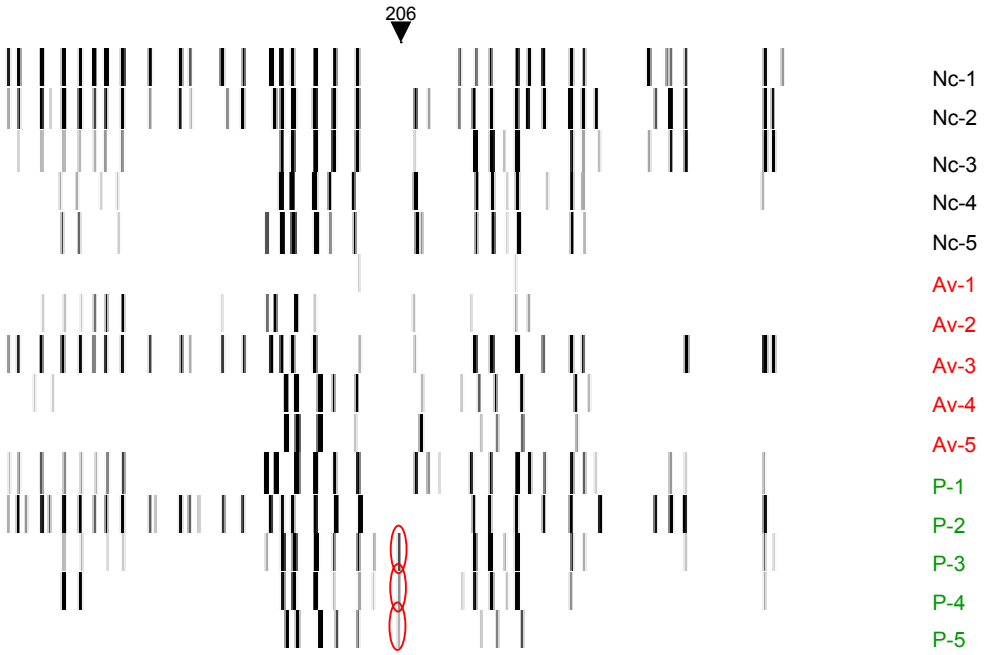

Figure 1. CE-SSCP fingerprints with universal primers (Hex labelling) obtained from ileal pools (6 individuals). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

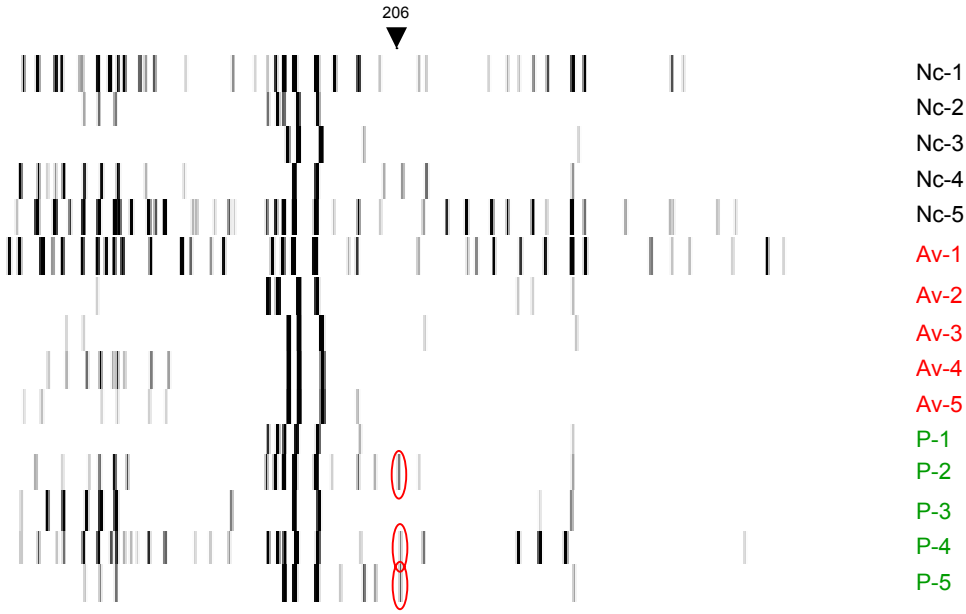

Figure 2. CE-SSCP fingerprints with universal primers (Hex labelling) obtained from cloacal pools (6 individuals). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

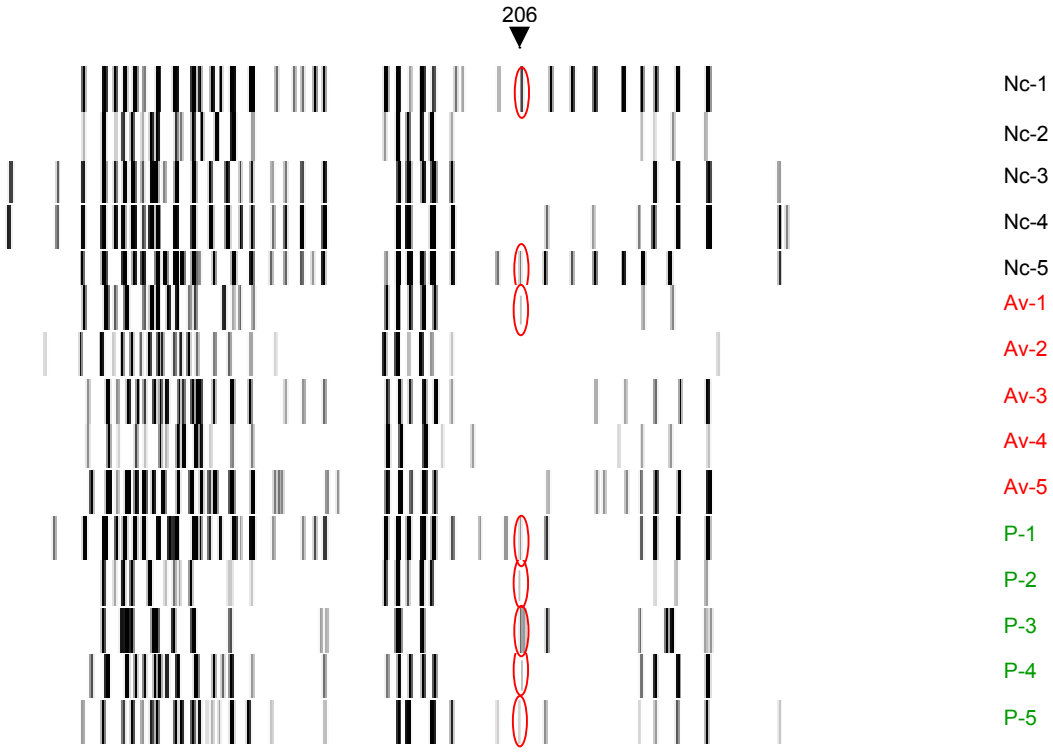

Figure 3. CE-SSCP fingerprints with universal primers (Hex labelling) obtained from caecal pools (6 individuals). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

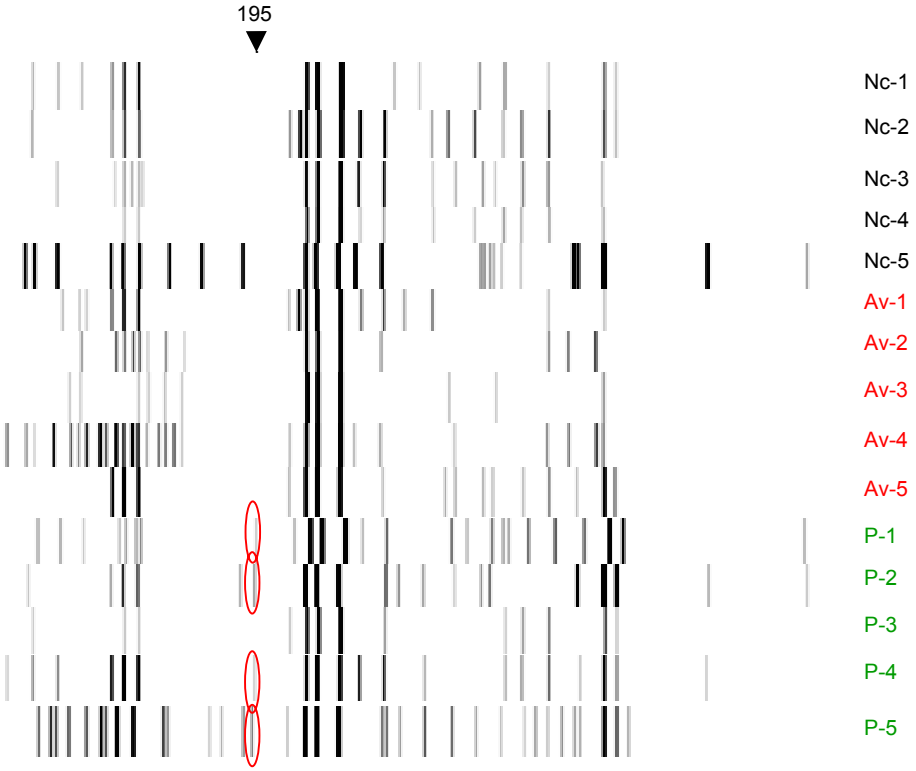

Figure 4. CE-SSCP fingerprints with universal primers (Hex labelling) obtained from fresh dropping pools (6 fresh droppings). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

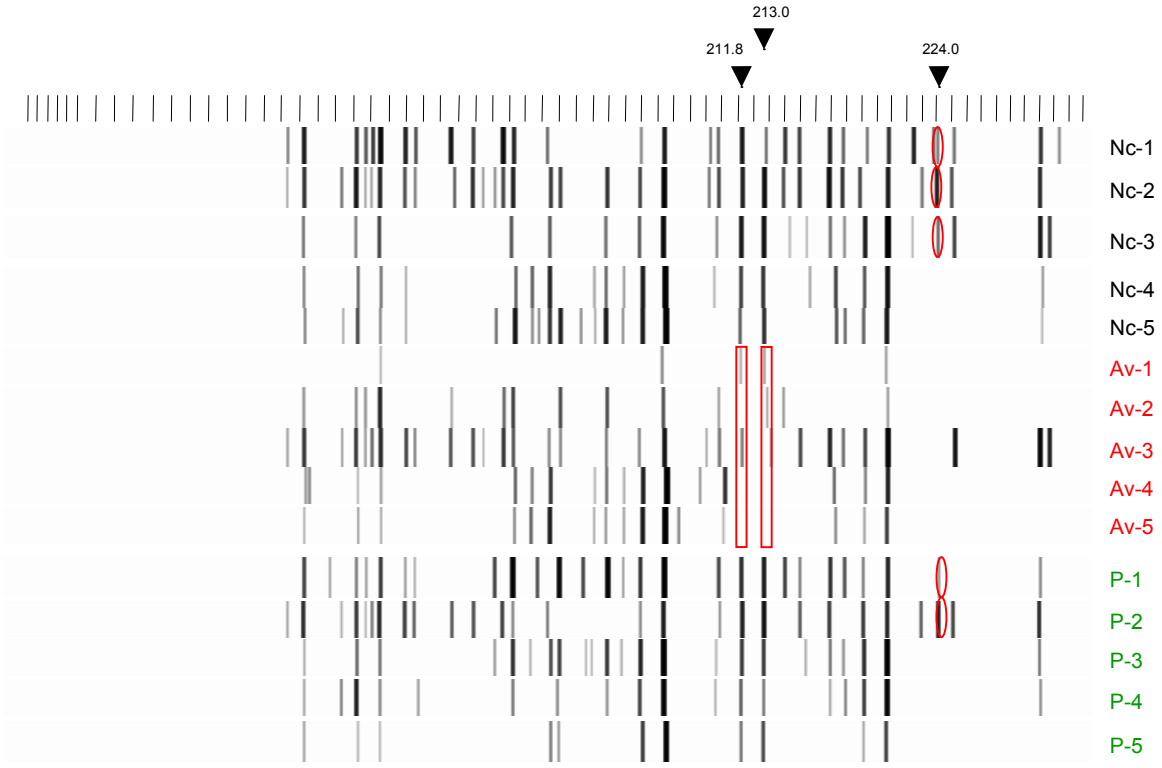

Figure 5. CE-SSCP fingerprints with universal primers (6-Fam) obtained from ileal pools (6 individuals). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

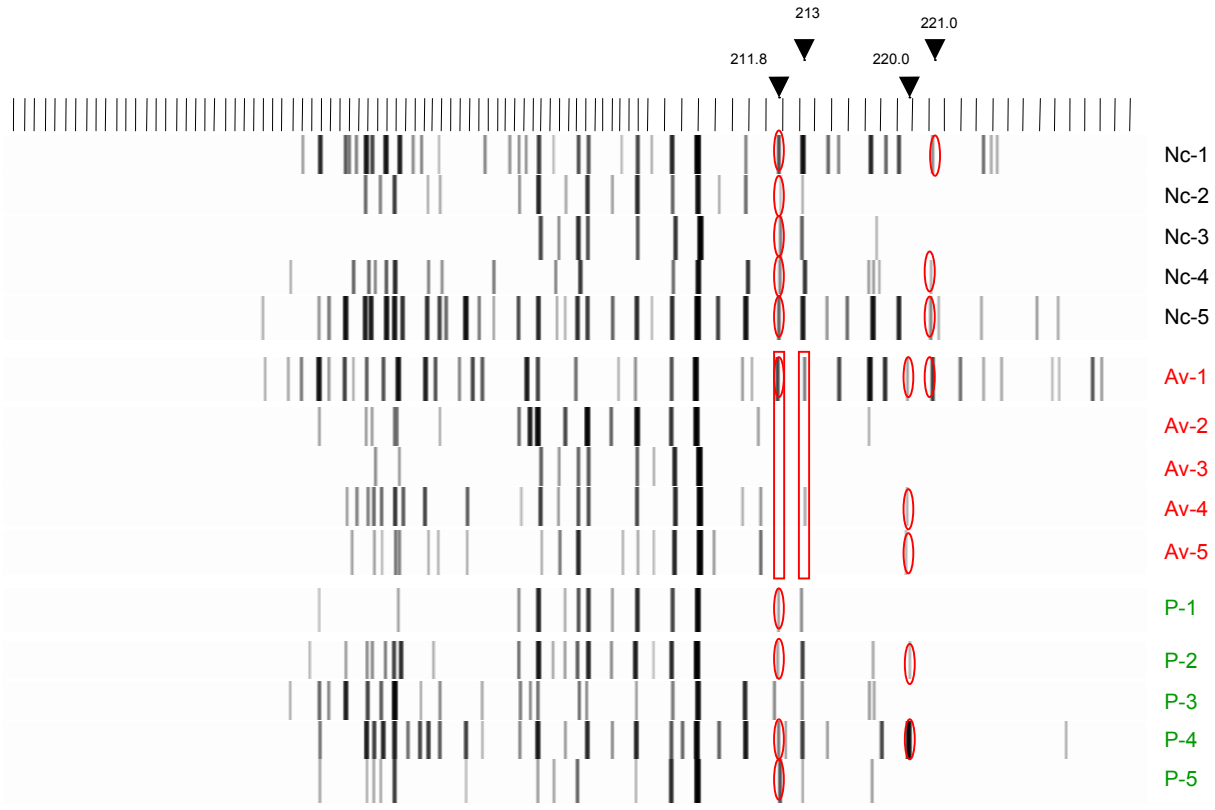

Figure 6. CE-SSCP fingerprints with universal primers (6-Fam) obtained from cloacal pools (6 individuals). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

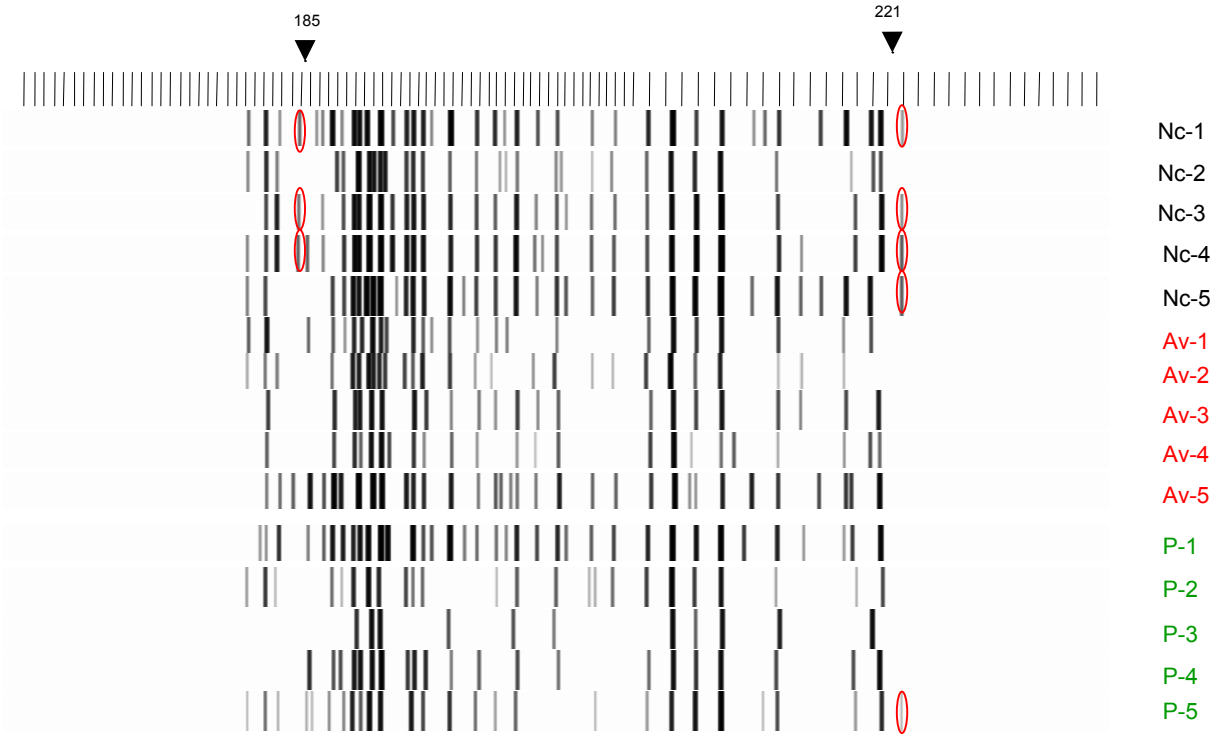

Figure 7. CE-SSCP fingerprints with universal primers (6-Fam) obtained from caecal pools (6 individuals). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

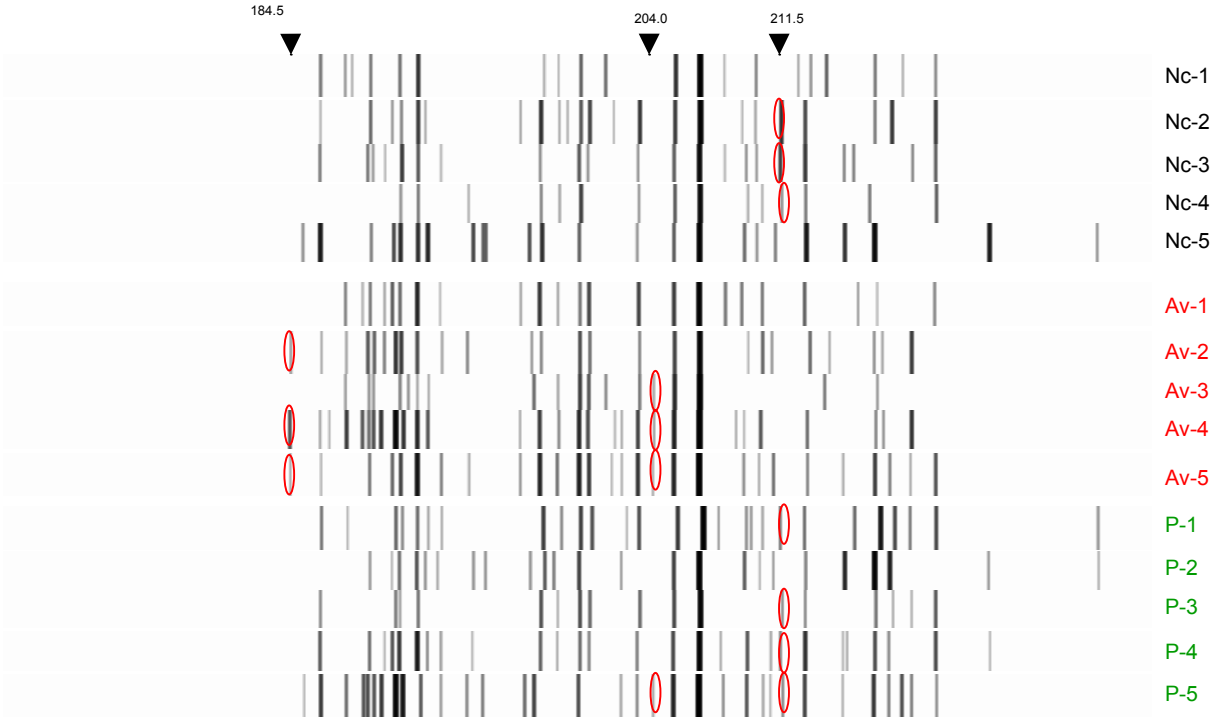

Figure 8. CE-SSCP fingerprints with universal primers (6-Fam) obtained from fresh dropping pools (6 fresh droppings). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

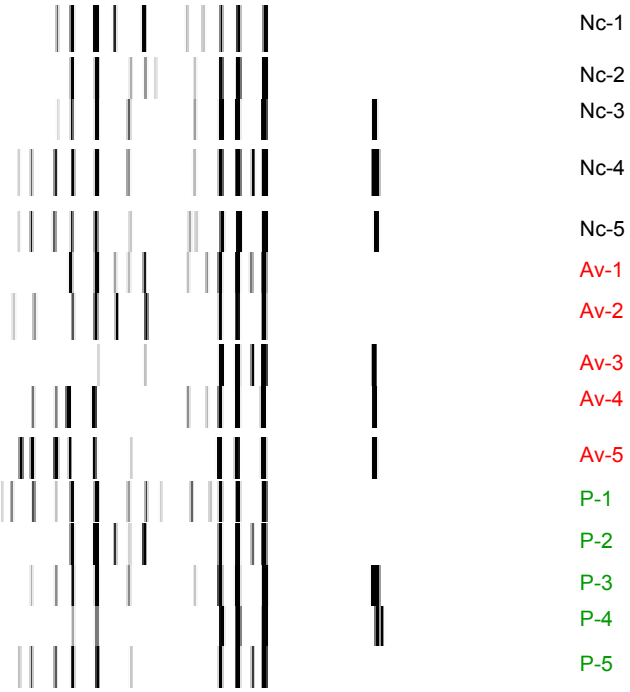

Figure 9. CE-SSCP fingerprints with primers of Lactic Acid Bacteria (Hex labelling) obtained from ileal pools (6 individuals). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

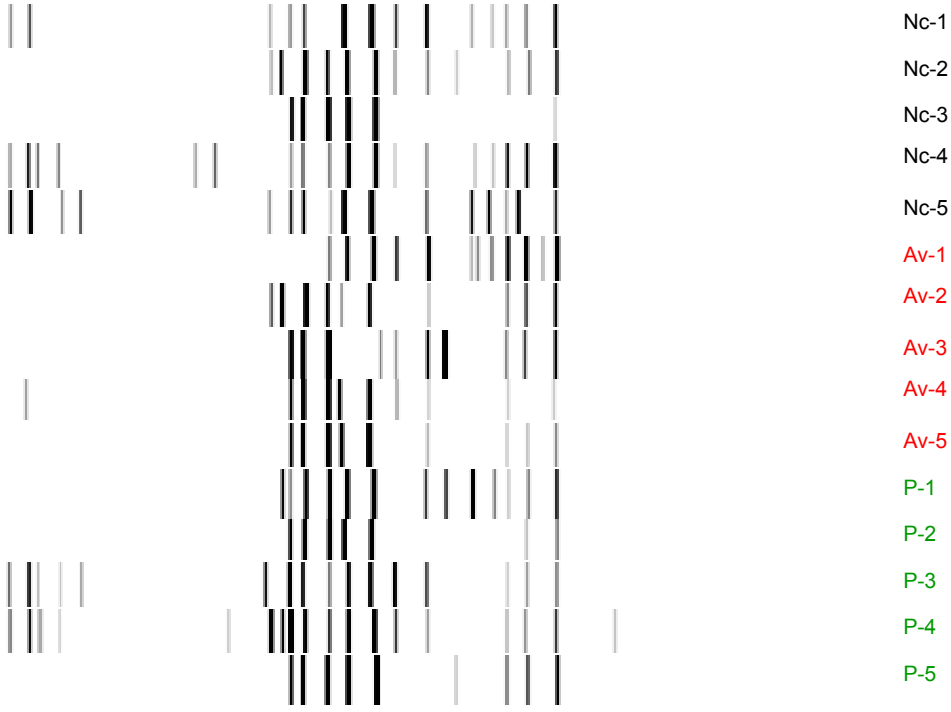

Figure 10. CE-SSCP fingerprints with primers of Lactic Acid Bacteria (Hex labelling) obtained from cloacal pools (6 individuals). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

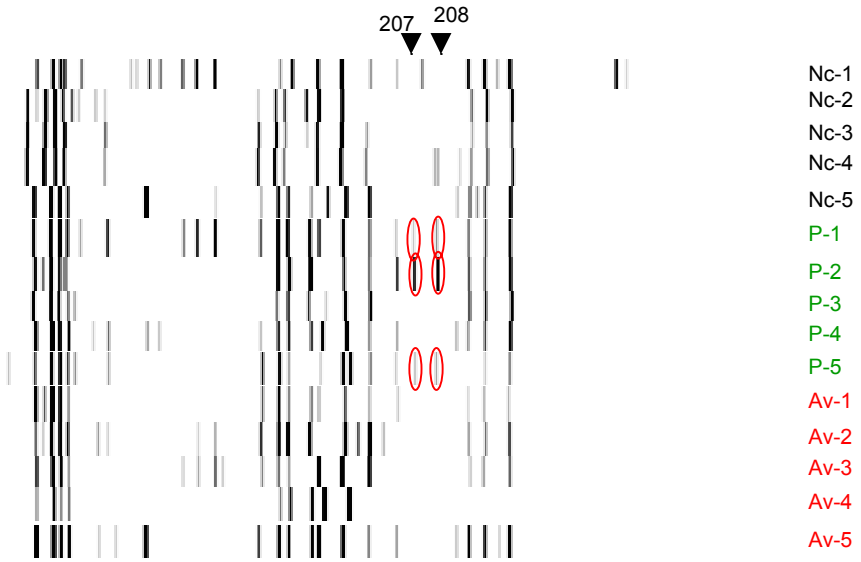

Figure 11. CE-SSCP fingerprints with primers of Lactic Acid Bacteria (Hex labelling) obtained from caecal pools (6 individuals). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

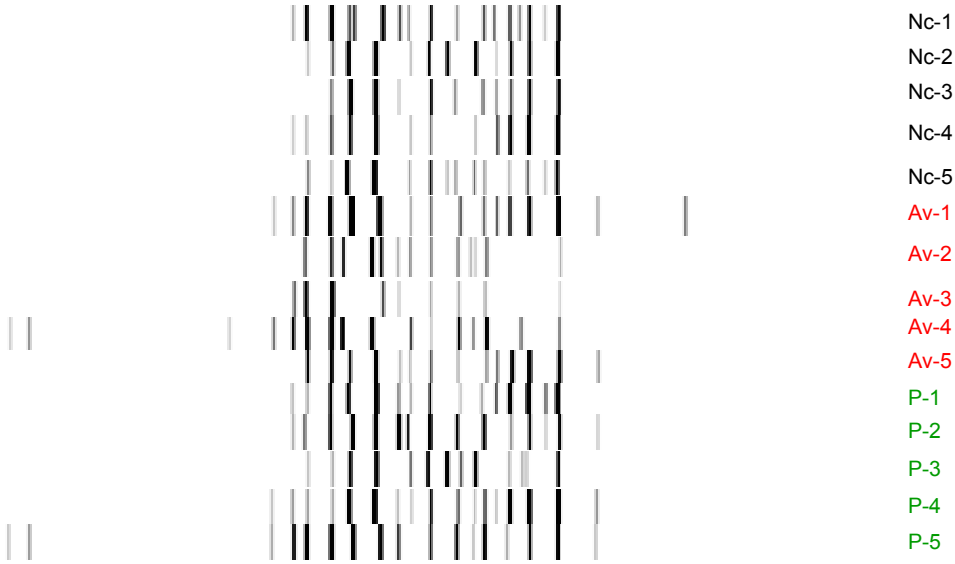

Figure 12. CE-SSCP fingerprints with primers of Lactic Acid Bacteria (Hex labelling) obtained from fresh dropping pools (6 fresh droppings). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

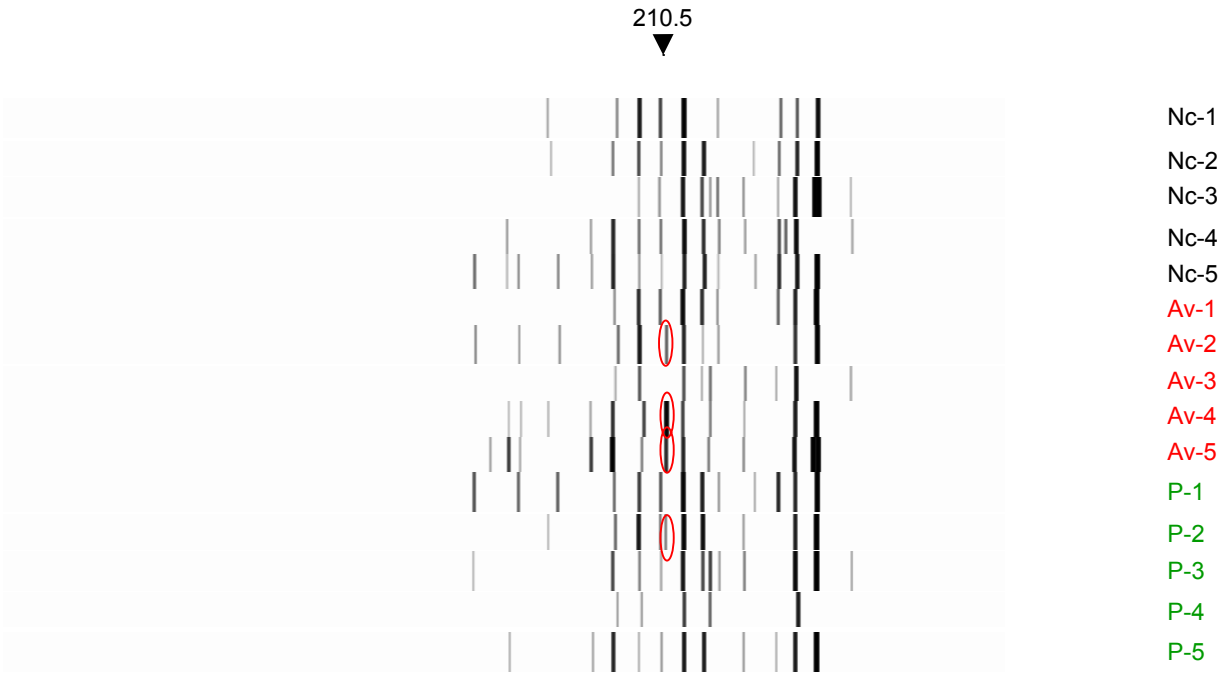

Figure 13. CE-SSCP fingerprints with primers of Lactic Acid Bacteria (6-Fam) obtained from ileal pools (6 individuals). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

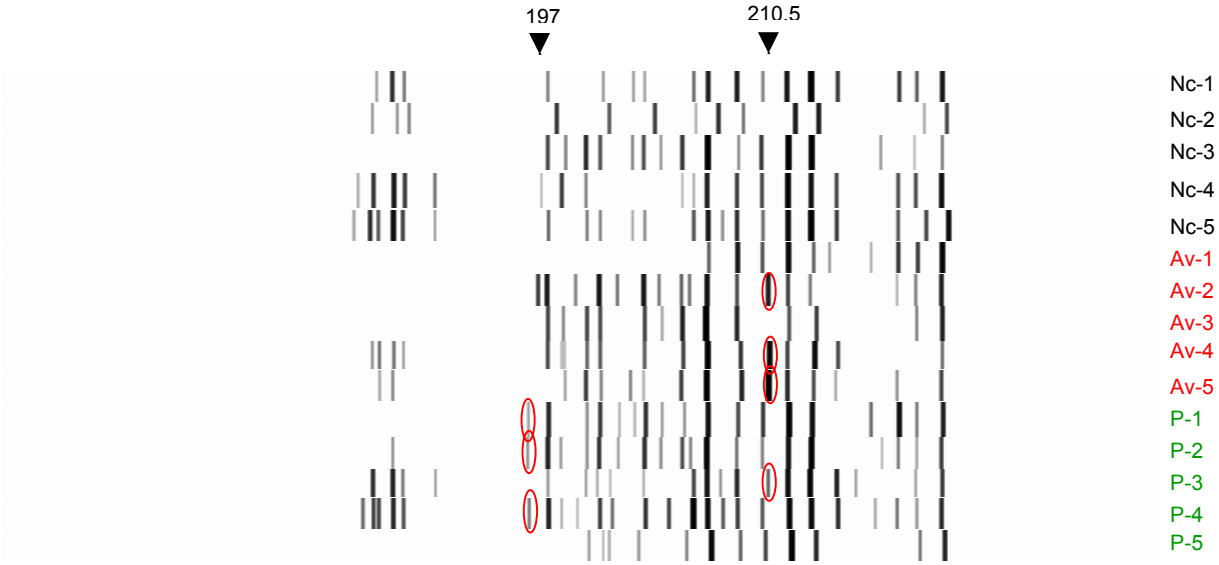

Figure 14. CE-SSCP fingerprints with primers of Lactic Acid Bacteria (6-Fam) obtained from cloacal pools (6 individuals). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

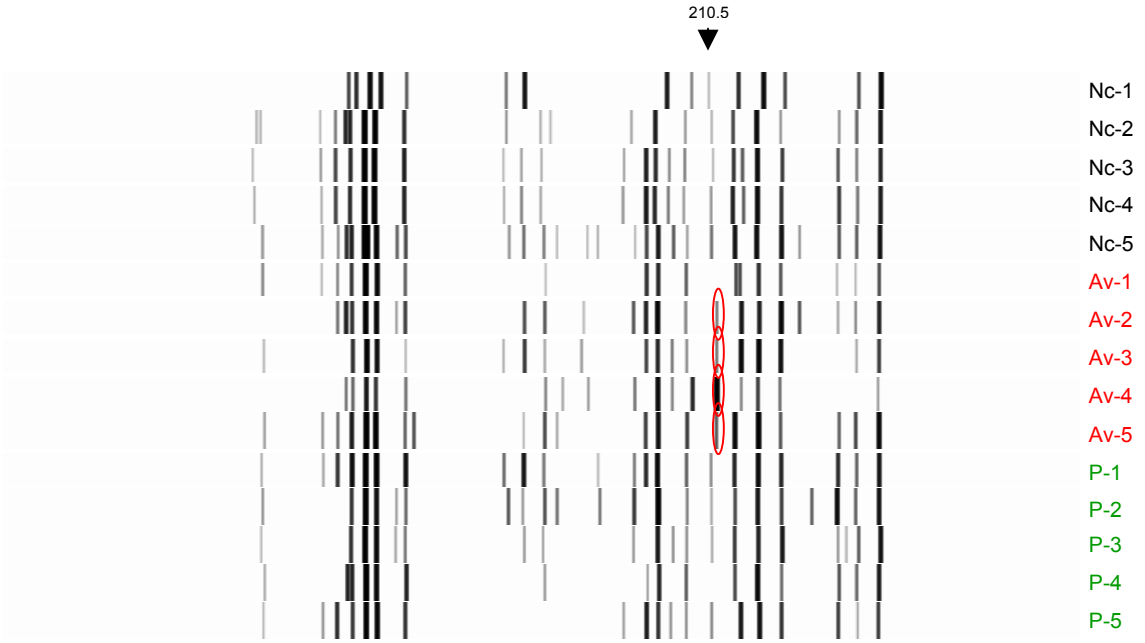

Figure 15. CE-SSCP fingerprints with primers of Lactic Acid Bacteria (6-Fam) obtained from caecal pools (6 individuals). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

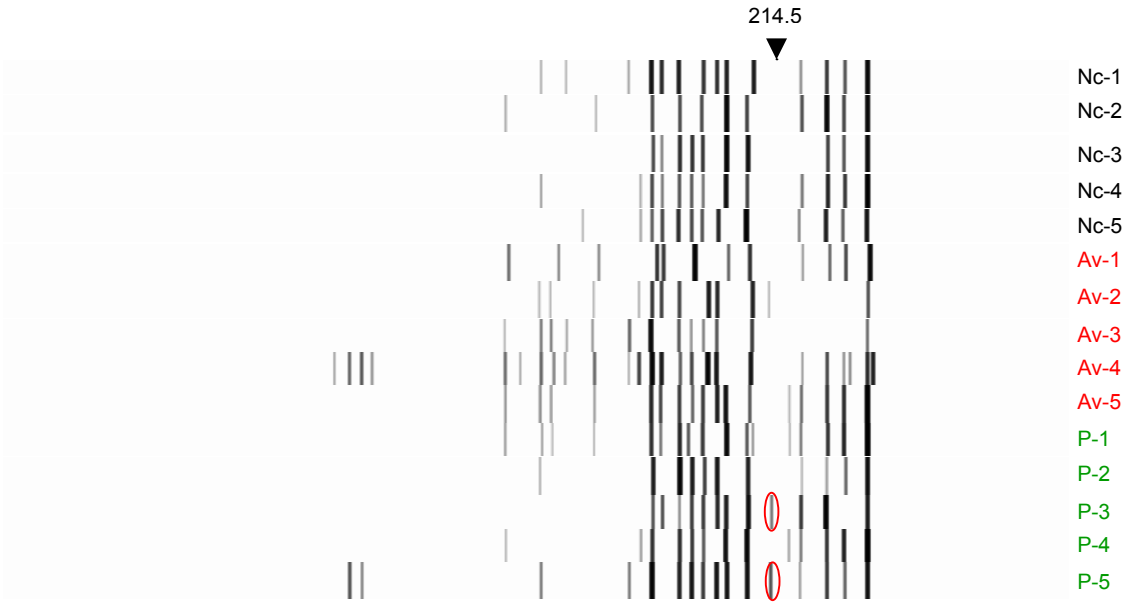

Figure 16. CE-SSCP fingerprints with primers of Lactic Acid Bacteria (6-Fam) obtained from fresh dropping pools (6 fresh droppings). Nc : negative control; Av : avilamycin; P: probiotic. Arrows indicated specific bands.

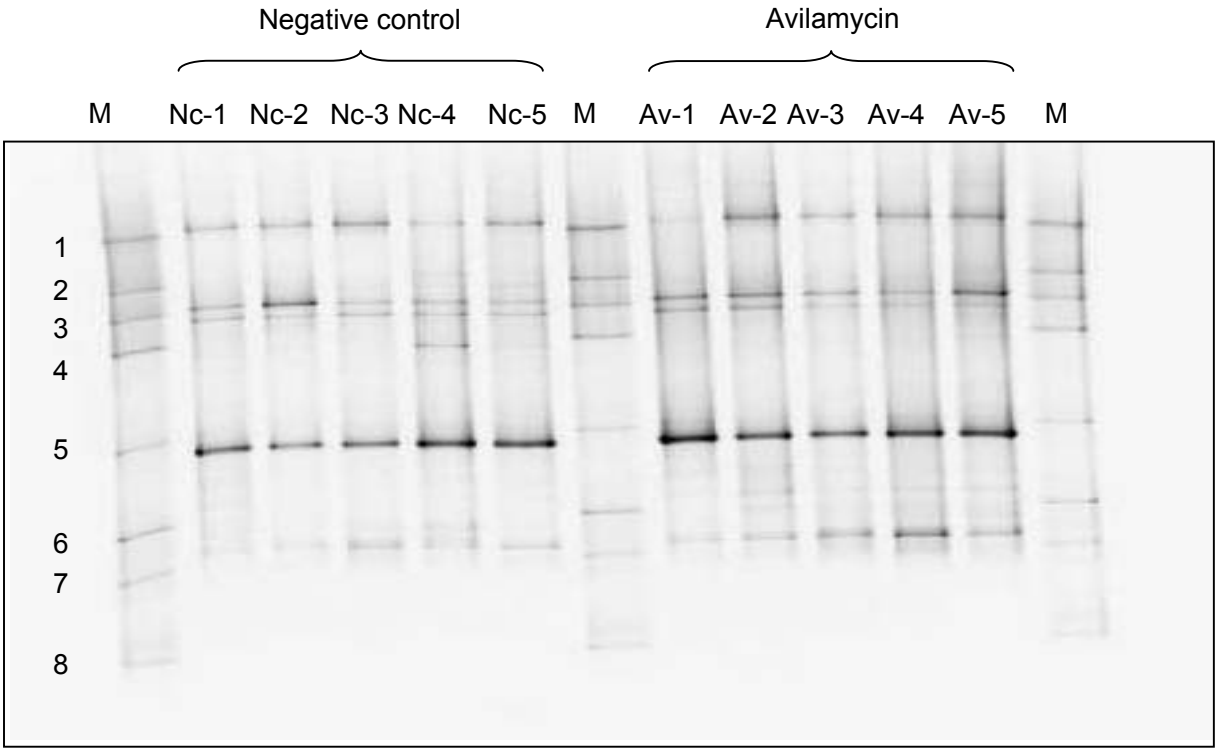

Figure 17. Fingerprints of TTGE with universal primers obtained from ileal pools (6 individuals) from negative control and avilamycin fed birds. Arrows indicated specific bands. M: marqueurs (1 to 8) of TTGE ladder.

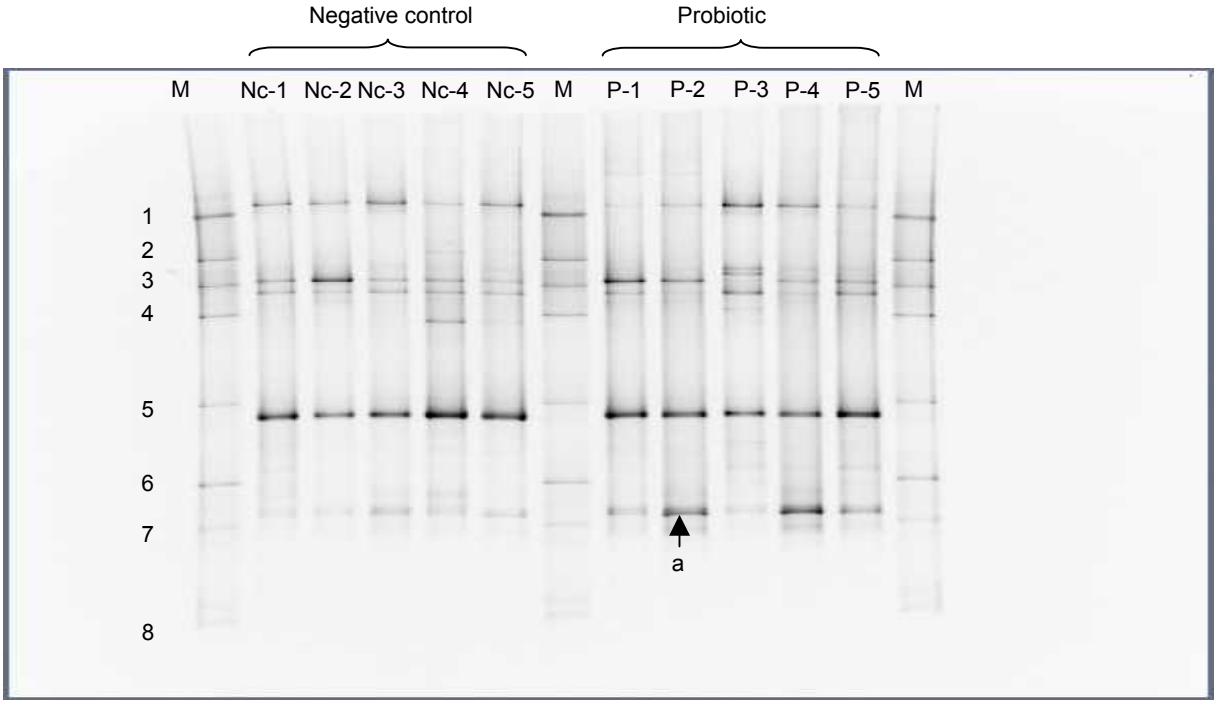

Figure 18. Fingerprints of TTGE with universal primers obtained from ileal pools (6 individuals) from negative control and probiotic fed birds. Arrows indicated specific bands. M: marqueurs (1 to 8) of TTGE ladder.

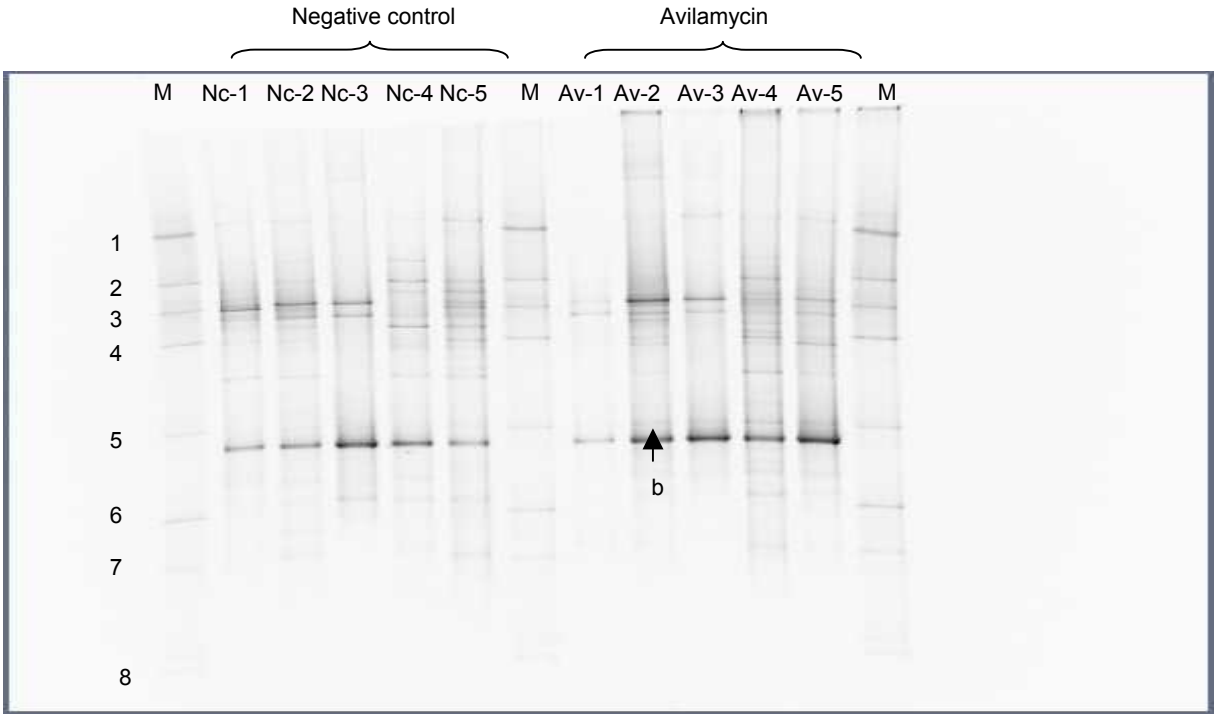

Figure 19. Fingerprints of TTGE with universal primers obtained from cloacal pools (6 individuals) from negative control and avilamycin fed birds. Arrows indicated specific bands. M: marqueurs (1 to 8) of TTGE ladder.

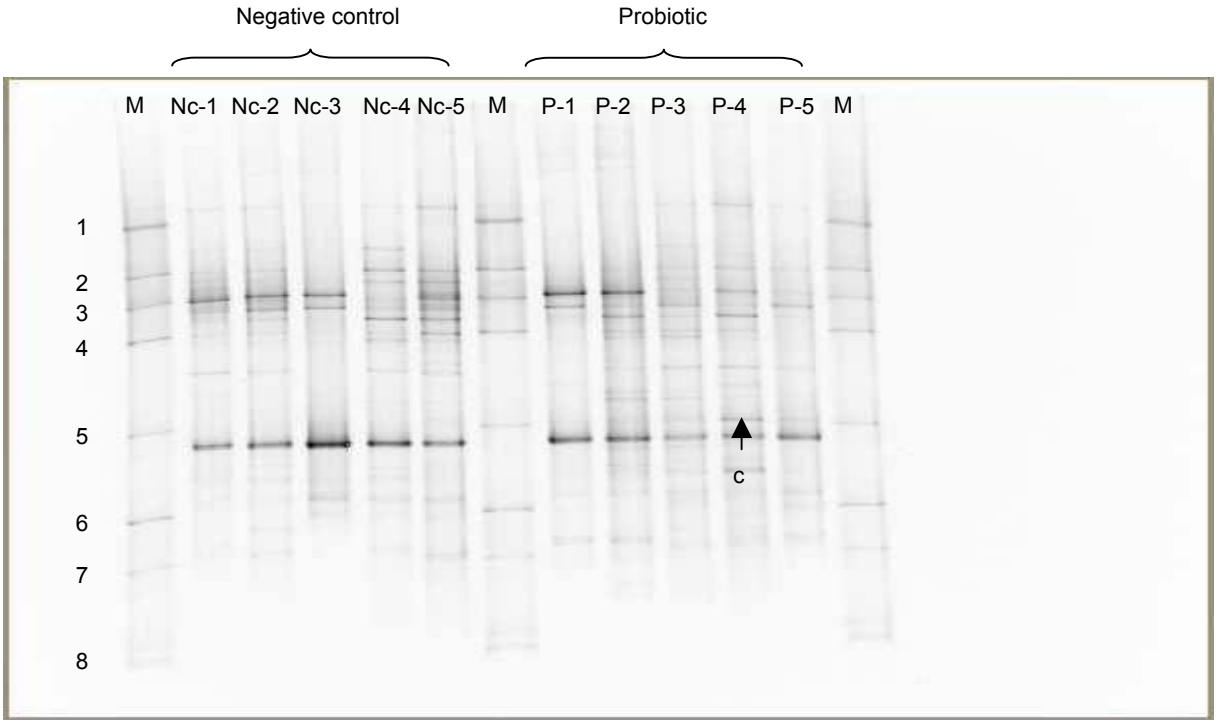

Figure 20. Fingerprints of TTGE with universal primers obtained from cloacal pools (6 individuals) from negative control and probiotic fed birds. Arrows indicated specific bands. M: marqueurs (1 to 8) of TTGE ladder.

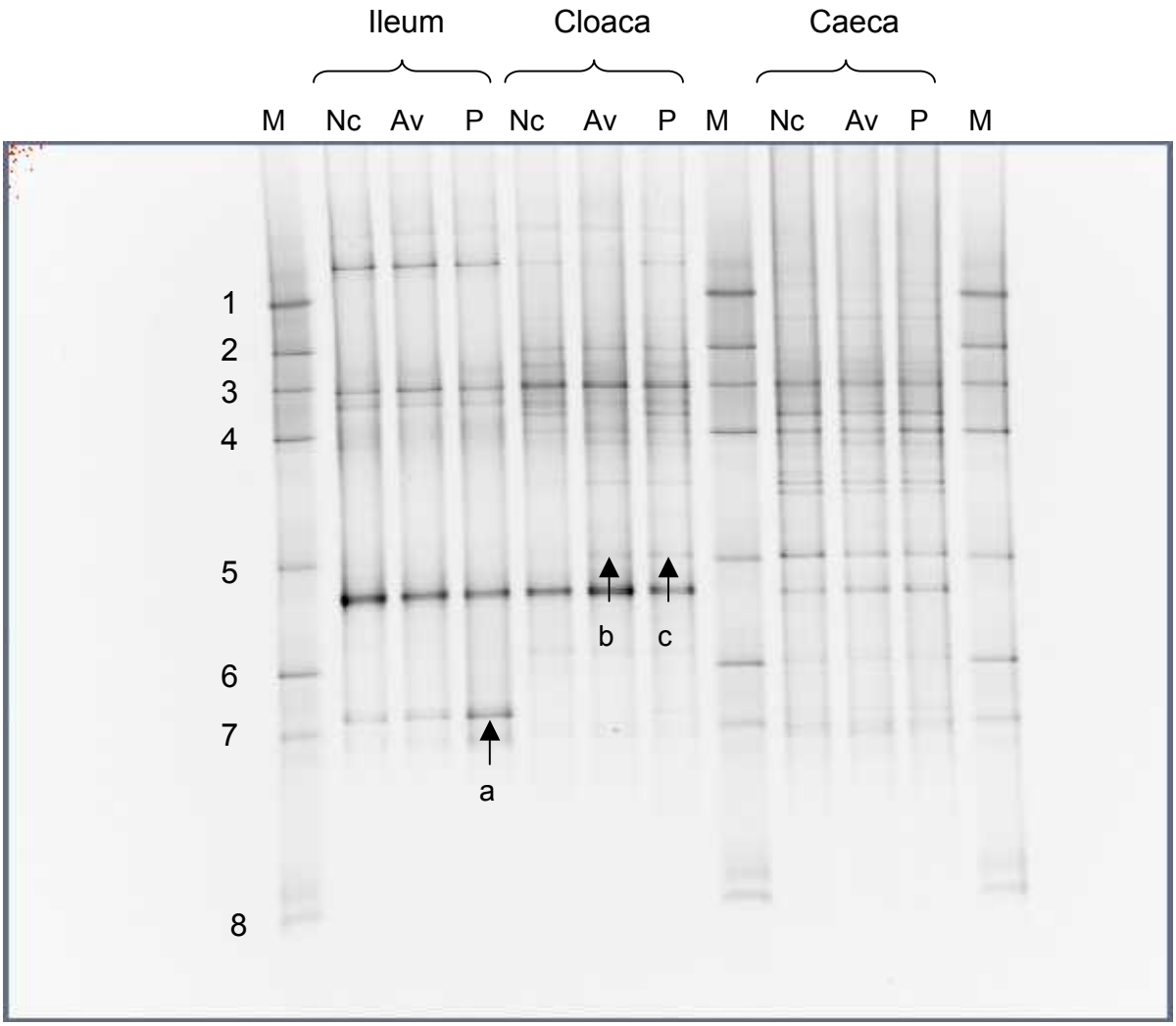

Figure 21. Fingerprints of TTGE with universal primers obtained from ileal, cloacal and caecal pools (30 individuals) of chickens fed the four dietary treatments. (Nc) : negative control; (Av) : avilamycin; (P) : probiotic. Arrows indicated specific bands. M: marqueurs (1 to 8) of TTGE ladder.

E. Effect of prebiotics (FOS) and diet structure (whole wheat instead of ground wheat) by INRA

E1. The effects of fructo-oligosaccharides or whole wheat on the performance and the digestive tract of broiler chickens (accepted for publication in British Poultry Science, January 2008)

Abstract

1. The objective of this experiment was to study two feeding methods, which could potentially act on the gut microflora, the structure and/or the function of the digestive tract and thereby improve the performance of broilers.
2. Four dietary treatments were studied: a negative control (wheat based) with no additives (C), a positive control with 0.01 g/kg avilamycin (AV), a treatment with 0.6 g/kg fructo-oligosaccharides (FOS) and a treatment with the same composition as treatment C but in which a part or all (400 g/kg) of the wheat was given as whole wheat and a concentrate complement (WW). The measurements were: the performance from 0 to 6 weeks, the bacterial counts at 3 weeks and 6 weeks, the digestive tract morphology and the activity of some intestinal enzymes at 3 weeks.
3. The birds fed AV had better daily live weight gain (DLWG) and FCR compared to treatment C. The birds fed FOS had a lower feed intake and a lower DLWG compared to the birds fed on treatment C, but their FCR was significantly improved. WW resulted in a numerically lower feed intake and a significant lower DLWG than treatment C. With AV, the number of aerobic mesophilic bacteria in the caeca was reduced at 3 weeks. With WW, gizzard and pancreas weights were higher and the surfaces of the ileal crypts were larger. An increased activity of leucine aminopeptidase (LAP) in the duodenum was found for treatments AV, FOS and WW.
4. In conclusion, in this study, treatments WW and FOS decreased the DLWG, which may be due to a lower feed intake during the whole period. With WW, the FCR was not affected maybe due to both positive and negative effects on digestive tract (higher gizzard and pancreas development and LAP activity; larger crypts). However, the FOS improved the FCR, which may be partly explained by the higher LAP activity.

1. Introduction

Since the 1950s subtherapeutical levels of antibiotic growth promoters (AGP) have been used in animal feed to improve the performance of animals by controlling the digestive microflora and thereby lower production costs (Thomke and Elwinger, 1998). However, the growing concern from consumers regarding antibiotic usage and the potential development of bacterial resistance, led to a ban of AGP from January 2006 in the European Union, which has resulted in a search for alternatives. Two potential alternatives in poultry production are fructo-oligosaccharides (FOS) and diets containing whole grains.

FOS are oligosaccharides, which are not hydrolysed by digestive enzymes, and may act as growth substrate for the intestinal flora (Monsan and Paul, 1995; Hartemink *et al.*, 1997). They are considered as prebiotics. They have been shown to have beneficial effects on the gut flora by stimulating the growth of beneficial bacteria such as bifidobacteria and lactobacilli, and by inhibiting potential pathogenic bacteria, i.e. *Salmonella* and *E. coli* (Bailey *et al.*, 1991; Waldroup *et al.*, 1993; Xu *et al.*, 2003). Furthermore, they stimulate the activity of some digestive enzymes. For example, Xu *et al.* (2003) found a higher activity of amylase and protease with the inclusion of FOS. The use of this prebiotic has also shown to improve the intestinal structure in broilers, by an increase in villus height in the ileum and a decrease in crypt depth in the jejunum and ileum (Xu *et al.*, 2003). The beneficial effects on the flora and the digestive physiology found with FOS could contribute to the observed improvements

in the performance in poultry (Monsan and Paul, 1995; Orban *et al.*, 1997; Patterson and Burkholder, 2003; Xu *et al.*, 2003).
Another type of feeding, which potentially modifies the intestinal flora, is the inclusion of whole grains in the diet. A lower number of *E. coli* (Gabriel *et al.*, 2003b), a reduction in lactose negative enterobacteria and an increase in the number of certain lactobacilli have been reported (Engberg *et al.*, 2004). These modifications of the flora could be due to a reduction in the pH (0.5-1 unit) in the gizzard, caused by an increased secretion of hydrochloric acid in the proventriculus (Gabriel *et al.*, 2003a; Engberg *et al.*, 2004). The inclusion of whole wheat has also shown to improve the development and maturity of the intestinal mucosa (Gabriel *et al.*, 2007). These modifications may explain the improvement in the performance of broilers observed in several studies (Preston *et al.*, 2000; Hetland *et al.*, 2002; Plavnik *et al.*, 2002; Gabriel *et al.*, 2003a). Furthermore, the inclusion of whole grains is an attractive alternative. It meets the consumer requirements for a more "natural" production system and it reduces the feed costs due to less transport and processing and thereby lower production costs (Hetland *et al.*, 2002; Svihus *et al.*, 2004).
The objectives of this experiment were to study the effects of these two potential alternatives to AGP, FOS and whole wheat, on the performance, the gut flora, the intestinal structure and function of broiler chickens.

2. Materials and methods

2.1. Experimental diets

The birds were allocated to four dietary treatments: 1) a negative control (wheat based) with no additives (C), 2) a positive control containing 0.01 g/kg avilamycin (AV), 3) a treatment containing 0.6 g/kg of short chain fructo-oligosaccharides (FOS) and 4) a treatment with the same composition as treatment C but in which a part or all (400 g/kg) of the wheat was given as whole wheat and a concentrate complement (WW).
The feeding program consisted of four different diets for each treatment: a starter diet (from 1 to 11 days of age), a grower diet (from 12 to 25 days of age), a finisher diet (from 26 to 36 days of age) and a withdrawal diet (from 37 to 42 days of age). The composition of the basal diets (the negative control diets) is shown in table 1. The diets were steam pelleted (2.5 mm in diameter, at 55 to 66°C).
For the treatments FOS and AV, the fructo-oligosccharides and the avilamycin were incorporated at the expense of the same amount of maize. For treatment WW, a part or all of the ground wheat of the basal diets was replaced by the same amount of coarsely ground or whole wheat, and was mixed with pelleted concentrate complements. These complements were calculated from the basal diets without the part of wheat given as coarse particles or whole grains. To accustom the chickens to whole grain, the coarsely ground or the whole wheat was gradually incorporated in the diet. Until day 7 the birds allocated to treatment WW received the same diet as treatment C. On day 8 and 9, 200 g/kg coarsely ground wheat was mixed with a pelleted concentrate complement (basal diet without 200g/kg of wheat). On day 10 and 11, 200 g/kg whole wheat was incorporated to the same concentrate complement and from day 12 onwards 400 g/kg whole wheat was added to concentrate complements (basal diets without 400g/kg of wheat).
The feed and the water were supplied *ad libitum*.

2.2. Birds and housing

A total of 864 Ross PM3 male broiler chickens vaccinated against infectious bronchitis were obtained from a commercial hatchery (Sicamen, Volnay, France). The chickens were raised in 3 m^2 floor pens with a stocking density of 12 birds/m^2 with 6 replicates per treatment. From day 1 to 5 the lighting programme consisted of 23 hours light and 1 hour dark (0-1 am), from day 6 to 11, it consisted of 20 hours light and 4 hours dark (0-4 am) and from day 12 to the end of the experiment 18 hours light and 6 hours dark (0-6 am). The temperature was 32°C

from day 1 to 6 , it was reduced to 31°C at day 7, 29°C at day 14, 28°C at day 21, 24°C at day 28, 22°C at day 37 and 18°C at day 42.

2.3. Experimental protocol and sample collection

The experiment was carried out in accordance with the specific guidelines for experiments on animals (Decree, 2001).
At arrival, the birds were randomly distributed with 36 birds in each pen with a similar weight per pen. After 6 hours of fasting, the birds were individually weighed on day 11, 25, 36 and 42. The feed intake in each pen was measured at the same age and the FCR calculated. The actual proportion of whole wheat intake was determined after measuring the whole grains in feed refusals. The mortality was checked daily. Feed intake was expressed as animal present each day (i.e. dead birds were not included). To calculate Daily Live Weight Gain (DLWG) any females and dead birds were taken out of the calculation, but they were included in the FCR calculation.
At 3 weeks of age, 6 chickens representative of their pens were selected (according to their weight) from each pen. They were killed by intravenous injection of sodium pentobarbital. For treatments C and WW, the gizzards were emptied, trimmed for excess fat and weighed, and the pancreases were collected and weighed. The weights were expressed as percentage of live weight.
For all 4 treatments, the digestive tract was removed from the beginning to the end of the intestine. The small intestine was divided into three segments: the duodenum (from gizzard to pancreo-biliary ducts), the jejunum (from pancreo-biliary ducts to Meckel's diverticulum) and the ileum (from Meckel's diverticulum to ileo-caecal junction). For histological analysis, the middle part (1.5 cm long) of the duodenum and ileum was taken, from 3 of the 6 sampled animals per pen. The samples were opened longitudinally, rinsed with cold saline (NaCl 9 g/l) and fixed in a buffered formaline solution overnight. They were then rinsed and stored in ethanol/water (70/30, v/v) and stored at 4°C until further analysis.
The cloacal content was obtained by abdominal pressure on the birds before they were slaughtered. The content from the ileum and caeca was collected by gentle pressure. These digestive content samples were pooled from the 6 animals per pen and stored at -70°C until further microbial analysis.
For the determination of intestinal enzymatic activities, samples were taken from the 3 animals per pen used for histological analysis. The middle section (one third) of each intestinal segment (duodenum, jejunum and ileum) was split longitudinally, rinsed with cold saline, wiped on a paper towel and the mucosa scrapped off before freezing in liquid nitrogen and stored at -70ºC.
At 6 weeks of age, 6 chickens representative of each pen were selected. The cloacal contents were collected as previously described, then the birds were killed. For treatments C and WW, the gizzard and pancreas were removed and weighed as described at 3 weeks. For all 4 treatments, the ileal and caecal contents were sampled and processed as described previously.

2.4. Histological analysis

The intestinal samples (duodenum and ileum) were analysed as described by Goodlad *et al.* (1991). A 0.5 cm sample was cut off and kept in ethanol/acetic acid (75/25, v/v) for 24 hours, followed by a rehydration in ethanol/water (50/50, v/v) and then in distilled water. Thereafter, the samples were stained by the Feulgen reaction: first a hydrolysis in hydrochloric acid 1 N at 60ºC for 6 minutes, then rinsed with distilled water and thereafter stained with Schiff reagent for 30 minutes. Finally, the samples were rinsed in distilled water and stored in acetic acid/water (45/55 v/v) at 4ºC until analysis.
For histological measurements, villi with their attached crypts of Lieberkühn were individually dissected under a dissecting microscope then mounted between a slide and a cover slip in an aqueous mounting agent (Aquatex, Merck). They were measured under the magnification

of 40 for crypts and 10 for villi, using an optical microscope (Leitz, Laborux), a camera (Scion corporation, CFW 1308C) and an image analysis software (Visilog 6.3, Noesis). The length and width of 10 villi and the depth and width of 20 crypts were measured from each segment of each bird. The surface area was calculated for each villi and crypt. An average value was calculated for each bird intestinal segment. Villus to crypt length and surface ratios were then calculated.

2.5. Enzyme activity assays

The intestinal samples (duodenum, jejunum and ileum) were analysed for enzymatic activity of alkaline phosphatase (AP) (EC 3.1.3.1) and of the digestive enzymes maltase (EC 3.2.1.20) and leucine aminopeptidase (LAP) (EC 3.4.11.2).
The frozen intestinal tissues were homogenised at a ratio of 50 mg/ml in phosphate buffer saline (pH 7.4) using an Ultra-turrax® (IKA) for 3 x 10 seconds, and centrifuged (10 000g, 15 min, 4°C). The supernatants were stored at -70°C until further analysis.
For measuring the different enzymatic activities, continuous methods with 96-well microplates were used. For the AP activity, the homogenate was diluted (1/20 for duodenum and jejunum and 1/10 for ileum) and 0.1 ml of the dilution was mixed with 0.2 ml of substrate (8.8 µmole of *p*-nitrophenyl phosphate (Sigma N 4645) per ml of glycine buffer 93 mM containing 50 mM $MgCl_2$, pH 8.8). Readings were carried out at 5 minutes intervals for 30 minutes with a multiscan spectrophotometer (Argus 300 Microplate reader) at 405 nm (at 37°C) using a standard curve with *p*-nitrophenol (Sigma N 7660).
For the LAP activity, the samples were diluted (1/2 for all segments) and 0.03 ml was mixed with 0.25 ml of substrate (1 µmol of L-leucine *p*-nitroanalide (Sigma L 2158) per ml of phosphate buffer 0.1 M, pH 7.2). The plate was read at 405 nm (37°C) at 2 minutes interval for 10 minutes. P-nitroaniline (Sigma N 2128) was used for the standard curve.
Maltase was measured as described by Giorgi *et al.* (1992). The samples were diluted (1/5 for all the samples). 0.05 ml of the sample was mixed with 0.15 ml of substrate 15 mM of maltose (Sigma M5885) in maleate buffer 60 mM containing 11 mM $MgCl_2$ pH 6.8, 342 000 IU/l mutarotase (Biozyme, MUR1), 5 025 IU/l hexokinase (Roche 11 426 362 001), 1.6 mmol/l ATP (Roche 10 519 979 001), 1.3 mmol/l NADP (Roche 10 128 0314 001) and 1 200 IU/l glucose 6-phosphate dehydrogenase (Roche 10 127 671 001). The plate was read at 366 nm at 37°C at 2 minutes interval for 15 minutes. Glucose was used for the standard curve.

2.6. Bacteriology

The samples for bacterial analysis were successively diluted at 1/10 in 9 g/l NaCl and analysed for coliform, lactic acid bacteria and aerobic mesophilic bacteria. The lactic acid bacteria were counted after being plated onto MRS agar (Man, Rogosa, Sharpe) and incubated for 48 hours, the coliforms were plated onto Drigalski agar and incubated for 24 hours and the aerobic mesophilic bacteria on brain heart infusion agar and incubated for 48 hours. All the plates were incubated aerobically at 37°C. The results were expressed as $\log_{10}$ colony forming units (CFU)/g of digestive contents.

2.7. Statistical analysis

The data were analysed using Statview® software programme (Abacus Concepts, Berkeley, CA, USA) by one-way analysis of variance (ANOVA), and significant differences between treatments were determined by Student Newman-Keuls test ($P < 0.05$). The proportion of whole wheat for treatment WW was compared to the expected value with a one-tailed T-test ($P < 0.05$). These results were presented in the text as mean ± standard error.

3. Results

3.1. Performance

During the whole experiment, the mortality was not significantly different between dietary treatments, 4.6% for C, 4.6% for AV, 3.2% for FOS and 4.7% for WW.
For the treatment AV, a significantly higher feed intake was seen from day 26 to 36 compared to the negative control treatment, a significantly higher DLWG was found at each period and throughout the experiment (day 1 to 42). A better FCR was also observed from day 26 to 36 and throughout the experiment (Table 2).
For the treatment FOS, the feed intake and the DLWG were significantly reduced from day 1 to 25 and for the whole period (Table 2). However, the FCR was significantly improved for the treatment FOS compared to the control from day 26 to 36 and throughout the experiment.
The feed intake with the treatment WW was numerically lower during the whole experiment (-5 %). The actual proportion of whole wheat intake in the treatment WW, during the first two days of introduction (from day 10 to 11), was lower than the amount included in the feed, 138 ± 6 g/kg instead of the 200 g/kg, but thereafter the actual proportion of whole wheat intake was only slightly different than the targeted one (400 g/kg): 381 ± 3 g/kg from day 12 to 25 and 388 ± 3 g/kg from day 26 to 36, and 405 ± 1 g/kg from day 37 to 42. The DLWG was lower for the treatment WW compared to the control from day 12 to 36 and for the entire period (Table 2). The FCR was not significantly affected apart from day 1 to 11, where an improvement was observed with WW.

3.2. Digestive microflora

The microflora was not affected by dietary treatments at 3 weeks of age in the ileum and the cloaca. However, in the caeca the number of aerobic mesophilic bacteria was lower for the treatment AV, but none of the other treatments influenced the bacterial counts at this age (Table 3). At 6 weeks of age, none of the dietary treatments affected the number of aerobic mesophilic bacteria, lactic acid bacteria or coliform in the ileum, caeca and cloaca (data not presented).

3.3. Digestive tract morphology and enzyme activities

For the treatment WW, the gizzard and the pancreas weights (Figure 1) were significantly higher compared to treatment C, both at 3 and 6 weeks.
At 3 weeks of age, the different treatments did not affect the gut morphology in the duodenum. The villus height, width and surface were not affected by dietary treatments in the ileum. However, for treatment WW, a numerically higher crypt depth (+ 12 %) was found and a significantly larger crypt surface.
For the intestinal enzyme activity at 3 weeks of age, LAP was significantly higher for the treatments AV, FOS and WW in the duodenum, but no effect was observed in the other segments. The AP and the maltase activities were not significantly affected by dietary treatments in any of the intestinal segments. However, it should be noticed that a numerically higher level of LAP (+ 18 %) and maltase (+ 20 %) occurred for the treatment AV in the jejunum, and for maltase (+ 24 %) for the treatment FOS in the ileum (Table 5).

4. Discussion

4.1. Effect of the AGP avilamycin

A significantly lower number of bacteria was observed in the caeca of birds fed on the treatment AV. This could be expected as AGP reduce the number of bacteria in the digestive tract (Thomke and Elwinger, 1998; Engberg *et al.*, 2000). Avilamycin in particular acts by

interfering with the polypeptides-synthesizing functions and it is mainly active against gram positive bacteria (Wolf, 1973; Butaye *et al.*, 2003), the most numerous bacteria in the digestive tract (Gabriel *et al.* 2006). This reduction in the digestive flora may partly explain the improved performance observed with AV. Indeed, a decrease in the microflora may lead to a lower stimulation of the immune system (Gabriel *et al.*, 2006), which could prevent a depression in feed intake (Klasing *et al.*, 1987) as observed in our study. This increased feed intake may have contributed to the higher weight gain. Moreover the lower digestive microflora resulted in less competition for nutrients (Gabriel *et al.*, 2006) and could partly explain the improved FCR.
This improved FCR could also be due to an increased activity of the digestive enzyme LAP in the duodenum and the numerically higher level of maltase and LAP in the jejunum, which may have contributed to a better feed digestion.
AGP positively affect the intestinal structure. They reduce the weight of the small intestine by thinning the intestinal wall (Coates *et al.*, 1955; Jukes *et al.*, 1956), and this has been suggested to improve the nutrient absorption and thereby the performance. The changes in intestinal morphology (villus and crypt size) depend on the type of AGP (Miles *et al.*, 2006). With avilamycin, higher villus surface area in the jejunum and lower crypt depth in the jejunum and ileum were reported (Sarica *et al.*, 2005; Hernandez *et al.*, 2006). These modifications improve the intestinal function. However, in the current experiment, the inclusion of avilamycin did not affect the gut morphology in the duodenum and the ileum, as previously reported by Catala-Gregori *et al.* (2007).

4.2. Effect of the prebiotic FOS

In the current study, FOS resulted in a lower feed intake. This has also previously been observed in broilers (Demir *et al.*, 2005) as well as in layers (Li *et al.*, 2007), but not in all studies. For example, Juskiewicz *et al.* (2006) reported no effects on the feed intake in turkeys, and Orban *et al.* (1997) reported a higher feed intake when including sucrose thermal oligosaccharide caramel, which is a complex mixture containing fructose-rich oligosaccharides and difructose di-anhydrides.
The lower feed intake observed in our study could have been caused by a stimulation of the intestinal immune system (Klasing *et al.*, 1987), as seen with FOS (Perrin *et al.*, 2001; Bornet and Brouns, 2002) due to bacterial stimulation. Indeed with FOS, a change in the digestive flora could be expected, as oligosaccharides increase the production of volatile fatty acids and lower the pH of the digestive content (Djouzi and Andrieux, 1997; Iji and Tivey, 1998; Perrin *et al.*, 2001; Bornet and Brouns, 2002), which promotes the growth of beneficial bacteria and suppresses the growth of certain pathogenic bacteria (Snel *et al.*, 2002). Thus, with conventional culturing methods Xu *et al.* (2003), when including 2 g/kg FOS, found an increase in the number of lactobacilli and a reduction in the number of *E. coli* in the caeca. With 4 g/kg FOS, they observed more differences in the digestive flora: an increase in the number of lactobacilli and bifidobacteria and a reduction in the number of *E. coli* in both the small intestine and the caeca. Similarly, Orban *et al.* (1997) reported an increase in the number of bifidobacteria in the caeca of broilers, but a reduction in lactobacilli in one study and no effect on either of them in another when using a sucrose thermal oligosaccharide caramel. In their second study they also noticed a reduction in the number of coliforms in the caeca. In the current experiment the inclusion of FOS in the diet did not affect the bacterial counts as observed by Catala *et al*. (2007) with the same inclusion rate of FOS (0.6 g/kg). This low inclusion rate in these studies might explain the lack of response, especially since effects on the intestinal bacterial counts have been noticed with inclusion rates of 2.0 g/kg, but mainly with inclusion rates of 4.0 g/kg (Griggs and Jacob, 2005). However, with low inclusion levels of FOS, modifications of the microflora can occur. Thus, with molecular techniques, which are more exhaustive methods than the standard microbiological cultures, Massias *et al.* (2006) reported changes in the bacterial populations with FOS incorporated at 0.6 g/kg and in particular for lactobacilli.

The effects of the inclusion of FOS in poultry diets on weight gain are not consistent. In our study, a lower weight gain was found, whereas Demir *et al.* (2005) reported no effects in broilers and Juskiewicz *et al.* (2006) in turkeys. On the contrary, Orban *et al.* (1997) reported a higher weight gain with sucrose thermal oligosaccharide caramel in broilers as did Catala-Gregori *et al.* (2007) with an inclusion of 0.6 g/kg FOS. These contradictory results, particularly between the current study and that of Catala-Gregori *et al.* (2007) could be explained by the rearing conditions of the birds, the effects of oligosaccharides are likely to be more beneficial when the chickens are raised in less than ideal conditions (Orban *et al.*, 1997). For example stocking density in Catala-Gregori *et al.* (2007) was 15 birds/m^2 and was only 12 birds/m^2 in our study, and the density was reduced during the experiment by the birds taking out for analyses. With 15 birds/m^2, their raising conditions were more compromised than those in the current study (12 birds/m^2). In the current study, the lower weight gain could have been caused by the lower feed intake.
The inclusion of FOS in the current study improved the FCR in agreement with other studies in broilers (Ammerman *et al.*, 1988; Orban *et al.*, 1997; Xu *et al.*, 2003) or in layers (Respondek and Rudeaux, 2005; Li *et al.*, 2007), while others have reported no significant effects, for example Demir *et al.* (2005) in broilers and Juskiewicz *et al.* (2006) in turkeys. The improved FCR observed in this study might partly be explained by the increased intestinal enzymatic activity with the FOS (a higher LAP activity in the duodenum and a numerically higher level of maltase activity in the ileum). Higher enzymatic activity of protease and amylase has previously been reported with FOS by Xu *et al.* (2003).
In the current study, the intestinal structure was not affected by the inclusion of FOS in the diet, in agreement with Catala-Gregori *et al.* (2007). However, Xu *et al.* (2003) reported higher villi in the ileum and shorter crypts depths in the jejunum and ileum with the inclusion of 4 g/kg FOS. But with the inclusion of 2 g/kg FOS, these authors only observed an increase in the ratio between the villus height/crypt depth in the ileum. The lower inclusion rate of FOS used in the current study might explain the lack of response, maybe due to lower modification of microflora as previously explained.

4.3. Effect of the diet structure: Whole wheat

A lower weight gain after the introduction of whole wheat was observed with whole wheat, which may have been caused by the numerically lower feed intake due to the different structure of the feed compared to the control diet. The reduced feed intake in the beginning of WW introduction may be due to a limited capacity for grinding whole wheat grains in the gizzard and the resulting slower transit rate in the digestive tract. Although, the gizzard adapted fast, as seen by the higher gizzard weight as early as one week after whole wheat introduction, the lower feed intake in the young bird led to a lower growth rate and thus a lower intake thereafter. Otherwise, a reaction towards the new form of diet was noted by the lower proportion of whole wheat grains intake in the first two days after their introduction (138 g/kg actually eaten compared to 200 g/kg included in the diet). However, the animals adapted quickly to this type of feeding in the experiment, as it was seen by the higher proportion of whole wheat after the first two days of introduction, where the actual intake was close to the amount mixed in the feed. A lower feed intake with whole wheat has already been reported by Engberg *et al.* (2004) and Hetland *et al.* (2002), who included moderate 125 to 300 g/kg or high 300 to 440g/kg rates of whole grains. However, other studies showed no difference in feed intake (Preston *et al.*, 2000; Plavnik *et al.*, 2002; Svihus *et al.*, 2002). Several studies have reported no effect on weight gain (Preston *et al.*, 2000; Bennett *et al.*, 2002; Svihus *et al.*, 2004), some have observed a higher weight gain (Plavnik *et al.*, 2002), and others as in the current study have reported a lower weight gain (Hetland *et al.*, 2002).
Although whole wheat improved the FCR in the starting period (day 1 to 11), it was not affected during the whole period. This is in agreement with previous studies (Hetland *et al.*, 2002; Gabriel *et al.*, 2003a; Svihus *et al.*, 2004). However, Plavnik *et al.* (2002) and Wu *et al.* (2004) have reported an improvement in FCR with the inclusion of 200 g/kg whole wheat. On

the contrary, a poorer FCR has been reported particularly with high inclusion level of whole grain (Bennett *et al.*, 2002; Engberg *et al.*, 2006).
In our study, the inclusion of whole wheat in the diet did not significantly affect the bacterial count in the intestine. However, other studies have shown a decrease in the number of aerobic mesophilic bacteria, coliforms and lactose-negative enterobacteria and higher counts of some *Lactobacillus* species (Gabriel *et al.*, 2003b; Engberg *et al.*, 2004; Gabriel *et al.*, 2007). Although no changes in the microflora were observed in the current study with conventional cultivation methods, other bacterial population could have been modified. This may be observed by using molecular tools, as previously explained for the FOS. These modifications of the digestive flora could be due to a decreased pH in the gizzard (Gabriel *et al.*, 2003a). Moreover, the higher activity of this organ, as indicated by its higher weight observed in our study and in previous studies (Jones and Taylor, 2001; Plavnik *et al.*, 2002; Gabriel *et al.*, 2003a; Engberg *et al.*, 2004), may increase digestion of all dietary compounds. The higher pancreas weight observed in this study and in previous studies (Banfield *et al.*, 2002; Engberg *et al.*, 2004; Wu *et al.*, 2004) may be responsible for the increased amylase activity in the jejunum content, which may contribute towards a higher ileal starch digestibility (Svihus and Hetland, 2001; Svihus *et al.*, 2004). This higher digestibility of nutrients leads to less available substrate for the microflora.
In the duodenum, in the current experiment, the feeding of whole wheat had no effect on morphological parameters, contrary to results obtained in a previous study (Gabriel *et al.*, 2007) showing a reduction in the crypt depth. However, an increased intestinal enzyme activity was observed in this experiment as well as in the previous study. Thus in our study a higher activity of LAP was observed, and in the previous study, a higher activity of AP.
In the ileum, although previous studies showed no effect of feeding whole wheat on the intestinal structure or enzymatic activity (Wu *et al.*, 2004; Gabriel *et al.*, 2007), we observed larger crypt surfaces. It may be related to an increase of the cellular renewal, as shown by the relation between the crypt depth and the activity of cellular proliferation (Brunsgaard and Eggum, 1995). This higher cell turn-over may lead to lower enterocyte maturity. However, no difference in AP activity, used as an indicator of enterocyte maturity (Weiser, 1973), was observed in our study. The increased crypt surfaces may also be due to a higher number of goblet cells particularly concentrated in the crypt, which can result in increased mucus secretion (Langhout *et al.*, 1999). The higher mucus production can decrease the nutrient absorption. In both the cases, the increase of cellular turn-over or the mucus production, this represents an increase of energy requirement for gut maintenance, which means the animal uses the nutrients for the functioning of the digestive tract instead of its growth.
Positive effects of whole wheat feeding were observed at the beginning of the digestive tract (increase development of gizzard and pancreas, increase enzymatic activity in the duodenum), whereas a negative effect was observed at the end of the intestine (higher crypt development in the ileum). This may explain the lack of effect on FCR during most of the experiment.
In conclusion, the inclusion of avilamycin improved the performance of broilers, which could be explained by the lower bacterial load in the caeca and the increased activity of the digestive enzymes. With the inclusion of FOS in the diet, a reduction in weight gain was observed which may be explained by the lower feed intake. However, the FCR was improved, which might be due to the contribution of higher intestinal enzymatic activities. With whole wheat feeding, the effects both positive (increase development of gizzard and pancreas, increase enzymatic activity) and negative (higher crypt development) on digestive tract may explained the lack of effect on FCR during most of the experiment. The reduction of weight gain with this treatment may be explained by the numerically lower feed intake due to the different structure of the feed.

5. References

AMMERMAN, E., QUARLES, C. & TWINING, P. (1988). Broiler response to the addition of dietary fructooligosaccharides. *Poultry Science,* **67**: 46.

BAILEY, J. S., BLANKENSHIP, L. C. & COX, N. A. (1991). Effect of fructooligosaccharide on salmonella colonization of the chicken intestine. *Poultry Science,* **70**: 2433-2438.

BANFIELD, M. J., KWAKKEL, R. P. & FORBES, J. M. (2002). Effects of wheat structure and viscosity on coccidiosis in broiler chickens. *Animal Feed Science and Technology,* **98**: 37-48.

BENNETT, C., CLASSEN, H. & RIDDELL, C. (2002). Feeding broiler chickens wheat and barley diets containing whole, ground and pelleted grain. *Poultry Science,* **81**: 995-1003.

BORNET, F. R. J. & BROUNS, F. (2002). Immune-stimulating and gut health-promoting properties of short-chain fructo-oligosaccharides. *Nutrition Reviews,* **60**: 326-334.

BRUNSGAARD, G. & EGGUM, B. O. (1995). Small intestinal tissue structure and proliferation as influenced by adaptation period and indigestible polysaccharides. *Comparative Biochemistry and Physiology (Physiology),* **112A**: 365-377.

BUTAYE, P., DEVRIESE, L. A. & HAESEBROUCK, F. (2003). Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on gram-positive bacteria. *Clinical Microbiology Reviews,* **16**: 175-188.

CATALA-GREGORI, P., MALLET, S., TRAVEL, A. & LESSIRE, M. (2007). Un extrait de plantes et un prebiotique sont aussi efficaces que l'avilamycine pour ameliorer les performances du poulet de chair. *7e Journées de la Recherche Avicole*, Tours, France, pp.: 202-206.

COATES, M. E., DAVIES, M. K. & KON, S. K. (1955). The effect of antibiotics on the intestine of the chick. *British Journal of Nutrition,* **9**: 110-119.

DECREE (2001). Decree no. 2001-646 related to experiments carried out on vertebrate animals, May 29, 2001. French Code. *Official Journal:* 8682.

DEMIR, E., SARICA , S., ÖZCAN, M. A. & SUIÇMEZ, M. (2005). The use of natural feed additives as alternative to an antibiotic growth promoter in broiler diets. *Archiv für Geflügelkunde,* **69**: 110-116.

DJOUZI, Z. & ANDRIEUX, C. (1997). Compared effects of three oligosaccharides on metabolism of intestinal microflora in rats inoculated with a human faecal flora. *British Journal of Nutrition,* **78**: 313-324.

ENGBERG, R. M., HEDEMANN, M. S., LESER, T. D. & JENSEN, B. B. (2000). Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. *Poultry Science,* **79**: 1311-1319.

ENGBERG, R. M., HEDEMANN, M. S., STEENFELDT, S. & JENSEN, B. B. (2004). Influence of whole wheat and xylanase on broiler performance and microbial composition and activity in the digestive tract. *Poultry Science,* **83**: 925-938.

ENGBERG, R. M., STEENFELDT, S. & JENSEN, B. B. (2006). The influence of high dietary concentrations of whole wheat and different forms of coccidiosis control on broiler production, nutrient digestibility and on the composition of the intestinal microflora*. Proceedings of the 12th European Poultry Conference*, Verone, Italy.

GABRIEL, I., MALLET, S. & LECONTE, M. (2003a). Differences in the digestive tract characteristics of broiler chickens fed on complete pelleted diet or on whole wheat added to pelleted protein concentrate. *British Poultry Science,* **44**: 283-290.

GABRIEL, I., MALLET, S., LECONTE, M., FORT, G. & NACIRI, M. (2003b). Effects of whole wheat feeding on the development of coccidial infection in broiler chickens. *Poultry Science,* **82**: 1668-1676.

GABRIEL, I., LESSIRE, M., MALLET, S. & GUILLOT, J. F. (2006). Microflora of the digestive tract: critical factors and consequences for poultry. *World Poultry Science Journal,* **62**: 499-511.

GABRIEL, I., MALLET, S., LECONTE, M., TRAVEL, A. & LALLÈS, J. P. (2007). Effects of whole wheat feeding on the develpment of the digestive tract of broiler chickens. *Animal Feed Science and Technology,* **in press**.

GIORGI, M., VANNI, P. & PINZAUTI, G. (1992). A new continuous optical assay for maltase and sucrase. *Enzyme,* **46**: 299-303.

GOODLAD, R., LEVI, S., LEE, C., MANDIR, N., HODGSON, H. & WRIGHT, N. (1991). Morphometry and cell proliferation in endoscopic biopsies: Evaluation of a technique. *Gastroenterology,* **101**: 1235-1241.

GRIGGS, J. P. & JACOB, J. P. (2005). Alternatives to antibiotics for organic poultry production. *Journal of Applied Poultry Research,* **14**: 750-756.

HARTEMINK, R., VAN LAERE, K. M. J. & ROMBOUTS, F. M. (1997). Growth of enterobacteria on fructo-oligosaccharides. *Journal of Applied Microbiology,* **83**: 367-374.

HERNANDEZ, F., GARCIA, V., MADRID, J., ORENGO, J., CATALTE;, P. & MEGIAS, M. D. (2006). Effect of formic acid on performance, digestibility, intestinal histomorphology and plasma metabolite levels of broiler chickens. *British Poultry Science,* **47**: 50 - 56.

HETLAND, H., SVIHUS, B. & OLAISEN, V. (2002). Effect of feeding whole cereals on performance, starch digestibility and duodenal particle size distribution in broiler chickens. *British Poultry Science,* **43**: 416-423.

IJI, P. A. & TIVEY, D. R. (1998). Natural and synthetic oligosaccharides in broiler chicken diets. *World's Poultry Science Journal,* **54**: 129-143.

JONES, G. P. D. & TAYLOR, R. D. (2001). The incorporation of whole grain into pelleted broiler chicken diets: production and physiological responses. *British Poultry Science,* **42**: 477-483.

JUKES, H. G., HILL, D. C. & BRANION, H. D. (1956). Effect of feeding antibiotics on the intestinal tract of the chick. *Poultry Science,* **35**: 716-722.

JUSKIEWICZ, J., JANKOWSKI, J., ZDUNCZYK, Z. & MIKULSKI, D. (2006). Performance and gastrointestinal tract metabolism of turkeys fed diets with different contents of fructooligosaccharides. *Poultry Science,* **85**: 886-891.

KLASING, K. C., LAURIN, D. E., PENG, R. K. & FRY, D. M. (1987). Immunologically mediated growth depression in chicks: influence of feed intake, corticosterone and interleukin-1. *Journal of Nutrition,* **117**: 1629-1637.

LANGHOUT, D. J., SCHUTTE, J. B., VAN LEEUWEN, P., WIEBENGA, J. & TAMMINGA, S. (1999). Effect of dietary high-and low-methylated citrus pectin on the activity of the ileal microflora and morphology of the small intestinal wall of broiler chicks. *British Poultry Science,* **40**: 340-347.

LI, X., LIU, L., LI, K., HAO, K. & XU, C. (2007). Effect of fructooligosaccharides and antibiotics on laying performance of chickens and cholesterol content of egg yolk. *British Poultry Science,* **48**: 185 - 189.

MASSIAS, B., ARTURO-SCHAAN, A.-M., ELIE, K., BEBIN, K., HOCDE, V., DENAYROLLES, M. & URDACI, M. C. (2006). Effects of non-antibiotic additives on the microbial equilibrium of broiler chicken intestine. *Reproduction Nutrition Development,* **46**: S105.

MILES, R. D., BUTCHER, G. D., HENRY, P. R. & LITTELL, R. C. (2006). Effect of antibiotic growth promoters on broiler performance, intestinal growth parameters, and quantitative morphology. *Poultry Science,* **85**: 476-485.

MONSAN, P. F. & PAUL, F. (1995). Oligosaccharide feed additives., in: WALLACE, J. and CHESSON, A. (Eds) *Biotechnology in Animal feeds and Animal Feeding,* pp. 233-245 (Weinheim, VCH).

ORBAN, J., PATTERSON, J., SUTTON, A. & RICHARDS, G. (1997). Effect of sucrose thermal oligosaccharide caramel, dietary vitamin-mineral level, and brooding temperature on growth and intestinal bacterial populations of broiler chickens. *Poultry Science,* **76**: 482-490.

PATTERSON, J. & BURKHOLDER, K. (2003). Application of prebiotics and probiotics in poultry production. *Poultry Science,* **82**: 627-631.

PERRIN, P., PIERRE, F., PATRY, Y., CHAMP, M., BERREUR, M., PRADAL, G., BORNET, F., MEFLAH, K. & MENANTEAU, J. (2001). Only fibres promoting a stable butyrate producing colonic ecosystem decrease the rate of aberrant crypt foci in rats. *Gut,* **48**: 53-61.

PLAVNIK, I., MACOVSKY, B. & SKLAN, D. (2002). Effect of feeding whole wheat on performance of broiler chickens. *Animal Feed Science and Technology,* **96**: 229-236.

PRESTON, C. M., MCCRACKEN, K. J. & MCALLISTER, A. (2000). Effect of diet form and enzyme supplementation on growth, efficiency and energy utilisation of wheat-based diets for broilers. *British Poultry Science,* **41**: 324-331.

RESPONDEK, F. & RUDEAUX, F. (2005). Effets des fructooligosaccharides a courtes chaines sur les performances zootechniques de la poule pondeuse. *6e Journées de la Recherche Avicole*, St Malo, France, pp. 201-204.

SARICA, S., ERDOGAN, S., KOC, A. & ERDOGAN, Z. (2005). Addition of avilamycin, mannaoligosaccharide and organic acids mixture to corn-soyabean meal based broiler diets. *Indian Journal of Animal Sciences,* **75**: 961-964.

SNEL, J., HARNSEN, H. J. M., VAN DER WIELEN, P. W. J. J. & WILLIAMS, B. A. (2002). Dietary strategies to influence the gastrointestinal microflora of young animals, and its potential to improve intestinal health, in: BLOK, M. C., VAHL, H. A., DE LANGE, L., VAN DE BRAAK, A. E., HEMKE, G. and HESSING, M. (Eds) *Nutrition and Health of the Gastrointestinal Tract,* pp. 37-69 (Wageningen, Wageningen Academic Publishers**)**.

SVIHUS, B. & HETLAND, H. (2001). Ileal starch digestibility in growing broiler chickens fed on a wheat-based diet is improved by mash feeding, dilution with cellulose or whole wheat inclusion. *British Poultry Science,* **42**: 633-637.

SVIHUS, B., HETLAND, H., CHOCT, M. & SUNDBY, F. (2002). Passage rate through the anterior digestive tract of broiler chickens fed on diets with ground and whole wheat. *British Poultry Science,* **43**: 662-668.

SVIHUS, B., JUVIK, E., HETLAND, H. & KROGDAHL, A. (2004). Causes for improvement in nutritive value of broiler chicken diets with whole wheat instead of ground wheat. *British Poultry Science,* **45**: 55-60.

THOMKE, S. & ELWINGER, K. (1998). Growth promotants in feeding pigs and poultry. I. Growth and feed efficiency responses to antibiotic growth promotants *Annales de Zootechnie,* **47**: 85-97.

WALDROUP, A. L., SKINNER, J. T., HIERHOLZER, R. E. & WALDROUP, P. W. (1993). An evaluation of fructooligosaccharide in diets for broiler-chickens and effects on salmonellae contamination of carcasses. *Poultry Science,* **72**: 643-650.

WEISER, M. M. (1973). Intestinal epithelial cell surface membrane glycoprotein synthesis. *The Journal of Biological Chemistry,* **248**: 2536-2541.

WOLF, H. (1973). Avilamycin, an inhibitor of the 30 S ribosomal subunits function. *FEBS Letters,* **36**: 181-186.

WU, Y. B., RAVINDRAN, V., THOMAS, D. G., BIRTLES, M. J. & HENDRIKS, W. H. (2004). Influence of method of whole wheat inclusion and xylanase supplementation on the performance, apparent metabolisable energy, digestive tract measurements and gut morphology of broilers. *British Poultry Science,* **45**: 385-394.

XU, Z. R., HU, C. H., XIA, M. S., ZHAN, X. A. & WANG, M. Q. (2003). Effects of dietary fructooligosaccharides on digestive enzyme activities, intestinal microflora and morphology of male broilers. *Poultry Science,* **82**: 1030-1036.

6. List of tables

Table 1. Composition of basal diets (g/kg)

Period (days)	Starter 1-11	Grower 12-25	Finisher 26-36	Withdrawal 37-44
Ingredients				
Wheat	400.0	400.0	400.0	400.0
Soyabean meal	368.7	281.0	276.5	276.5
Maize	133.8	217.0	227.9	228.1
Rapeseed oil	59.0	50.0	49.0	49.0
Maize gluten meal		17.4	14.5	14.5
Dicalcium phosphate	16.4	14.4	13.8	13.8
Calcium carbonate	12.9	9.7	10.2	10.2
Vitamin/mineral premix[1]	4.0	4.0	4.0	4.0
Sodium chloride	3.0	3.0	3.0	3.0
Lysine	0.50	1.70		
Methionine	1.50	1.60	0.95	0.95
Anticoccidian (Clinacox ™)	0.2	0.2	0.2	
Calculated nutrient analysis				
ME [2] (MJ/kg)	12.6	12.8	12.8	12.8
Crude protein	220.0	200.0	195.0	195.0
Lysine	12.0	11.0	9.5	9.5
Methionine + cystine	8.5	8.2	7.5	7.5
Calcium	11.0	9.0	9.0	9.0
Available phosphorus	4.2	3.8	3.7	3.7

[1]The composition of the vitamin/mineral premix was (per kg diet): Co 0.6 mg, Cu 20 mg, I 2 mg, Se 0.2 mg, Zn 90 mg, Fe 50 mg, Mn 80 mg, retinyl acetate 5.2 mg, cholecalciferol 125 µg, D,L-□-tocopheryl acetate 100 mg, thiamine mononitrate 5 mg, menadione 5 mg, riboflavin 8 mg, pyridoxine 7 mg, cyanocobalamine 0.02 mg, calcium pantothenate 25 mg, folic acid 3 mg, biotin 0.3 mg, choline chloride 550 mg, niacin 100 mg, butylated hydroxy toluene 125 mg.

[2] ME = metabolisable energy

Table 2. Performance of broiler chickens fed the experimental diets from 1 to 42 days

	Treatment					
	C[1]	AV[2]	FOS[3]	WW[4]	S.E.M.[5]	P
Daily feed intake (g/animal/day)[6]						
Day 1-11	24.7^{ab}	25.6^{a}	22.5^{c}	23.7^{b}	0.36	<0.001
Day 12-25	79.7^{ab}	84.3^{a}	70.3^{c}	75.3^{b}	1.67	<0.001
Day 26-36	133.9^{b}	143.6^{a}	126.2^{b}	127.7^{b}	3.13	0.003
Day 37-42	181.8^{ab}	191.7^{a}	173.7^{b}	174.9^{b}	3.69	0.009
Day 1-42	91.1^{ab}	96.1^{a}	84.3^{c}	86.6^{bc}	1.82	0.001
Daily live weight gain (g/animal/day)[7]						
Day 1-11	19.9^{b}	21.2^{a}	17.8^{c}	20.1^{b}	0.22	<0.001
Day 12-25	53.6^{b}	58.7^{a}	49.3^{c}	50.1^{c}	0.65	<0.001
Day 26-36	78.4^{b}	84.3^{a}	76.0^{b}	72.5^{c}	0.95	<0.001
Day 37-42	100.1 ^{b}	106.9^{a}	97.3^{b}	97.8^{b}	1.41	<0.001
Day 1-42	58.8^{b}	63.5^{a}	55.7^{c}	55.8^{c}	0.59	<0.001
Feed conversion ratio[6]						
Day 1-11	1.25^{bc}	1.22^{ab}	1.28^{c}	1.19^{a}	0.013	<0.001
Day 12-25	1.50^{ab}	1.46^{a}	1.45^{a}	1.53^{b}	0.013	0.002
Day 26-36	1.77^{c}	1.73^{b}	1.69^{a}	1.80^{c}	0.013	<0.001
Day 37-42	1.85	1.83	1.81	1.79	0.018	NS

$^{a, b, c}$ = Means in the same row with no common superscript differ significantly ($P < 0.05$).

[1] C = negative control treatment.

[2] AV = positive control treatment containing 0.01 g/kg avilamycin.

[3] FOS = treatment containing 0.6 g/kg fructo-oligosaccharides.

[4] WW = treatment in which wheat is given as coarsely ground or whole grains.

[5] S.E.M. = standard error of the mean.

[6] Data represent the mean value of 6 replication pens.

[7] Data represent the mean value of 6 replication pens with 36 birds in each from the beginning of the experiment until the first slaughtering of birds (3 weeks old), and with 30 birds in each pen after first slaughtering of birds.

Table 3. Digestive flora (log $_{10}$ CFU/g intestinal content) of broiler chickens (3 weeks old) fed the experimental diets [1]

	Treatment					
	C[2]	AV[3]	FOS[4]	WW[5]	S.E.M.[6]	P
Ileum						
Aerobic mesophilic	7.52	6.62	8.06	7.60	0.358	0.065
Lactic acid bacteria	7.58	6.78	8.04	7.63	0.419	NS
Coliform	3.20	3.67	3.74	3.87	0.311	NS
Caeca						
Aerobic mesophilic	10.25[a]	8.78[b]	10.23[a]	10.09[a]	0.261	0.002
Lactic acid bacteria	10.67	10.53	10.72	10.15	0.169	NS
Coliform	6.70	6.85	6.92	6.90	0.154	NS
Cloaca						
Aerobic mesophilic	8.24[ab]	7.54[b]	9.15[a]	8.31[ab]	0.349	0.032
Lactic acid bacteria	8.34	7.83	9.18	8.36	0.401	NS
Coliform	5.35	5.38	5.40	5.06	0.237	NS

[a, b] = Means in the same row with no common superscript differ significantly ($P < 0.05$).
[1] Data represent the mean value of 6 replication pens with pools of 6 birds in each.
[2] C = negative control treatment.
[3] AV = positive control treatment containing 0.01 g/kg avilamycin.
[4] FOS = treatment containing 0.6 g/kg fructo-oligosaccharides.
[5] WW = treatment in which wheat is given as coarsely ground or whole grains.
[6] S.E.M.= standard error of the mean.

Table 4. Histological measurements of the intestinal wall of broiler chickens (3 weeks old) fed the experimental diets [1]

		Treatment					
		C[2]	AV[3]	FOS[4]	WW[5]	S.E.M.[6]	P
Duodenum							
Villus	Height (µm)	1548	1516	1441	1507	37.5	NS
	Width (µm)	681	670	663	643	24.5	NS
	Surface (µm^2)	1 055 137	1 035 221	955 297	976 309	48 393	NS
Crypt	Depth (µm)	118	114	121	120	2.6	NS
	Width (µm)	61	61	61	63	1.2	NS
	Surface (µm^2)	7 234	6 939	7 378	7 487	262.5	NS
Villus/crypt	Height	13.24	13.23	12.05	12.80	0.437	NS
	Surface	148	147	133	135	8.2	NS
Ileum							
Villus	Height (µm)	420	412	445	442	15.6	NS
	Width (µm)	504	505	503	471	17.6	NS
	Surface (µm^2)	212 527	208 967	226 681	209 866	12 582	NS
Crypt	Depth (µm)	102	102	105	114	3.8	0.094
	Width (µm)	70	71	72	75	1.6	NS
	Surface (µm^2)	7 258^{b}	7 207^{ab}	7 716^{ab}	8 684^{a}	403.6	0.042
Villus/crypt	Height	4.15	4.11	4.31	3.91	0.159	NS
	Surface	29.8	29.8	30.2	24.9	1.63	0.073

[a, b] = Means in the same row with no common superscript differ significantly ($P < 0.05$).
[1] Data represent the mean value of 18 birds (6 pens of replication x 3 birds/pen).
[2] C = negative control treatment.
[3] AV = positive control treatment containing 0.01 g/kg avilamycin.
[4] FOS = treatment containing 0.6 g/kg fructo-oligosaccharides.
[5] WW = treatment in which wheat is given as coarsely ground or whole grains.
[6] S.E.M. = standard error of the mean.

Table 5. Enzyme activity (U/g tissue) in the intestine of broiler chickens (3 weeks old) fed the experimental diets [1]

	Treatment					
	C[2]	AV[3]	FOS[4]	WW[5]	S.E.M.[6]	P
Duodenum						
AP[7]	4.29	4.85	4.14	5.19	0.350	NS
LAP[8]	2.68[b]	3.36[a]	3.14[a]	3.41[a]	0.156	0.006
Maltase	3.55	3.84	3.73	3.44	0.211	NS
Jejunum						
AP	2.69	3.13	2.46	3.18	0.256	NS
LAP	2.76	3.25	2.85	2.77	0.153	0.087
Maltase	4.10	4.94	3.79	3.99	0.307	0.054
Ileum						
AP	0.60	0.58	0.65	0.58	0.036	NS
LAP	2.52	2.63	2.62	2.45	0.131	NS
Maltase	1.80	1.62	2.24	1.98	0.177	0.092

[a, b] = Means in the same row with no common superscript differ significantly ($P < 0.05$).
[1] Data represent the mean value of 18 birds (6 replicate pens x 3 birds/pen).
[2] C = negative control treatment.
[3] AV = positive control treatment containing 0.01 g/kg avilamycin.
[4] FOS = treatment containing 0.6 g/kg fructo-oligosaccharides.
[5] WW = treatment in which wheat is given as coarsely ground or whole grains.
[6] S.E.M. = standard error of the mean
[7] AP = alkaline phosphatase
[8] LAP = leucine aminopeptidase

7. List of figures

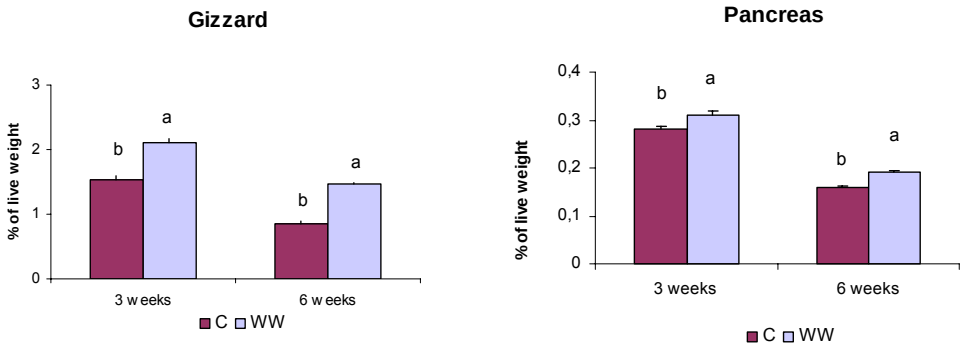

Figure 1. Empty weight of gizzard and pancreas for broiler chickens (3 and 6 weeks old) fed the control (C) or the whole wheat (WW) treatments. Means ± SE with different letters for an age or an organ are significantly different (n=36 birds, P<0.05).

E2. The effects of fructo-oligosaccharides or whole wheat on the digestive bacterial community of broiler chickens using fingerprint methods

Abstract

The objective of this study was to evaluate the effect of two alternatives to antibiotic growth promoters on digestive microflora of broiler chickens. The two potential alternatives were fructo-oligosaccharides (FOS) and diets containing whole grains. For this four experimental diets were used : 1) a negative control (wheat based) with no additives (C), 2) a positive control containing 0.01 g/kg avilamycin (AV), 3) a treatment containing 0.6 g/kg of short chain fructo-oligosaccharides (FOS) and 4) a treatment with the same composition as treatment C but in which a part or all (400 g/kg) of the wheat was given as whole wheat and a concentrate complement (WW). The birds were reared in pens, with 6 replicates per dietary treatments. At 3 weeks of age, 6 chickens per pen were sampled to obtain ileal, cloacal and caecal contents, which were pooled per pen. Digestive microflora was studied by fingerprint techniques with universal primers: Single-Strand Conformation Polymorphism (SSCP), Capillary Electrophoresis Single-Strand Conformation Polymorphism (CE-SSCP) and Temporal Temperature Gradient gel Electrophoresis (TTGE).
Analyses of ileal samples by SSCP showed no effect of dietary treatments. Analyses by CE-SSCP and TTGE showed no cluster in relation with a specific treatment whatever the digestive content (ileum, cloaca and ceca contents). However some differences between diets were observed in ileum and cloaca with TTGE, and in the three segments with CE-SSCP. Significant higher similarity coefficient between digestive contents of birds fed antibiotic or the two studied alternative was observed compared to control diet. With Avilamycin, CE-SSCP showed disappearance of several bands in the three digestive contents compared to control diet, and in the caeca, a band absent in the control diet, was observed. In the cloacal content, with TTGE, a band corresponding to a long segmented filamentous micro organism, not present with control diet was detected. The use of the prebiotic FOS led to the appearance of specific bands compared to control diet, as well as with CE-SSCP and TTGE. In ileum, compared to control diet, a band corresponding to segmented filamentous bacterium was detected. The use of WW in diet led to modifications of digestive microflora, as well as appearance or disappearance of bands compared to control diet. In the ileal content, with TTGE a band corresponding to a segmented filamentous bacterium and a *Lactobacillus salivarius* which was no present with control diet, was observed. In addition to these bands detected with WW and not with control diet, a band corresponding to *Escherichia coli* disappeared in the cloaca of WW fed birds.
Thus the fingerprint techniques CE-SSCP and TTGE were able to detect difference in digestive microflora due to dietary treatments.

1. Introduction

Since the 1950s subtherapeutical levels of antibiotic growth promoters (AGP) have been used in animal feed to improve the performance of animals by controlling the digestive microflora and thereby lower production costs (Thomke and Elwinger, 1998). However, the growing concern from consumers regarding the antibiotic usage and the potential development of bacterial resistance, led to a ban of AGP from January 2006 in the European Union, which has resulted in a search for alternatives. Among the alternatives that have been proposed, there are prebiotic and probiotic (Patterson and Burkholder, 2003). Two potential alternatives in poultry production are fructo-oligosaccharides (FOS) and diets containing whole grains.
FOS are oligosaccharides, which are not hydrolysed by digestive enzymes, which may act as growth substrate for the intestinal flora (Monsan and Paul, 1995; Hartemink *et al.*, 1997). They are considered as prebiotics. They have been shown to have beneficial effects on the gut flora by stimulating the growth of beneficial bacteria such as bifidobacteria and

lactobacilli, and by inhibiting potential pathogenic bacteria, i.e. *Salmonella* and *E. coli* (Bailey *et al.*, 1991; Waldroup *et al.*, 1993; Xu *et al.*, 2003). The beneficial effects on the flora found with FOS could contribute to the observed improvements in the performance in poultry (Monsan and Paul, 1995; Orban *et al.*, 1997; Patterson and Burkholder, 2003; Xu *et al.*, 2003).

Another type of feeding, which potentially modifies the intestinal flora, is the inclusion of whole grains in the diet. A lower number of *E. coli* (Gabriel *et al.*, 2003b), a reduction in lactose negative enterobacteria and an increase in the number of certain lactobacilli have been reported (Engberg *et al.*, 2004). These modifications of the flora could be due to a reduction in the pH (0.5-1 unit) in the gizzard, caused by an increased secretion of hydrochloric acid in the proventriculus (Gabriel *et al.*, 2003a; Engberg *et al.*, 2004). These modifications may explain the improvement in the performance of broilers observed in several studies (Preston *et al.*, 2000; Hetland *et al.*, 2002; Plavnik *et al.*, 2002; Gabriel *et al.*, 2003a). Furthermore, the inclusion of whole grains is an attractive alternative. It meets the consumer requirements for a more “natural” production system and it reduces the feed costs due to less transport and processing and thereby lower production costs (Hetland *et al.*, 2002; Svihus *et al.*, 2004).

Most of the studies performed on effect of alternatives to antibiotics on microflora have been performed by using conventional culturing methods. However, a great part of bacteria (up to 90% according to some estimations) are unable to grow under these conditions (Lan *et al.,* 2002). Therefore, standard microbiological methods only very partially reflect the digestive ecosystem. In order to solve this problem, molecular techniques have been developed. They enable microorganisms to be revealed using their 16 S ribosomal DNA, whatever their viability conditions. These techniques give a more precise and complete image of the microbial diversity than cultures. Thus fingerprinting methods as single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) or temporal temperature gradient gel electrophoresis (TTGE), restriction fragment length polymorphism (RFLP), terminal restriction fragment length polymorphism (T-RFLP) can be used to show difference due to various factors. Each of these methods has advantages and disadvantage in terms of resolution, throughput, possibility for identifying bands. Methods as DGGE or TTGE have been widely used to study the effect of dietary treatment, because of their easily application (Knarreborg et al, 2002; Humblot et al, 2005; Massias et al, 2006; Pedroso et al, 2006; Zhou et al, 2007). SSCP although not common as been used to study modification of digestive microflora (Ott et al, 2004). Capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) is a promising tool for profiling complex bacterial community as digestive community (Pissavin et al, 2006). Indeed a major advantage of CE-SSCP over gel-based techniques is the high-throughput nature that allows parallel processing of multiple samples. However identification of band is laborious, as it consist to analyse a clone library generated from the same sample in parallel and sequence clones that correspond to bands in the community.

The objective of this work was to study the effects of an antibiotic growth promoter (AGP) and two potential alternatives to AGP, FOS and whole wheat, on the gut flora of broiler chickens, by the use of three fingerprint methods, two methods performed on gel, SSCP and TTGE, allowing band identification, and a high-throughput method CE-SSCP.

2. Materials and methods

2.1. Experimental diets

The birds were allocated to four dietary treatments as described previously (Williams et al, 2008): 1) a negative control with no additives (C) mainly composed of wheat (400 g/kg), soyabean meal and maize; 2) a positive control containing 0.01 g/kg avilamycin (AV); 3) a treatment containing 0.6 g/kg of short chain fructo-oligosaccharides (FOS) ; 4) a treatment with the same composition as treatment C but in which a part or all of the wheat was given

as whole wheat and a concentrate complement (WW). From day 8, 200 g/kg coarsely ground wheat was used, from day 10, 200 g/kg whole wheat was incorporated, from day 12 onwards 400 g/kg whole wheat was used.
The feeding program consisted of two different diets for each treatment: a starter diet (from 1 to 11 days of age) and a grower diet (from 12 to 25 days of age). The diets were steam pelleted. The feed and the water were supplied *ad libitum*.

2.2. Animal housing and sample collection

The experiment was carried out in accordance to the specific guidelines for experiments on animals (Decree, 2001).
A total of 864 Ross PM3 male broiler chickens vaccinated against infectious bronchitis were obtained from a commercial hatchery (Sicamen, Volnay, France). The chickens were raised in 3 m^2 floor pens with a stocking density of 12 birds/m^2 with 6 replicates per treatment. From day 1 to 5 the lighting programme consisted of 23 hours light per day, from day 6 to 11, it consisted of 20 hours light per day and from day 12 to the end of the experiment 18 hours light per day. The temperature was gradually decreased from 32°C (day 1) to 28°C (day 21).
During 3 days, in the fourth week of the birds (day 22 to 24), 6 chickens representative of their pens were selected (according to their weight) from each pen (2 replicate pens per treatment per day). The cloacal content was obtained by abdominal pressure on the birds before they were slaughtered. They were killed by intravenous injection of sodium pentobarbital. The digestive tract was removed from the ileum (from Meckel's diverticulum to ileo-caecal junction) to the end of the intestine. The contents from the ileum and caeca were collected by gentle pressure. These digestive content samples were pooled from the 6 animals per pen. These pooled samples were sub sampled for the 3 further microbial analyses. During sampling, pooling and splitting, samples were kept at 4ºC (crushed ice). For each of the 3 microbial analyses, 1 g of sample was mixed in 3 ml ethanol 96%. Samples for SSCP and CE-SSCP were sent with dry ice (-80°C), and all the samples were stored at -20°C until further DNA extraction.

2.3. Fingerprint methods

DNA extraction: The ethanol was removed from samples (pools of digestive content of 6 birds) after centrifugation (9 000 g) and the pellet was rinsed three times with physiological water. DNA was extracted from 200 mg samples using the QIAamp DNA Stool Mini Kit (Qiagen) as described by the manufacturer. An additional treatment with lysozyme was performed in order to improve the extraction of Gram positive bacteria DNA. After the step of incubation of samples with ASL buffer during 5 minutes at 95°C, and before the use of InhibitEX tablets, 140µl of a 10 mg/mL of lysozyme (Sigma L-7651) in Tris-EDTA pH 8 (Tris 10 mM, EDTA 1 mM) was added to each extraction tube. Samples with lysozyme were incubated at 37°C during 30 min. At the end of the procedure, the purified DNA was stabilised with the addition of 4µL of 40 mg/ml BSA (Bovine Serum Albumin, Sigma B-4287) plus 2 µl of Ribonuclease–A (Sigma R-4642) and maintained at -20°C until used. The concentration and integrity of nucleic acids were determined by electrophoresis on 1% agarose gel containing ethidium bromide. The DNA extracted from each of the 6 pools of digestive content of each dietary treatment was used to compared the 6 replicates per dietary treatment by SSCP, CE-SSCP and TTGE. Moreover the 6 DNA extracts per dietary treatment for each digestive content were pooled to compare the average profil of each dietary treatment by TTGE and to identify bacterial species specific of the dietary treatments.

SSCP

PCR reaction: The V4-V5 regions of the 16S rRNA gene was amplified with primers Com1 (CAG CAG CCG CGG TAA TAC) and Com2 (CCG TCA ATT CCT TTG AGT TT) (Schwieger and Tebbe, 1998).

SSCP: PCR products were denatured and approx. 0.5 µg DNA analysed on 7% non-denaturing PAGE at 100V for 17 h. Gels were silver stained by first fixing them in cold 10% ethanol containing 0.5% acetic acid for 5 min at room temperature. They were then silverstained for 30 minutes at room temperature with 0.15% silver nitrate containing 0.056% formaldehyde for 7 minutes and rinsed with deionized H_2O for 10 second. Color development was performed for 2-5 minutes with 1.5 % (15g/l) sodium hydroxide containing 0.07 % formaldehyde. The color reaction was then stopped by the addition of 10% ethanol containing 0.5 % acetic acid for 2 min at room temperature.

CE-SSCP analysis

PCR reaction: For total microflora analysis, PCR was performed according to Delbes *et al*., (2001) by amplification of the V3 region with the primers W49 (ACG GTC CAG ACT CCT ACG GG) and W104 (TTA CCG CGG CTG CTG GCA C). Primers W49 and W104 were labelled on the 5' end with hexachloro derivative of fluorescein (Hex) and 5'-fluorescein-CE phosphoramidite (6-Fam) respectively. The amplification of the V3 region was performed by using the *pfu* Turbo enzyme (Stratagene, La Jolla, CA). After a step of DNA denaturing 10 minutes at 94°C, 25 cycles composed of 30 sec. at 94°C, 30 sec. at 61°C (W49-W104) and 30 sec. at 72°C were run. After PCR, amplified DNA was loaded onto a 2% agarose gel and stained with 0.5 mg/ml ethidium bromide. Images were captured with a Biocapt camera (Bioblock Scientific).
CE-SSCP electrophoresis: DNA was mixed with formamide and Genescan 400 HD-Rox standard (Applied Biosystems, France) according to the ratios 1:18.5:0.5. After a denaturing step at 95°C during 10 minutes, the mix was quickly cooled on ice. The 96-well plate containing the samples was placed into a ABI Prism Genetic Analyzer 3100-*Avent* (Applied Biosystems, France). The non-denaturing polymer matrix used was 5.6% CAP polymer (Applied Biosystems, France) - 10% Glycerol - 1x TBE. The electrophoresis was performed in 1x TBE buffer - 10% Glycerol. The samples were run at 15 kV at 32°C. The data were collected with a Gene Mapper V4.0 software. A normalisation was performed by using the internal standard 400 HD-Rox.

TTGE

PCR reaction: Primers Bact 968-GC-f (5' GCclamp- AAC GCG AAG AAC CTT AC) and Bact 1401-r (5' CGG TGT GTA CAA GAC CC) were used to amplify the V6-V8 region of bacterial 16S rRNA genes (Nubel et al., 1996). PCR was performed with the reaction mixture described by Gerard et al (2004) with a Thermal Cycler with the following program: 95° C for 15 min, 30 cycles of 97°C for 1 min, 58°C for 1 min, 72°C for 1 min 30 s and finally 72°C for 15 min. PCR products were analyzed by electrophoresis on 1% agarose gels containing ethidium bromide to check their size (473 bp) and estimate their concentration.
TTGE: PCR products were separated by Temporal Temperature Gradient gel Electrophoresis (TTGE) using the Dcode Universal Mutation Detection System (Biorad, Paris, France). Electrophoresis was run for 17 hours at a fixed voltage corresponding to 63 mA, an initial temperature of 66°C, and a ramp rate of 0.2°C/h. For better resolution, the voltage was fixed at 20 V for 15 min at the beginning of the electrophoresis. On each gel, the 5 replicates of control diet were compared to the 5 replicates of one of the other dietary treatments (Avilamycin, FOS or whole wheat). A TTGE ladder consisting of a PCR amplicon mix of 8 cloned rDNAs from different bacterial species was used to normalize the profiles (Suau et al., 1999; Johansen et al, 2006). Gels were stained in the dark by immersion for 30 min in a solution of SYBR green I (nucleic acid gel stain, Roche Diagnostics, GmbH, Mannheim, Germany) and the fluorescence was read with a UV camera (Gel DOC XR, Biorad).
Identification of bacteria by sequencing TTGE fragment: DNA fragments of interest were excised aseptically from the polyacrylamide gel and placed in 200µl of water. The samples were heated 10 mn 50°C to allow elution of the DNA. After centrifugation (8 000 rpm, 4 min),

the supernatant was purified using the QIAquick PCR Purification Kit (Qiagen Ref 28104). Prior to sequencing, the samples were amplified by PCR with Primers Bact 968-GC-f and Bact 1401-r, and were checked by TTGE with the original sample from which the band was excised. Only products that migrated as single band and to the same position with respect to the original sample were used for sequencing. When products resulting from excision were not composed of single band, the band of interest was excised and processed as previously described. For sequencing, PCR amplification was performed using primers without the GC clamp. Sequencing was carried out by Genome Express (Grenoble, France). The sequences retrieved were compared with the GenBank database using BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). Positive identifications of unknown sequences were considered significantly similar when sequences were more than 98% identical to BLAST database sequences. The RDP II database was used to obtain classification with the classification algorithm of Wang et al (2007) for unidentified bacteria with the GenBank database (http://rdp.cme.msu.edu/classifier/classifier.jsp).

2.4. Statistical analysis:

The profiles obtained by each of the fingerprint methods (SSCP, CE-SSCP and TTGE) were compared using Bionumerics or GelCompar II software (Applied Maths, Belgium). For TTGE, only samples run on the same gel were compared. The profiles were normalized by means of TTGE ladder. The comparisons of profiles obtained by the three fingerprint methods were based on the Pearson similarity coefficient (SCp) which tooks into account the number of bands, their position, and their intensity. Similarity coefficient, calculated for each pair of profiles, yielded a similarity matrix. A dendrogram was constructed from this matrix by using the unweighted pair group method using arithmetic averages (UPGMA).
The data were analysed using Statview® software programme (Abacus Concepts, Berkeley, CA, USA) by one-way analysis of variance (ANOVA), and significant differences between treatments were determined by Student Newman-Keuls test ($P < 0.05$).

3. Results

3.1. SSCP fingerprint

Ileal samples were studied by SSCP. Number of band detected was about 5 to 9 (data not shown). Dendrograms were drawn according to the similarity percentage between the patterns, but no cluster in relation with a specific treatment was observed. In order to know if the dietary treatments (antibiotic, prebiotic, whole wheat) led to characteristic bands compared to control diet, that is led to appearance or disappearance of some bacteria species, we compared profiles obtained for the four dietary treatments. No specific difference between dietary treatments was observed.

3.2. CE-SSCP fingerprint

The fingerprints of the ileum, cloaca and caeca contents are presented in the figures 1 to 6. Number of band detected in ileum, cloaca and caeca was about 10 to 30, 20 to 40 and 25 to 40 respectively. Dendrograms were drawn according to the similarity percentage between the patterns, but no cluster in relation with a specific treatment was observed (data not shown). However, in order to know if the dietary treatments (antibiotic, prebiotic, whole wheat) led to characteristic bands compared to control diet, that is led to appearance or disappearance of some bacteria species, we compared the six profiles obtained for the four dietary treatments for each intestinal segment. Several modifications were observed (Figures 1 - 6; Table 1).
In the ileum pools, with 6-Fam labelling, one band (position 186) was observed in four pools among the six from animals fed negative control (P1, P8, P15, P18) or prebiotic (P3, P6, P13, P20) and three pools from animals fed whole wheat (P4, P10, P17), but only in one pool

and with low intensity for animal fed avilamycin (P2) (Figure 1). Another band (position 233) was observed only in three pools of birds fed whole wheat (P4, P10, P17). With Hex labelling, we note two bands (position 182.5 and 191.5) that were present in some pools of all the diets but not with avilamycin (Figure 2).
In pools of cloaca contents, with 6-Fam labelling, one band (position 230) was detected in three samples from animal fed prebiotic (P6, P9, P23) and from one positive control (P16) but not in samples of negative control and whole wheat (Figure 3). With Hex labelling, one band (position 201.2) was observed in three samples of birds fed negative control diet (P1,P15,P18) or with whole wheat (P10, P14, P17), and also in two samples of birds fed prebiotic diet (P3,P13), but was not observed in samples of birds fed Avilamycin (Figure 4). We noted a band (position 224.2) that was present in some pools of all the diets but not with avilamycin.
With the caecal samples, with 6-Fam labelling, one band (position 195.5) was observed in three pools from animals fed negative control diet (P8,P11,P15), and one pool from animals fed prebiotic (P23) or whole wheat (P4), but this band was not observed in samples of birds fed Avilamycin (Figure 5). A band (position 205.2) was observed in three pools from animals fed prebiotic (P6, P9, P23) and in one pool of birds fed whole wheat (P10) but not in pools of birds fed negative control or avilamycin. One band (position 209.5) was detected in the fingerprint of four samples from animals fed prebiotic (P3, P9, P13, P23) and one sample of birds fed whole wheat (P10), but not in pools of birds fed negative control or avilamycin. One band (Position 221.5) was present for three pools of animals fed negative control (P8, P11, P18) or whole wheat (P4,P14,P24), and for two pools of animals fed prebiotic (P6,P20), but not with avilamycin. With Hex labelling, a band (position 206.5) was observed in three pools from animals fed prebiotic (P6, P9, P23) but not with the other diets (Figure 6). A band (position 210.2) was observed for four pools of birds fed avilamycin (P2, P12, P16, P21), three pools of birds fed whole wheat (P7,P14,P24), and one pool of birds fed prebiotic (P13) but not in samples of birds fed negative control diet.

3.3. TTGE fingerprint

The TTGE profiles of bacterial community of digestive content of pools of 6 chickens are shown in figures 7 to 12. Number of band detected in ileum, cloaca and caeca was about 6 to 8, 10 to 15, and 15 to 20 respectively.
With the pools of 6 birds of ileal, cloacal and caecal samples, when comparing control and avilamycin, control and FOS, or control and whole wheat diet, the samples did not cluster according to treatment (data not shown). However, significant higher similarity coefficient between microflora profiles of digestive contents of birds fed antibiotic or the two studied alternative was observed compared to control diet (Table 2). Thus with antibiotic, higher similarity was observed in caecal content; with FOS, higher similarity was observed in ileum and caeca, and with whole wheat higher similarity was observed in all the digestive contents.
As for SSCP and CE-SSCP, in order to know if the dietary treatments (antibiotic, prebiotic, whole wheat) led to characteristic bands compared to control diet, we compared profiles obtained for each intestinal segment. In the ileal content, no difference was observed between control diet and avilamycin (Figure 7). With the FOS diet, a band (a) which was no present with control diet, was present in 4 of the 6 pools of the FOS diet with moderate (P3) or low intensity (P6, P9, P23) (Figure 8). With the whole wheat diet, a band (b), which was not present with the control diet, was present in 4 of the 6 pools of the whole wheat diet with high (P4), moderate (P14) or low intensity (P10, P17) (Figure 9). Moreover, another band (c) was observed in 4 of the 6 pools of the whole wheat diet with high (P4, P7, P17) or low intensity (P10), wheras it was observed only in one pool of control fed birds and with low intensity (P8). In the cloacal content, with avilamycin, a band (d) not present with control diet was detected in 4 of the 6 pools with high (P2, P16) or low intensity (P5, P19) (Figure 10). Moreover, a band (h) was observed with a moderate intensity in one of the pool of Avilamycin (P2) diet, and with a low intensity in another pool (P19), and only with a very low intensity in 4 of the 6 pools of control diet (P1, P8, P11, P15). With FOS diet, a band (i) was

observed with a high intensity in one of the 6 pools (P3) and with low intensity in 2 pools (P6, P9), whereas it was observed only with a very low intensity in 4 of the 6 pools of control diet (P1, P8, P11, P15) (Figure 11). With whole wheat, a band (e) observed with control diet with high intensity in three pools (P8, P15, P22) and low intensity in the three other pools (P1, P11, P18), showed only a very low intensity (Figure 12). A band migrating at the same level that this band (e) was also observed with avilamycin (band f) with high intensity in two pools (P5, P16) and low intensity in an other pool (P19) (Figure 10), and with prebiotic (band g) with high intensity in two pools (P20, P23), moderate intensity in three pools (P3, P6, P9), and low intensity in one pool (P13) (Figure 11). Moreover, with whole wheat, a band of moderate intensity (j) observed for 5 of the 6 pools (P4, P7, P10, P14, P24), was observed only with very low intensity with control diet (P1, P8, P11, P15) (Figure 12). In the caecal content, no difference between dietary treatments was observed (data not showed).

In order to characterize the specific band of dietary treatment observed with TTGE, for each digestive segment, the samples of all birds were pooled for each dietary treatment (36 birds). As observed previously with pools of six birds, we observed specific bands of dietary treatments (Figure 13). In the ileal content, with the FOS diet, the band (a) which was not present with control diet, was observed. With the whole wheat diet, the band (b) migrating at the same level than the band (a) of FOS diet was also observed. Moreover, another band (c) not detected in control diet, was observed with whole wheat diet. In the cloacal content, with avilamycin, the band (d) not present with control diet was detected. The three bands (e,f,g) migrating at the same level, were detected in control, avilamycin and FOS diets respectively, with no band at the same level in whole wheat diet. In the same manner, the three bands (h, i, j) migrating at the same level, were detected in avilamycin (with a very low intensity), FOS and whole wheat diets respectively, with no band at the same level in control diet.

The characteristic TTGE bands of dietary treatments observed with pools of 36 birds were sequenced and assigned to a species or a genus in the GenBank database using BLAST algorithm (Table 3). Identification of band showed that the bands (a) and (b) observed in the ileum of FOS and whole wheat fed birds, but not with control and avilamycin diet, were similar (100%) and were affiliated to a segmented filamentous bacterium. The band (c) present in the ileum of whole wheat fed birds corresponded to a *Lactobacillus salivarius*. The band (d) present in the cloaca of avilamycin group, corresponded to a long segmented filamentous microorganism. The bands (e, f, g) not detected in the cloaca of whole wheat fed birds were homologous (98 to 100%) and corresponded to *E. coli*. The band (h, i, j) not detected in the cloaca of control diet, but in the cloaca of the three other experimental diets, were homologous (100%) and corresponded to an uncultured firmicutes bacteria according to the GenBank database, and was classified as a bacteria belonging to the order Clostridiales with the RDP II database.

4. Discussion

4.1. Comparison of fingerprint techniques

The two fingerprint techniques performed on gel, SSCP and TTGE, have lower resolution than CE-SSCP, as show by the detection of less bands 6 to 8 for TTGE or 5 à 9 for SSCP in ileum and 15 to 20 for TTGE in caeca, instead of about 10 to 30 in ileum and 25 to 40 in caeca for CE-SSCP. This is in agreement with previous studies (Hong et al, 2007). Although SSCP and TTGE have similar low resolution, whereas SSCP was not able to show difference between dietary treatments, TTGE was able to show difference. In fact SSCP is able to observe difference between management systems (Frederike Hilbert, PoultryFlorGut, 2nd Activity Report) but was not able to show difference between dietary treatments that may be less important.
As CE-SSCP has higher resolution than TTGE, and due to its double labelling, CE-SSCP allowed to detect band with one label or the other, this fingerprint technique could be expected to shown more difference than TTGE, which was the case (Table 1). Thus in the caeca, where the two techniques led to the detection of more bands than in the small intestine due to the higher diversity of digestive microflora in this segment (Gong et al, 2002; Lu et al, 2003), whereas TTGE was not able to show difference between dietary treatments, CE-SSCP shown difference as well as for the antibiotic and for the two potential alternative to AGP compare to control diet. In the same manner, CE-SSCP was able to detect an effect of avilamycin compare to control diet in the ileum, whereas no difference was observed with TTGE. However, TTGE was able to detect differences that CE-SSCP did not show. Thus, in cloaca, whereas CE-SSCP shown the disappearance of two bands with avilamycin, TTGE shown the appearance of two bands with this AGP. In the same manner, in the cloaca, TTGE shown the apparition of one band with FOS, which was not the same that the one detected by CE-SSCP as this latest one was not detected by CE-SSCP with WW, whereas it was observed by TTGE with this diet. Moreover, in some cases, whereas TTGE show difference between diet, CE-SSCP shown no difference. Thus whereas no difference were observed by CE-SSCP with FOS in ileal content, and with whole wheat in cloacal content, TTGE detected differences. Moreover, whereas CE-SSCP shown with WW in ileal content, the appearance of only one bands, TTGE was able to shown the appearance of two bands.
Thus these two fingerprint techniques used in this study allowed detecting differences between dietary treatment, and these differences were not the same. Moreover CE-SSCP is a high throughput method allowing comparing numerous samples, contrary to the limitation of the method on gel as TTGE where only samples on the same gel can be compared. However, TTGE allows to easily identifying bands on the gel. Thus these techniques appear to be complementary. The difference of detection of characteristic of dietary treatment is not only due to the resolution of the technique as shown by the detection of difference between diets by TTGE that were not detected by CE-SSCP. The choice of primers for PCR and mode of separation of DNA fragment are probably implied. Indeed in this study, we do not use the same universal primers for the two techniques. For CE-SSCP, PCR was performed with the primers W49 and W104 to amplify the V3 region according to Delbes *et al*., (2001), and for TTGE primers Bact 968-GC-f and Bact 1401-r were used to amplify the V6-V8 region of bacterial 16S rRNA genes (Nubel et al., 1996). Whereas during CE-SSCP, DNA fragments are denatured before being separated on a capillary, during TTGE, DNA fragments are denatured during their migration on an acrylamide gel. Thus the use of these two complementary techniques allows detecting difference in the digestive microflora due to the AGP avilamycin and two alternatives to AGP, a prebiotic (FOS) and a modification of the structure of the diet, introduction of whole wheat, whereas conventional methods with selective medium of culture have difficulties to show difference (Gabriel et al, 2008; Williams et al, 2008).

4.2. Effect of avilamycin

Avilamycin appeared to have a regulatory effect on digestive microflora as showed by the higher similarity between microflora TTGE profiles of caecal samples of birds fed avilamycin compared to profiles of birds fed the control diet.
As observed by CE-SSCP, the treatment with Avilamycin lead to disappearance of several bands in the three digestive contents compared to control diet. In ileum, a band (position 186, 6-Fam labelling) that was observed in four pools of the control diet was observed only in one pool and with low intensity with avilamycin. Moreover, two bands that were observed in all the diets, were not observed with avilamycin (Position 182.5 and 191.5, Hex labelling). In the cloaca, this diet led to the disappearance of one band (position 201.2, Hex labelling), and one bands that was observed in all the diets, it was not observed with avilamycin (position 224.2, Hex labelling). In the caeca, two band disappeared (position 195.5 and 221.5, 6-Fam labelling). Thus avilamycin led t the disappearance of some bacterial species. This is in agreement with the significantly lower number of bacteria observed in the caeca of birds fed on the treatment AV (Williams et al, 2008). A was. This could be expected as AGP reduce the number of bacteria in the digestive tract (Thomke and Elwinger, 1998; Engberg *et al.*, 2000). Avilamycin in particular acts by interfering with the polypeptides-synthesizing functions and it is mainly active against gram positive bacteria (Wolf, 1973; Butaye *et al.*, 2003), the most numerous bacteria in the digestive tract (Gabriel *et al.* 2006). We note that the position of the bands were not the same according to the gut compartment. In consequence, it did not seem to be the same bacterial populations.
However, appearance of some bands was also observed with this AGP compared to control diet, as well as with SSCP and TTGE. Thus, with CE-SSCP, in the caeca, a band absent in the control diet, was observed with avilamycin (position 210.2, Hex labelling). With TTGE, in cloaca, a modification of profile was observed, as previously reported by Pedroso et al (2006) with TTGE. A band corresponding to long segmented filamentous bacterium was detected. These bacteria are also referred to segmented filamentous organisms (Klaasen et al, 1992). They are commonly found attached to the small intestinal wall of many animals (Klaasen et al, 1993), and were also found in the caeca of birds (Glick et al, 1978). They are usually found in young animals as chicken less than four weeks (Goodwin et al, 1991; Allen, 1992). They are gram-positive, anaerobic, spore-forming bacteria that are distantly related to members of the genus Clostridium (Snel et al, 1995). They are non-pathogenic. They are associated with a healthy digestive tract in animals as they play an important role in the development of mucosal immune system of intestine (Meyerholz et al, 2002; Suzuki et al, 2004). These organisms may play a role in disease prevention by inhibiting colonization by pathogens such as *Escherichia coli* and *Salmonella enterica* (Garland et al, 1982; Heczko et al, 2000). The stimulating effect of avilamycin observed in this study is opposed to the negative effect observed by Allen (1992) with virginiamycin on development of long segmented filamentous bacterium. This may be due to the fact that different AGP can have different effect on bacteria, as shown by Pedroso et al (2006).

4.3. Effect of FOS

A regulatory effect of FOS on digestive microflora was detected as showed by the higher similarity between microflora TTGE profiles of ileal and caecal samples of birds fed this prebiotic compared to profiles of birds fed the control diet.
Thus, the use of this prebiotic led to the appearance of specific bands compared to control diet, as well as with SSCP and TTGE. In ileum, compared to control diet, a band corresponding to segmented filamentous bacterium was detected with FOS by TTGE. In the cloaca, one band (position 230, 6-Fam labelling) was detected by CS-SSCP. TTGE also detected a band with this diet. In the caeca content, 3 bands were detected by CE-SSCP (position 205.2 and 209.5 with 6-Fam labelling, position 206.5 with Hex labelling). As previously observed with avilamycin, the position of the bands was not the same according to the gut compartment. The effect of this prebiotic on microflora profiles is in accordance with

previous study performed with DGGE (Massias *et al.* (2006). The detection of new band with FOS is in accordance with the stimulation of some bacteria as lactobacilli and bifidobacteria with this prebiotic (Xu *et al.,* 2003; Griggs and Jacob, 2005).

4.4. Effect of whole wheat

As for the two other dietary treatments studied, a higher similarity between microflora TTGE profiles of samples from birds fed WW was observed compared to profiles of birds fed the control diet, showing a regulatory effect of this mode of feeding on digestive microflora. This regulatory effect was previously observed in the microflora of the caeca in another study (Gabriel et al, 2007). It seems particularly important, as contrarily to the other dietary treatments where one or two digestive segments were concerned, in the case of WW, the three segments were concerned.
The use of WW in diet led to modifications of digestive microflora, as well as appearance or disappearance of bands compared to control diet. Thus in the ileum, one band was observed by CE-SSCP (position 233 with 6-Fam labelling), and two bands with TTGE. With the latest fingerprint technique, identification of these bands led to a segmented filamentous bacterium and a *Lactobacillus salivarius*. A low abundance of *L. salivarius* in ileal content of birds of 3 weeks fed wheat based diet containing ground wheat was observed (Knarreborg et al, 2002). However previous work on effect of whole wheat on *L. salivarius* counts in all the digestive tract has shown no effect (Engberg et al, 2004). This discrepancy with our study may be due to the lower incorporation rate of whole wheat in this study (300 g/kg) instead of 400 g/kg in our study. It may also be due to the age of the birds as in the study of Engberg et al (2004) chickens were in their fifth week, whereas in our study birds were in their fourth week, and it was observed that *L. salivarius* is a dominant lactic acid bacteria in broiler intestinal content of chicken in their fifth week (Engberg et al (2000) and become abundant at day 35 compare to day 21 (Knarreborg et al, 2002), and whole wheat effect may be different according to abundance of this Lactobacillus. Lactobacilli are generally considered to be beneficial for the host due to the acidification of the digestive content limiting the growth of potential pathogenic bacteria, and their production of bacteriocin. However they produced bile salt hydrolases which might impair lipid absorption and lead to reduce weight gain (Tannock et al, 1994). In particular *L. salivarius* was reported to be associated with deconjugation of bile salts in the ileum and reduced broiler productivity (Engberg et al 2000; Guban et al, 2006). In the cloaca, contrary to control diet, a band was detected by TTGE, and in the caeca, a band (position 210.2 with Hex labelling) was observed by CE-SSCP. In addition to these bands detected with WW and not with control diet, a band corresponding to *Escherichia coli* disappeared in the cloaca of WW fed birds. This is in accordance with decrease of *Escherichia coli* counts previously reported with this mode of feeding (Glünder et al, 2002; Gabriel et al, 2003). The decrease of some other bacterial populations has been previously reported with the use of whole grains, such as anaerobic bacteria in the gizzard, lactose - negative enterobacteria in the gizzard and intestine and a trend to decrease of C*lostridium perfringens* in ileum and caeca (Engberg et al., 2004). This decrease of bacterial population could be due to the lower pH of the gizzard content (Gabriel et al., 2003a; Engberg et al., 2004). Moreover the more functional gizzard (Gabriel et al., 2003a, Engberg et al., 2004) and its positive effects on digestion (Svihus and Hetland, 2001) may lead to less substrate for microbial growth. The faster intestinal passage rate with whole wheat (Svihus et al., 2002) may also be implicated.
The detection of segmented filamentous bacterium and *L. salivarius* associated with an healthy digestive tract and disappearance of a band identified as *E. coli* associated with negative effect, show a positive effect of this mode of feeding on digestive microflora.

In conclusion, CE-SSCP and TTGE fingerprint methods were two complementary methods to show some modification of bacterial community according to diet. The AGP avilamycin was show to led to disappearance of several bands in the three digestive segments studied (ileum, cloaca and caeca). However appearance of some bands was also observed with this

AGP compared to control diet. Thus, a band identified as a long segmented filamentous bacterium was detected in the cloaca. With the prebiotic FOS, specific bands appeared in the three segments. In the ileum, one band was identified as a segmented filamentous bacterium, associated with an healthy gut. With whole wheat, new bands also appeared in the three segments. Thus in ileum, a segmented filamentous bacterium as with the FOS was detected. *L. salivarius* was also observed, but may represent a negative effect. However, the disappearance in cloaca of one band identified as *E. coli* was observed, which represent a positive effect for this mode of feeding.

5. References

Allen, P.C., 1992. Comparative study of long, segmented, filamentous organisms in chickens and mice. Lab Anim Sci. 42, 542-547.

Bailey, J.S., Blankenship, L.C., Cox, N.A., 1991. Effect of fructooligosaccharide on Salmonella colonization of the chicken intestine. Poultry Science 70, 2433-2438.

Butaye, P., Devriese, L.A., Haesebrouck, F., 2003. Antimicrobial growth promoters used in animal feed : effects of less well known antibiotics on gram-positive bacteria. Clin. Microbiol. Rev. 16, 175-188.

Delbes, C., Leclerc, M., Zumstein, E., Godon, J.J., Moletta, R., 2001. A molecular method to study population and activity dynamics in anaerobic digestors Water Science and Technology 43, 51-57.

Engberg, R.M., Hedemann, M.S., Leser, T.D., Jensen, B.B., 2000. Effect of zinc bactracin and salinomycin on intestinal microflora and performance of broilers. Poultry Science 79, 1311-1319.

Engberg, R.M., Hedemann, M.S., Steenfeldt, S., Jensen, B.B., 2004. Influence of whole wheat and xylanase on broiler performance and microbial composition and activity in the digestive tract. Poultry Science 83, 925-938.

Gabriel, I., Leconte, M., Guillon, J., Rideaud, P., Moreau-Vauzelle, C., Dupont, C., 2007. Individual variability in the digestive flora of the broiler chicken analysed by molecular fingerprint, 16th European Symposium on Poultry Nutrition, Strasbourg, France, pp. 305-308.

Gabriel, I., Lessire, M., Mallet, S., Guillot, J.F., 2006. Microflora of the digestive tract: critical factors and consequences for poultry. World's Poultry Science Journal 62, 499-511.

Gabriel, I., Mallet, S., Leconte, M., 2003. Differences in the digestive tract characteristics of broiler chickens fed on complete pelleted diet or whole wheat added to pelleted protein concentrate. British Poultry Science 44, 283-290.

Gabriel, I., Mallet, S., Leconte, M., Fort, G., Naciri, M., 2003. Effects of whole wheat feeding on the development of coccidial infection in broiler chickens. Poultry Science 82, 1668-1676.

Gabriel, I., Mallet, S., Leconte, M., Travel, A., Lalles, J.P., 2008. Effects of whole wheat feeding on the development of the digestive tract of broiler chickens. Animal Feed Science and Technology accepté le 25/06/07; doi:10.1016/j.anifeedsci.2007.06.036.

Garland, C.D., Lee, A., Dickson, M.R., 1982. Segmental filamentous bacteria in the rodent small intestine: their colonization of growing animals and possible role in host resistance to Salmonella. Microbial Ecology 8, 181-190.

Glick, B., Holbrook, K.A., Olah, I., Perkins, W.D., Stinson, R., 1978. A scanning electron microscope study of the caecal tonsil. The identification of a bacterial attachment to the villi of the caecal tonsil and the possible presence of lymphatics in the caecal tonsil. Poultry Science 57, 1408-1416.

Glünder, G., 2002. Influence of diet on the occurrence of some bacteria in the intestinal flora of wild and pet birds. Deutsche Tierarztliche Wochenschrift 109, 266-270.

Goodwin, M.A., Cooper, G.L., Brown, J., Bickford, A.A., Waltman, W.D., Dickson, T.G., 1991. Clinical, pathological, and epizootiological features of long-segmented filamentous organisms (bacteria, LSFOs) in the small intestines of chickens, turkeys, and quails. Avian Diseases 35, 872-876.

Griggs, J.P., Jacob, J.P., 2005. Alternatives to antibiotics for organic poultry production. J. Appl. Poult. Res. 14, 750-756.
Guban, J., Korver, D.R., Allison, G.E., Tannock, G.W., 2006. Relationship of dietary antimicrobial drug administration with broiler performance, decreased population levels of Lactobacillus salivarius, and reduced bile salt deconjugation in the ileum of broiler chickens. Poultry Science 85, 2186-2194.
Harrow, S.A., Ravindran, V., Butler, R.C., Marshall, J.W., Tannock, G.W., 2007. Real-Time Quantitative PCR Measurement of Ileal Lactobacillus salivarius Populations of Broiler Chickens to Determine the Influence of Farming Practices. Appl Environ Microbiol in press.
Hartemink, R., 1998. Non-digestible oligosaccharides: healthy food for the colon?, University of the North, Pietersburg, theNetherlands, p. 177.
Heczko, U., Abe A, F.B., 2000. Segmented filamentous bacteria prevent colonization of enteropathogenic Escherichia coli O103 in rabbits. J Infect Dis 181, 1027-1033.
Hetland, H., Svihus, B., Olaisen, V., 2002. Effect of feeding whole cereals on performance, starch digestibility and duodenal particle size distribution in broiler chickens. British Poultry Science 43, 416-423.
Hong, H., Pruden A, R.K., 2007. Comparison of CE-SSCP and DGGE for monitoring a complex microbial community remediating mine drainage. J Microbiol Methods. 2007 Apr;69(1):52-64
Humblot, C., Bruneau, A., Sutren, M., Lhoste, E.F., Dore, J., Andrieux, C., Rabot, S., 2005. Brussels sprouts, inulin and fermented milk alter the faecal microbiota of human microbiota-associated rats as shown by PCR-temporal temperature gradient gel electrophoresis using universal, Lactobacillus and Bifidobacterium 16S rRNA gene primers. BRITISH JOURNAL OF NUTRITION 93, 677-684.
Johansen, C.H., Bjerrum, L., Finster, K., Pedersen, K., 2006. Effects of a Campylobacter jejuni infection on the development of the intestinal microflora of broiler chickens. Poult. Sci. 85, 579-587.
Klaasen, H.L., Koopman JP, P.F., Beynen AC., 1992. Intestinal, segmented, filamentous bacteria. FEMS Microbiol Rev, 8, 165-180.
Klaasen, H.L., Van der Heijden PJ, S.W., Poelma FG, Koopman JP, Van den Brink ME, Bakker MH, Eling WM, Beynen AC., 1993. Apathogenic, intestinal, segmented, filamentous bacteria stimulate the mucosal immune system of mice. Infect Immun. 1993 Jan;61(1):303-6.
Knarreborg, A., Simon, M.A., Engberg, R.M., Jensen, B.B., Tannock, G.W., 2002. Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. Applied & Environmental Microbiology 68, 5918-5924.
Lan, P.T., Hayashi, H., Sakamoto, M., Benno, Y., 2002. Phylogenetic analysis of cecal microbiota in chicken by the use of 16S rDNA clone libraries. Microbiol Immunol 46, 371-382.
Massias, B., Arturo-Schaan, M., Elie, A.M., Bebin, K., Hocde, V., Denayrolles, M., Urdaci, M.C., 2006. Effects of non-antibiotic additives on the microbial equilibrium of broiler chicken intestine. Reprod. Nutr. Develop. 46, S105.
Meyerholz, D.K., Stabel TJ, C.N., 2002. Segmented filamentous bacteria interact with intraepithelial mononuclear cells. Infect Immun. 70, 3277-3280.
Monsan, P.F., Paul, F., 1995. Oligosaccharide feed additives, In: Wallace, R.J.C., A. (Ed.), Biotechnology in animal feeds and animal feeding, VCH Verlagsgesellschaft mbH; , Weinheim; Germany, pp. 233-245
Monsan, P.F., Paul, F., 1995. Oligosaccharide feed additives, In: Wallace, R.J.C., A. (Ed.), Biotechnology in animal feeds and animal feeding, VCH Verlagsgesellschaft mbH; , Weinheim; Germany, pp. 233-245
Nubel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W., Backhaus, H., 1996. Sequence heterogeneities of genes encoding 16S rRNAs in

Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. J. Bacteriol. 178, 5636-5643.
Orban, J.I., Patterson, J.A., Sutton, A.L., Richards, G.N., 1997. Effect of sucrose thermal oligosaccharide caramel, dietary vitamin-mineral level, and brooding temperature on growth and intestinal bacterial populations of broiler chickens. Poultry Science 76, 482-490.
Ott, S.J., Musfeldt, M., Wenderoth, D.F., Hampe, J., Brant, O., Folsch, U.R., Timmis, K.N., Schreiber, S., 2004. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. Gut 53, 685-693.
Patterson, J.A., Burkholder, K.M., 2003. Application of prebiotics and probiotics in poultry production. Poultry Science 82, 627-631.
Pedroso, A.A., Menten, J.F., Lambais, M.R., Racanicci, A.M., Longo, F.A., Sorbara, J.O., 2006. Intestinal bacterial community and growth performance of chickens fed diets containing antibiotics. Poult Sci 85, 747-752.
Pissavin, C., Gabriel, I., Burel, C., Mallet, S., Maurice, R., Lessire, M., Fravalo, P., 2006. Development of a molecular technique to study the equilibrium of poultry gut microbiota. Reprod. Nutr. Develop. 46, S26.
Plavnik, I., Macovsky, B., Sklan, D., 2002. Effect of feeding whole wheat on performance of broiler chickens. Animal Feed Science and Technology 96, 229-236.
Preston, C.M., McCracken, K.J., McAllister, A., 2000. Effect of diet form and enzyme supplementation on growth, efficiency and energy utilisation of wheat-based diets for broilers. British Poultry Science 41, 324-331.
Schwieger, F., Tebbe, C.C., 1998. A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. Appl. Environ. Microbiol. 64, 4870-4876.
Snel, J., Heinen, P.P., Blok, H.J., Carman, R.J., Duncan, A.J., Allen, P.C., Collins, M.D., 1995. Comparison of 16S rRNA sequences of segmented filamentous bacteria isolated from mice, rats, and chickens and proposal of "Candidatus Arthromitus". International Journal of Systematic Bacteriology 45, 780-782.
Suau, A., Bonnet, R., Sutren, M., Godon, J.J., Gibson, G.R., Collins, M.D., Dore, J., 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol 65, 4799-4807.
Suzuki, K., Meek B, D.Y., Muramatsu M, Chiba T, Honjo T, Fagarasan S., 2004. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proc Natl Acad Sci U S A 101, 1981-1986.
Svihus, B., Hetland, H., 2001. Ileal starch digestibility in growing broiler chickens fed on a wheat-based diet is improved by mash feeding, dilution with cellulose or whole wheat inclusion. British Poultry Science 42, 633-637.
Svihus, B., Hetland, H., Choct, M., Sundby, F., 2002. Passage rate through the anterior digestive tract of broiler chickens fed on diets with ground and whole wheat. Br. Poult. Sci 43, 662-668.
Svihus, B., Juvik, E., Hetland, H., Krogdahl, A., 2004. Causes for improvement in nutritive value of broiler chicken diets with whole wheat instead of ground wheat. BRITISH POULTRY SCIENCE 45, 55-60.
Thomke, S., Elwinger, K., 1998. Growth promotants in feeding pigs and poultry. I. Growth and feed efficiency responses to antibiotic growth promotants. Annales de Zootechnie 47, 85-91.
Waldroup, A.L., Skinner, J.T., Hierholzer, R.E., Waldroup, P.W., 1993. An evaluation of fructooligosaccharide in diets for broiler chickens and effects on salmonellae contamination of carcasses. Poultry Science 72, 643-650.
Wang, Q., Garrity, G.M., Tiedje, J.M., R., C.J., 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73, 5261-5267.

Williams, J., Mallet, S., Leconte, M., Lessire, M., Gabriel, I., 2008. The effects of fructo-oligosaccharides or whole wheat on the performance and the digestive tract of broiler chickens. British Poultry Science.

Wolf, H., 1973. Avilamycin, an inhibitor of the 30 S ribosomal subunits function. FEBS Letters 36, 181-186.

Xu, Z.R., Hu, C.H., Xia, M.S., Zhan, X.A., Wang, M.Q., 2003. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. Poultry Science 82, 1030-1036.

Zhou, H., Gong, J., Brisbin, J.T., Yu, H., Sanei, B., Sabour, P., Sharif, S., 2007. Appropriate chicken sample size for identifying the composition of broiler intestinal microbiota affected by dietary antibiotics, using the polymerase chain reaction-denaturing gradient gel electrophoresis technique. Poultry Science 86 2541-2549.

6. List of tables

Table 1. Specific bands of dietary treatments observed with CE-SSCP and TTGE [1]

Digestive segment	Dietary treatment	CE-SSCP [2]	TTGE
Ileum	Avilamycin	- Absence band 186 (6-Fam) (except P 2) - Absence band 182.5 and 191.5 (Hex))	
	Prebiotic		- Presence band a (P 3,6,9,23)
	Whole wheat	- Presence band 233 (6-Fam) (P 4,10,17)	- Presence band b (P 4,10,14,17) - Presence band c (P 4, 7, 10, 17)
Cloaca	Avilamycin	- Absence band 201.2 (Hex) - Absence band 224.2 (Hex)	- Presence band d (P 2, 16)
	Prebiotic	- Presence band 230 (6-Fam) (P 6,9,23)	- Presence band i (P 3,6,9)
	Whole wheat		- Presence band j (P 4,7,10,14, 24) - Very low intensity of band e
Caeca	Avilamycin	- Presence band 210.2 (Hex) (P 2,12,16,21) - Absence band 195.5 and 221.5 (6-Fam)	
	Prebiotic	- Presence band 205.2 (6-Fam) (P 6,9,23) - Presence band 209.5 (6-Fam) (P 3,9,13,23) - Presence band 206.5 (Hex) (P 6,9,23)	
	Whole wheat		

ND : not determined

[1] Specific bands: absent or present bands with the dietary treatments (Avilamycin, prebiotic, whole wheat) compared to negative control diet.

[2] Hex : Hex labelling ; 6-Fam : 6-Fam labelling

Table 2. Similarity coefficient (Pearson correlations, %) obtained with TTGE for each alternative to antibiotic and each digestive segment [1]

Intestinal segment	Dietary treatments		SEM	Proba
	A: Control	B: Avilamycin		
Ileum	46	39	8,35	NS
Cloaca	34	47	7,72	NS
Caeca	92 b	94 a	0,71	0,024
	A: Control	C : FOS		
Ileum	45 b	67 a	6,97	0.030
Cloaca	47	52	6	NS
Caeca	93 b	98 a	0,53	<0,001
	A: Control	D : Whole wheat		
Ileum	43 b	62 a	6,13	0,038
Cloaca	44 b	59 a	4,93	0,041
Caeca	91 b	94 a	0,64	0,003

(a, b) : Means in the same row with no common letter differ significantly ($P < 0.05$). NS : not significant.
[1] Six profiles of microflora of pools of 6 birds were compared per dietary treatment

Table 3. Characterization of bands observed by TTGE affected by dietary treatments

Digestive segment	Dietary treatment	Band	Bacteria identified	Accession number	Similarity (%)
Ileum	Prebiotic	a	Segmented filamentous bacterium	X87244.1	98%
	Whole wheat	b	Segmented filamentous bacterium	X87244.1	98%
		c	Lactobacillus salivarius	EF519868.1	99%
Cloaca	Control	e	Escherichia coli	EU014689.1	99%
	Avilamycin	d	Long segmented filamentous organism	X80834.1	99%
		f	Escherichia coli	EU014689.1	99%
		h	Order Clostridiales	EF071188.1	98%
	Prebiotic	g	Escherichia coli	EU014689.1	99%
		i	Order Clostridiales	EF071188.1	99%
	Whole wheat	j	Order Clostridiales	EF071188.1	98%

7. List of figures

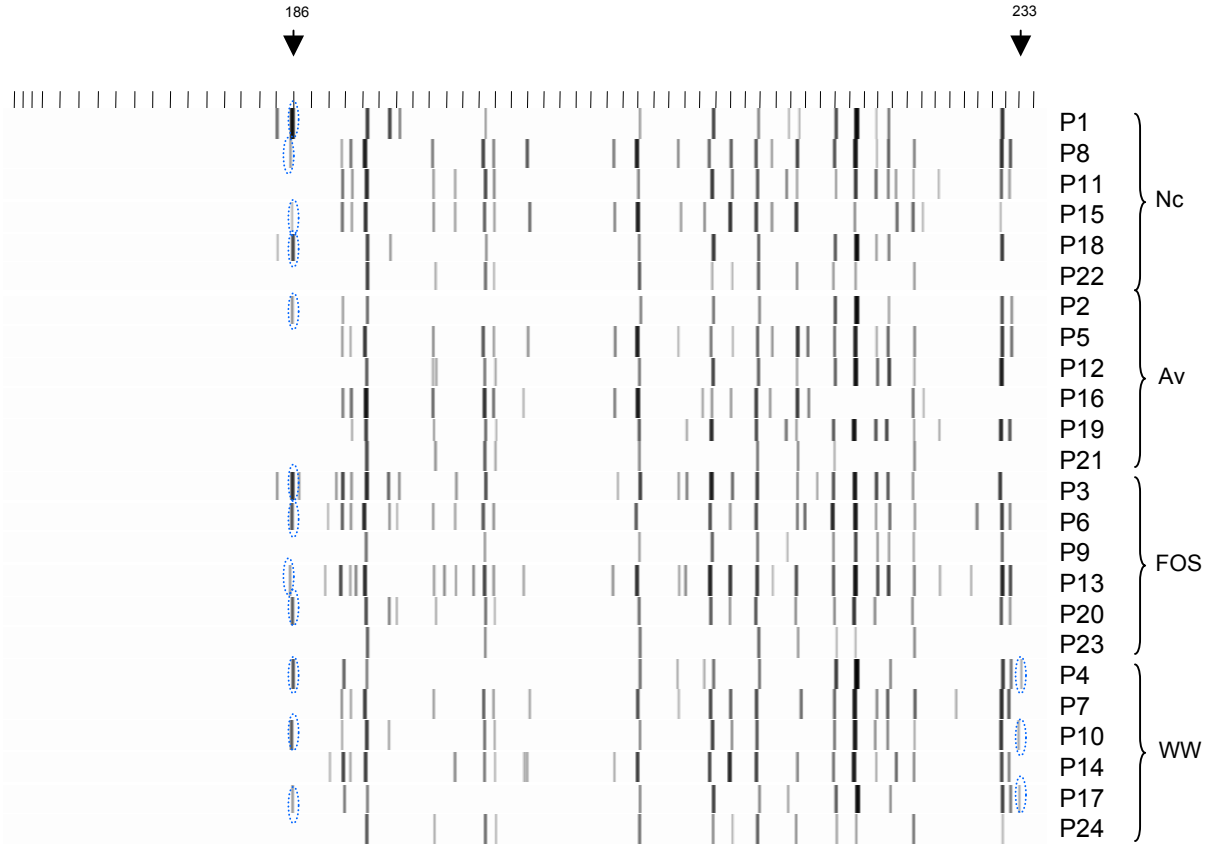

Figure 1. Fingerprints of CE-SSCP with universal primers (6-Fam labelling) obtained from ileal pools (6 individuals). Nc : negative control; Av : avilamycin; FOS : fructo-oligosaccharides; WW : whole wheat. Arrows indicated specific bands.

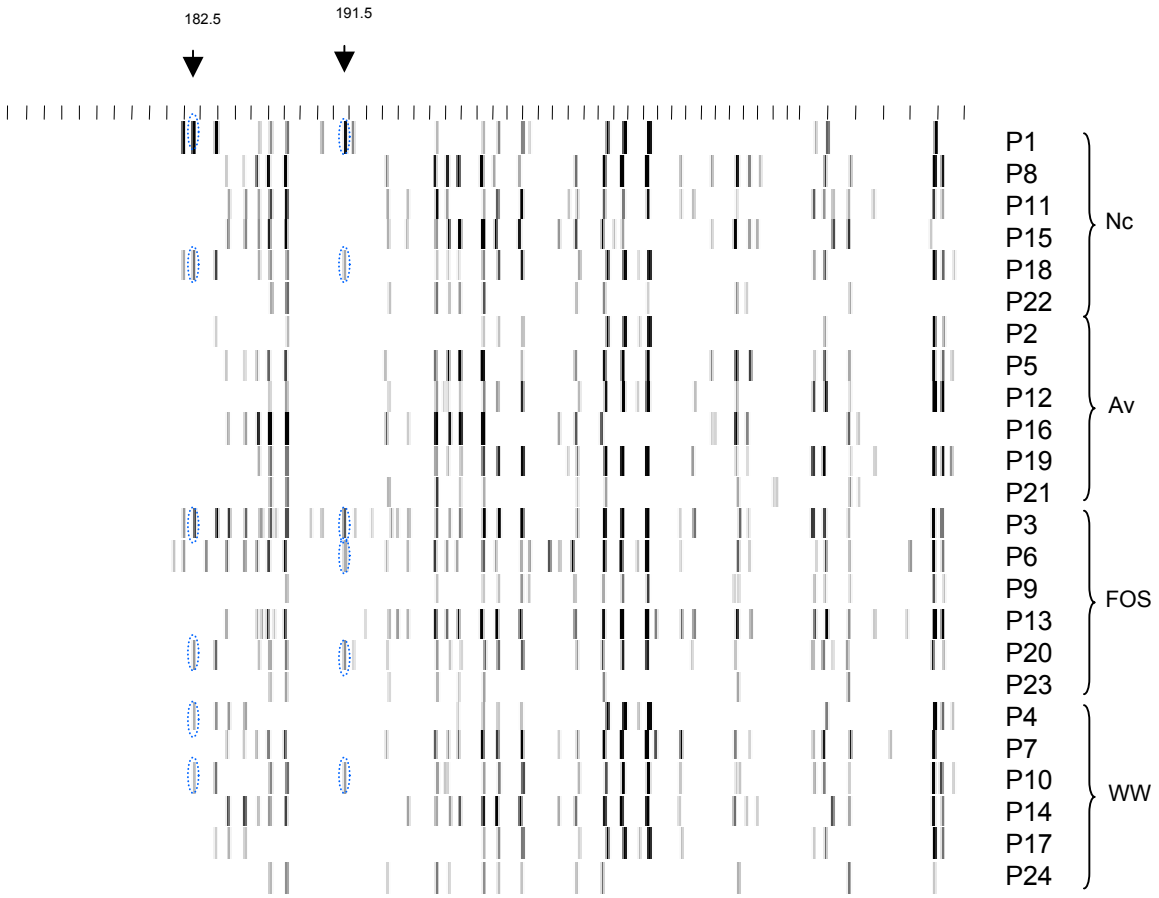

Figure 2. Fingerprints of CE-SSCP with universal primers (Hex labelling) obtained from ileal pools (6 individuals). Nc : negative control; Av : avilamycin; FOS : fructo-oligosaccharides; WW : whole wheat. Arrows indicated specific bands.

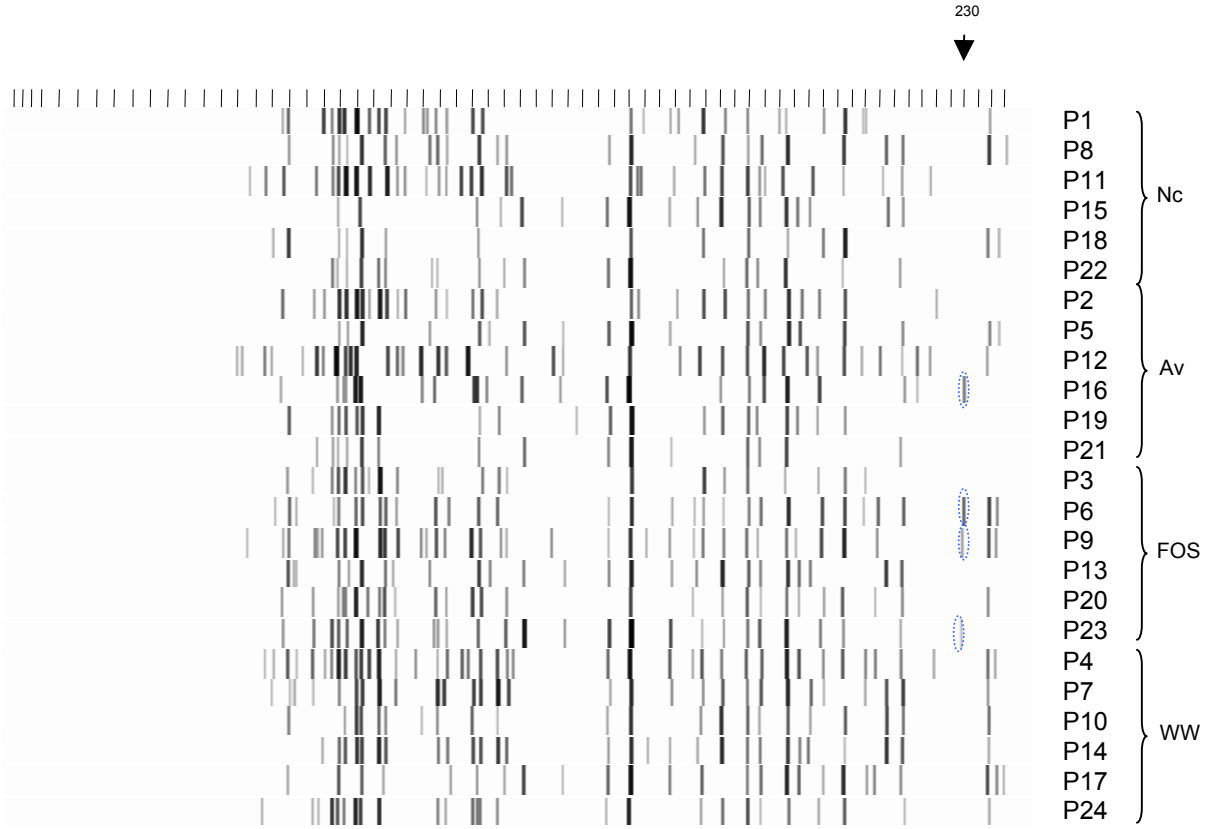

Figure 3. Fingerprints of CE-SSCP with universal primers (6-Fam labelling) obtained from cloacal pools (6 individuals). Nc : negative control; Av : avilamycin; FOS : fructo-oligosaccharides; WW : whole wheat. Arrows indicated specific bands.

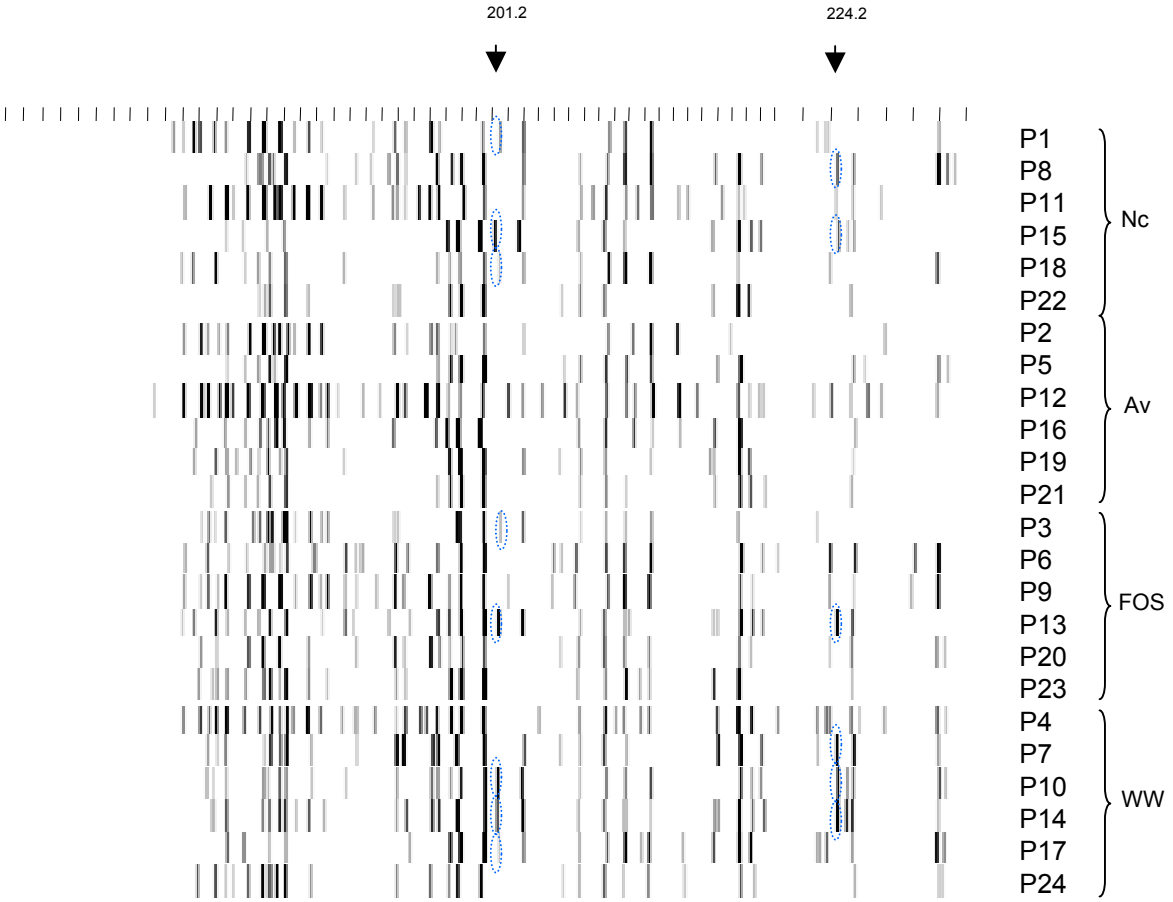

Figure 4. Fingerprints of CE-SSCP with universal primers (Hex labelling) obtained from cloacal pools (6 individuals). Nc : negative control; Av : avilamycin; FOS : fructo-oligosaccharides; WW : whole wheat. Arrows indicated specific bands.

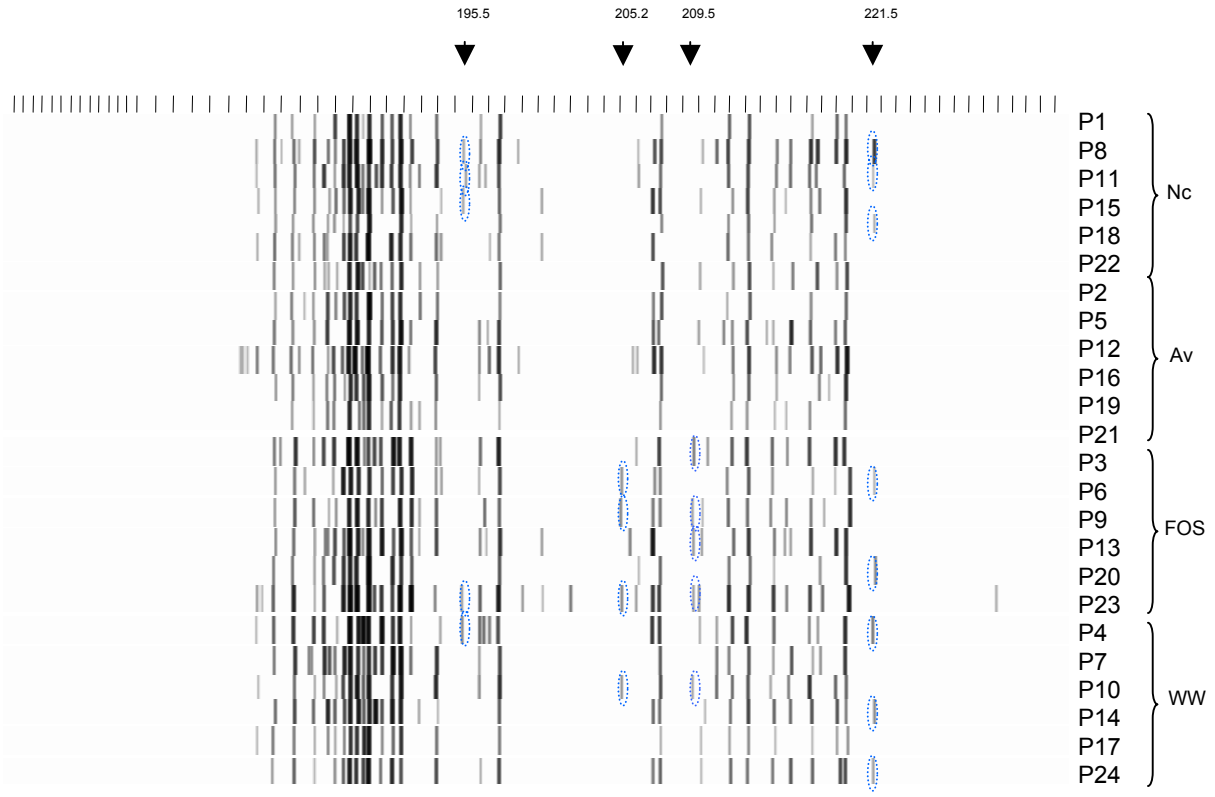

Figure 5. Fingerprints of CE-SSCP with universal primers (6-Fam labelling) obtained from caecal pools (6 individuals). Nc : negative control; Av : avilamycin; FOS : fructo-oligosaccharides; WW : whole wheat. Arrows indicated specific bands.

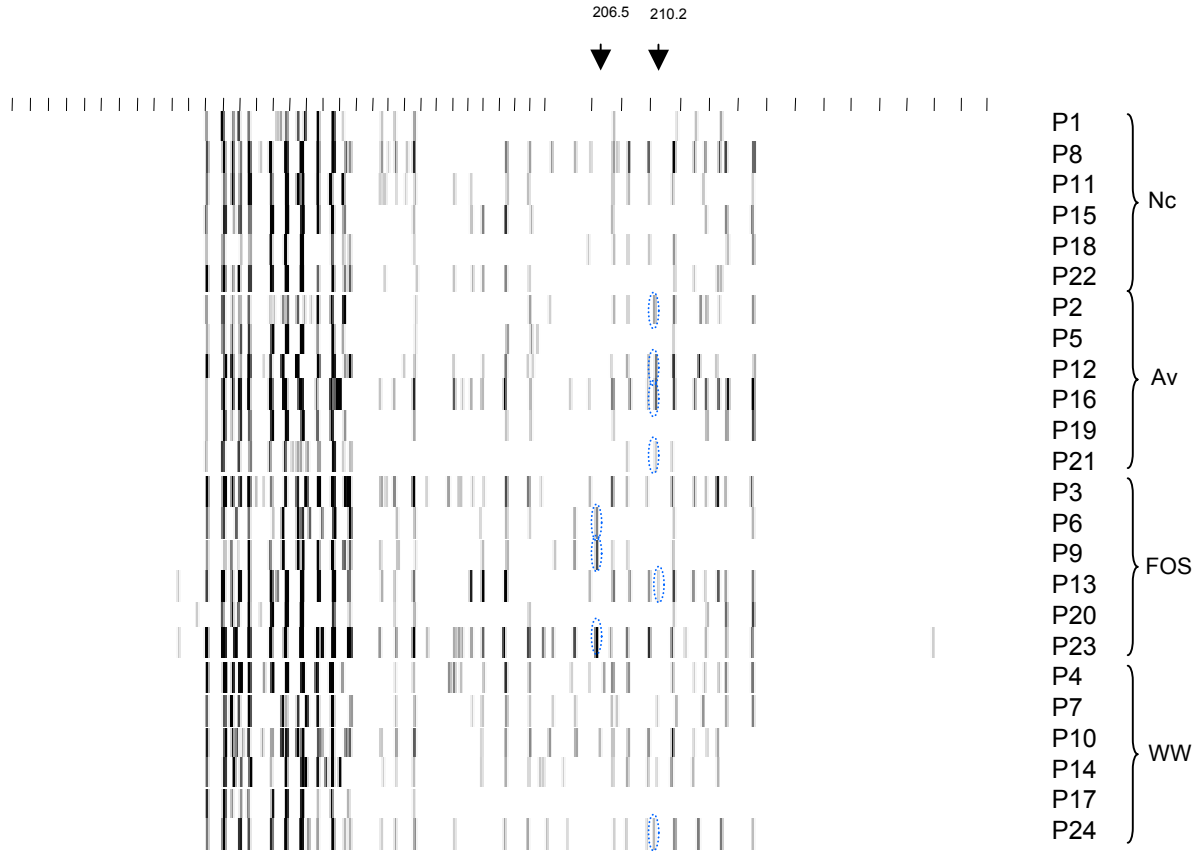

Figure 6. Fingerprints of CE-SSCP with universal primers (Hex labelling) obtained from caecal pools (6 individuals). Nc : negative control; Av : avilamycin; FOS : fructo-oligosaccharides; WW : whole wheat. Arrows indicated specific bands.

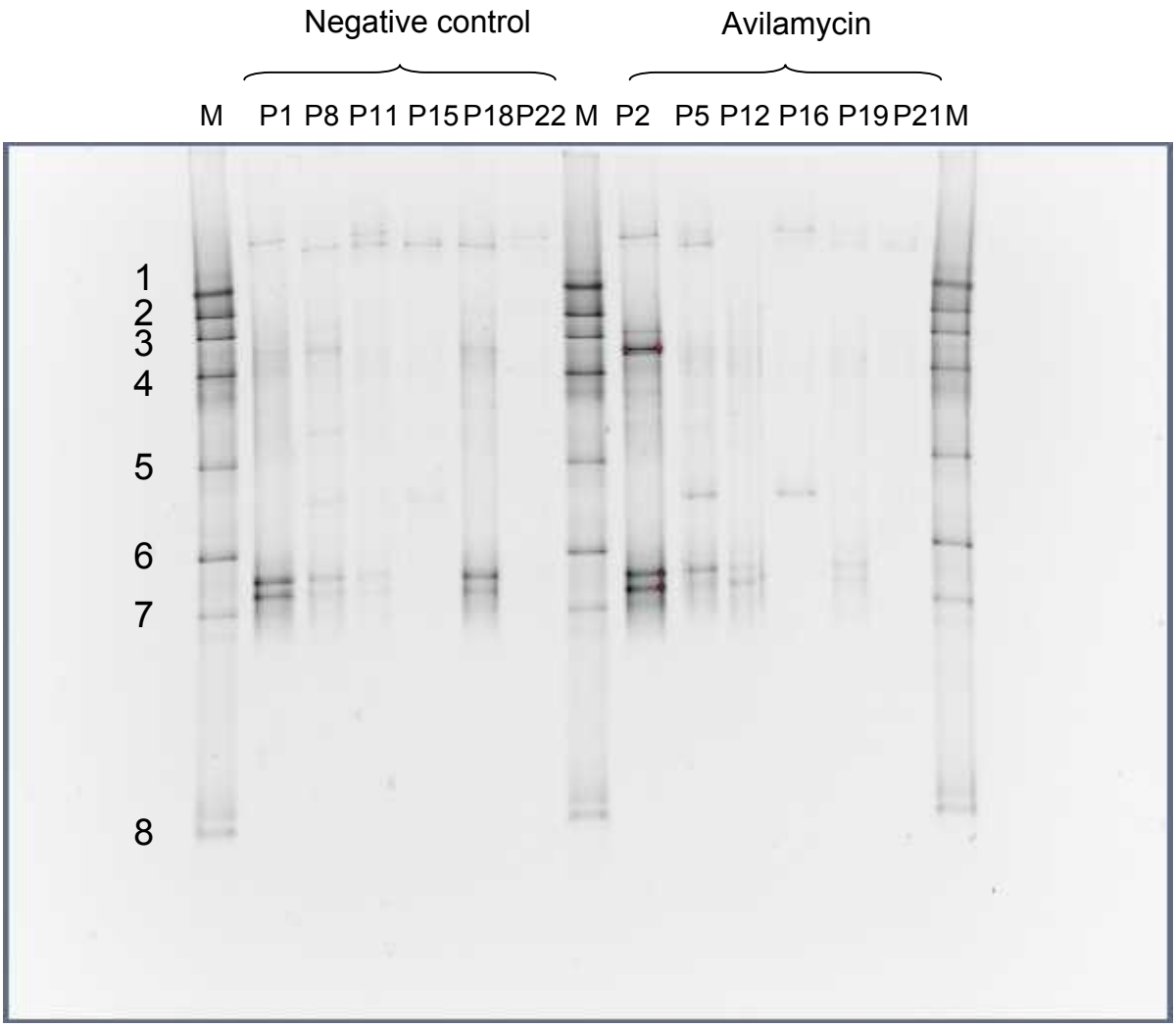

Figure 7. Fingerprints of TTGE with universal primers obtained from ileal pools (6 individuals) from negative control and avilamycin fed birds. Arrows indicated specific bands. M: marqueurs (1 to 8) of TTGE ladder.

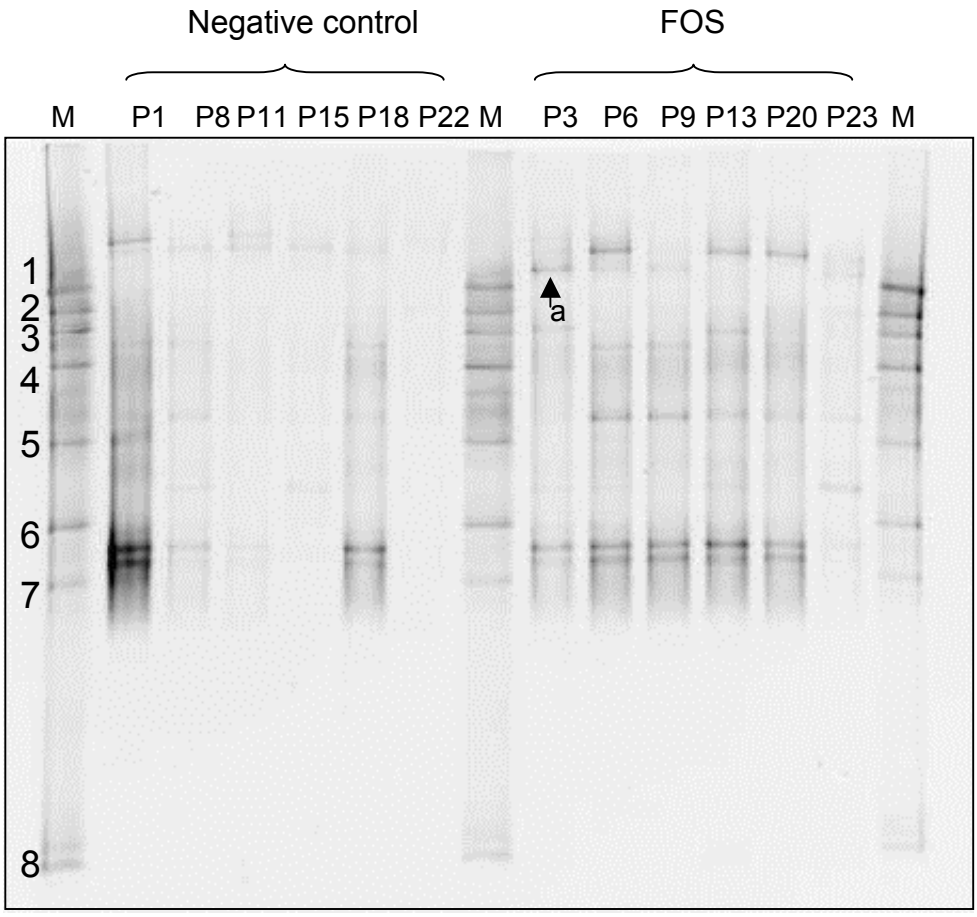

Figure 8. Fingerprints of TTGE with universal primers obtained from ileal pools (6 individuals) from negative control and prebiotic (FOS) fed birds. Arrows indicated specific bands. M: markers (1 to 8) of TTGE ladder.

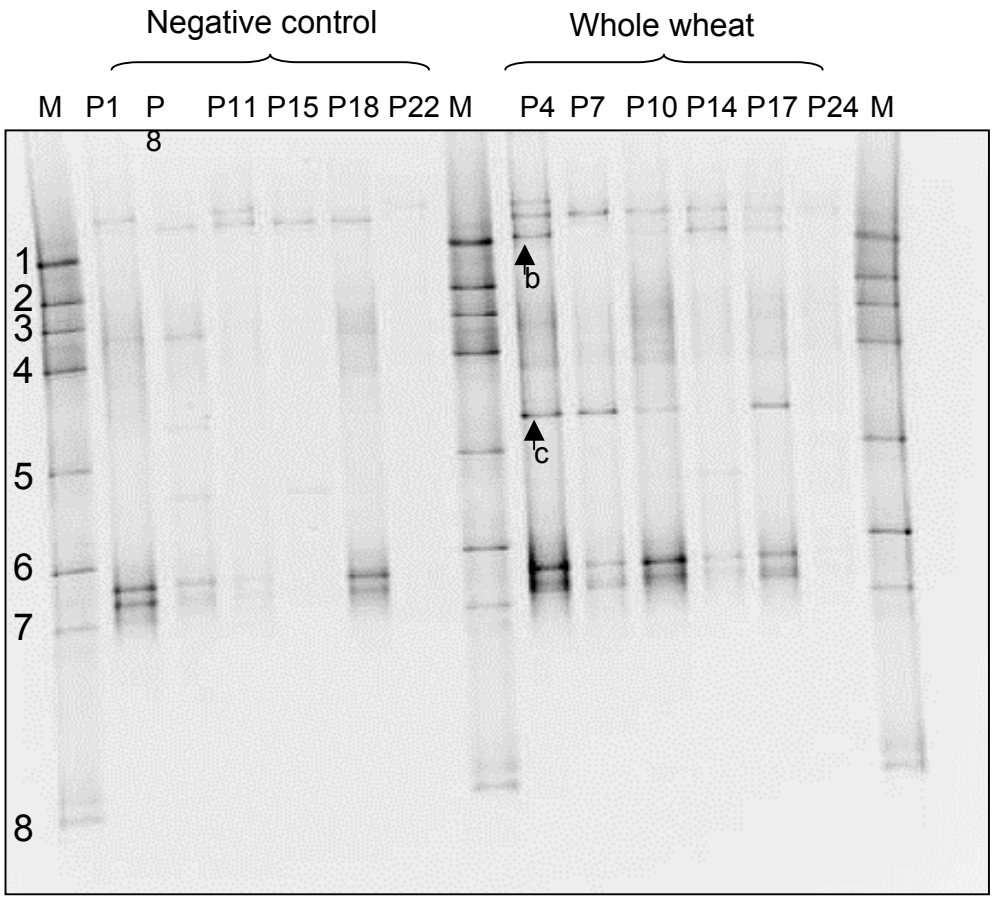

Figure 9. Fingerprints of TTGE with universal primers obtained from ileal pools (6 individuals) from negative control and whole wheat fed birds. Arrows indicated specific bands. M: markers (1 to 8) of TTGE ladder.

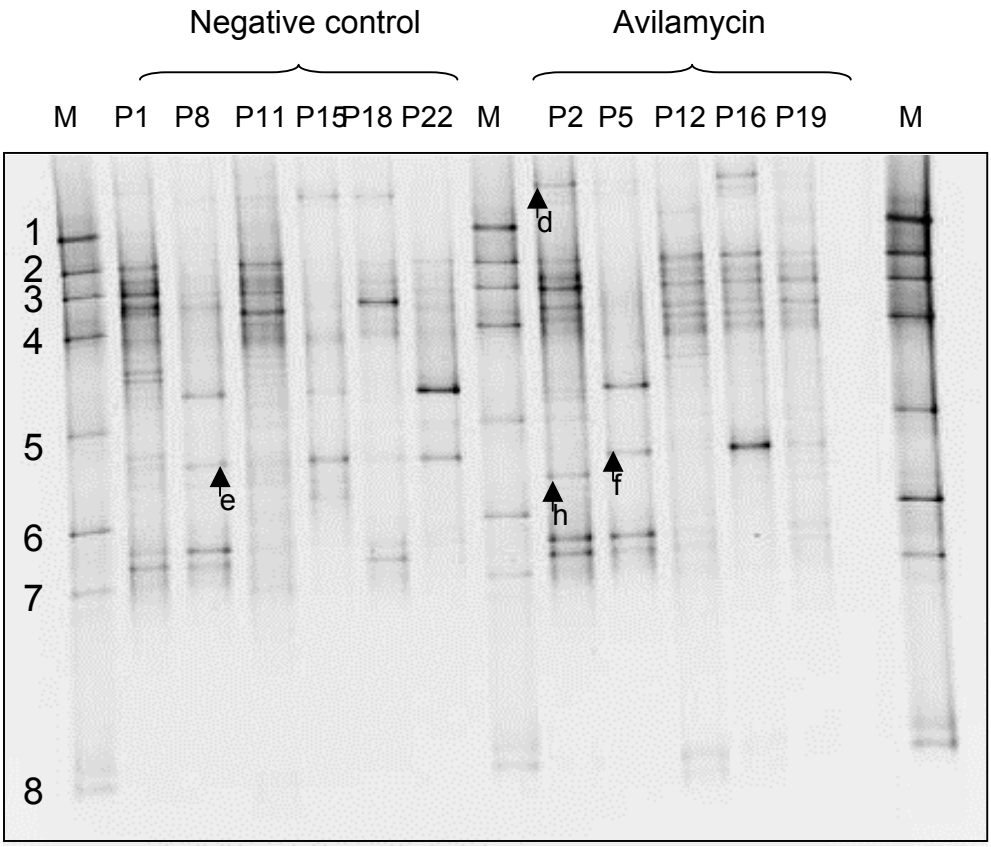

Figure 10. Fingerprints of TTGE with universal primers obtained from cloacal pools (6 individuals) from negative control and avilamycin fed birds. Arrows indicated specific bands. M: markers (1 to 8) of TTGE ladder.

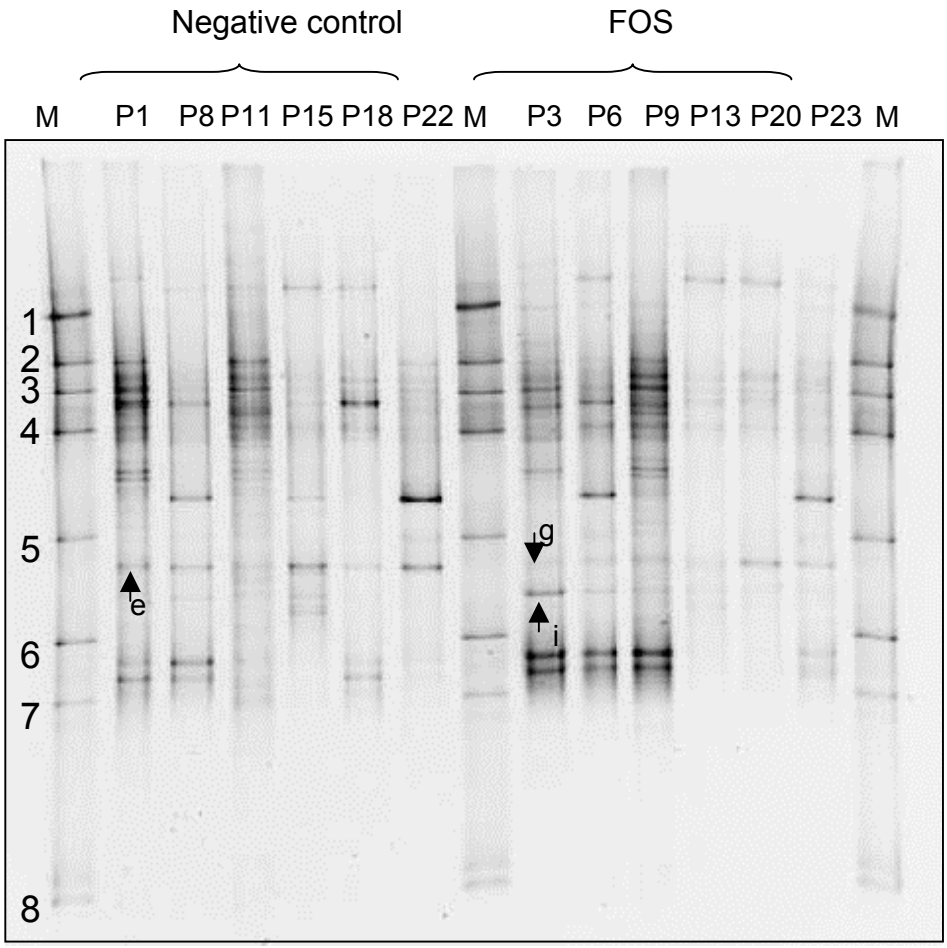

Figure 11. Fingerprints of TTGE with universal primers obtained from cloacal pools (6 individuals) from negative control and prebiotic (FOS) fed birds. Arrows indicated specific bands. M: markers (1 to 8) of TTGE ladder.

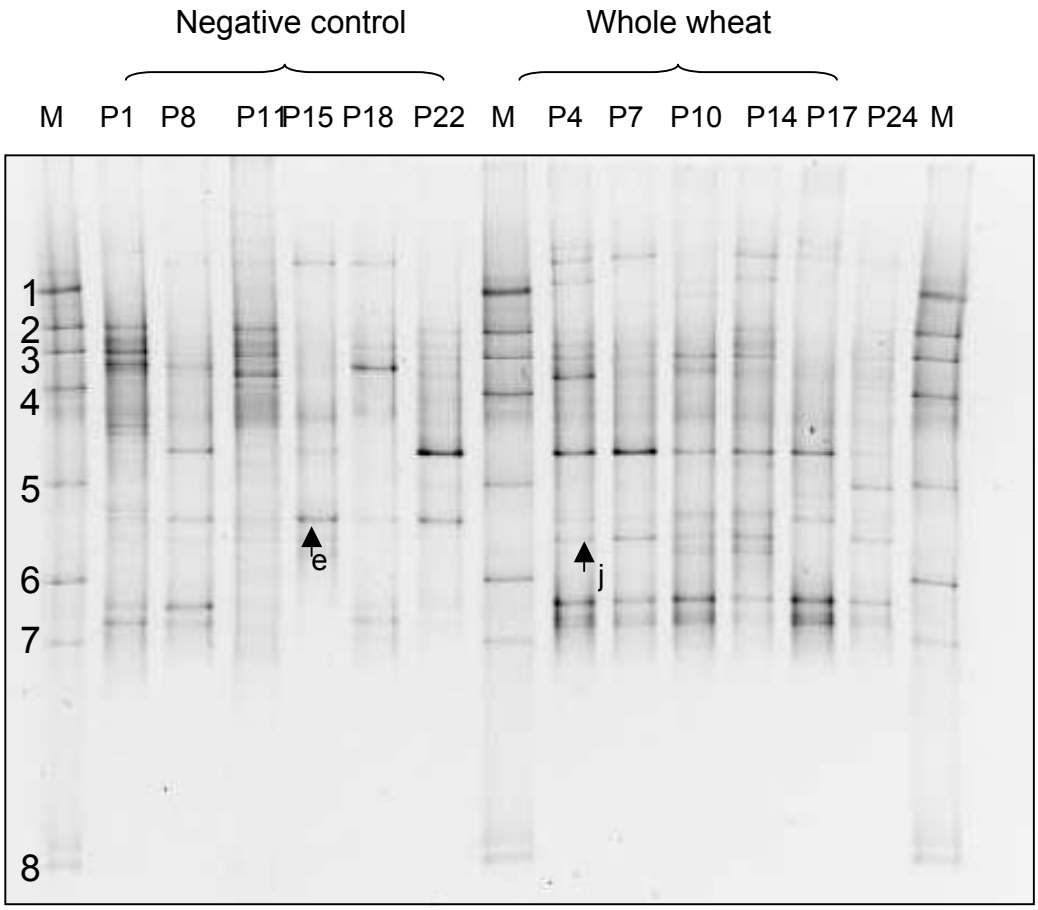

Figure 12. Fingerprints of TTGE with universal primers obtained from cloacal pools (6 individuals) from negative control and whole wheat fed birds. Arrows indicated specific bands. M: markers (1 to 8) of TTGE ladder.

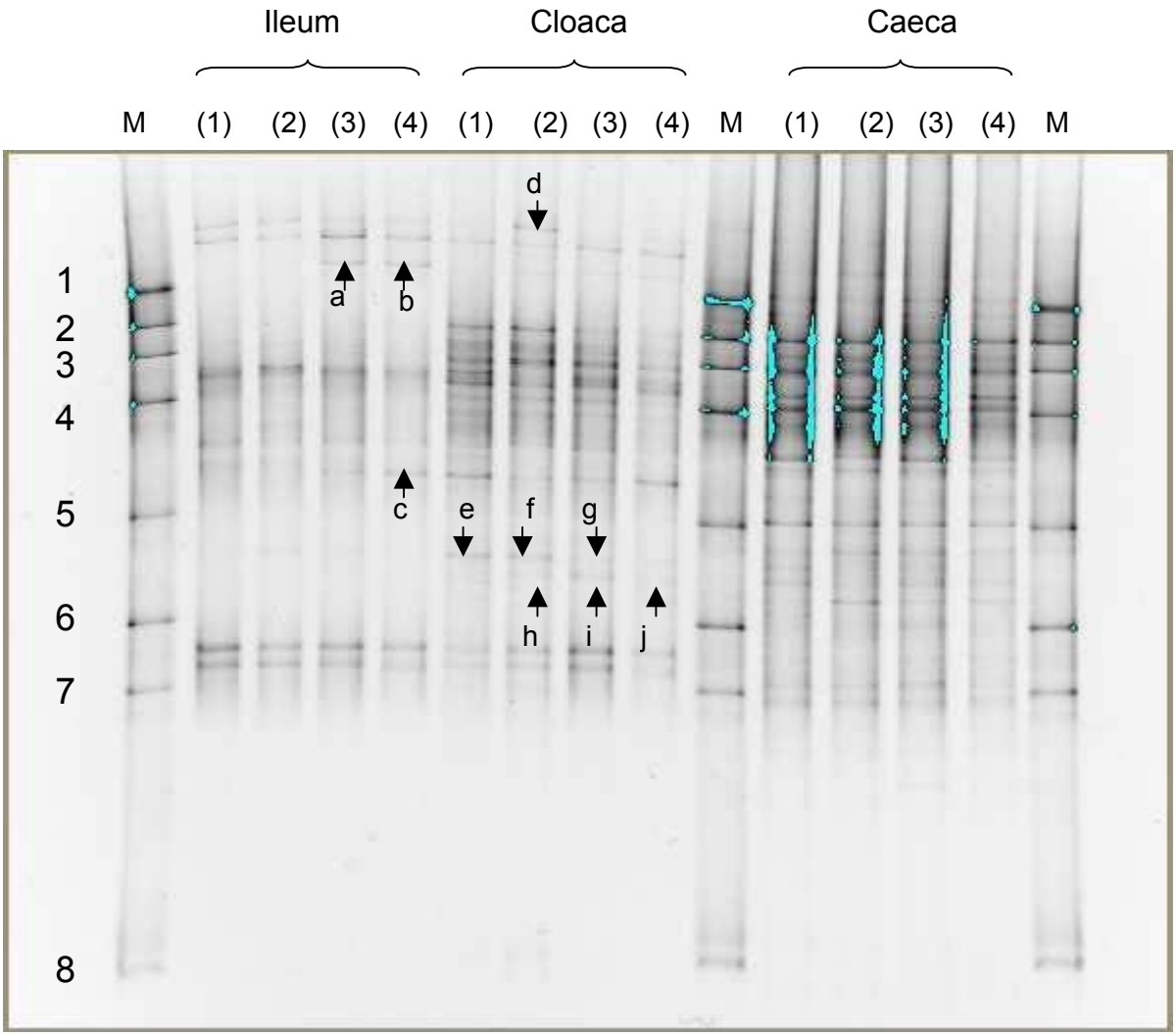

Figure 13. Fingerprints of TTGE with universal primers obtained from ileal, cloacal and caecal pools (36 individuals) of chickens fed the four dietary treatments. (1) : negative control; (2) : avilamycin; (3) : fructo-oligosaccharides ; (4) : whole wheat. Arrows indicated specific bands. M: markers (1 to 8) of TTGE ladder.

F. General discussion on the use of fingerprint techniques (CE-SSCP, RFLP, TTGE) to observe effect of antibiotic growth promoters and alternative to in-feed antibiotic on the digestive flora of poultry

When analysing microflora of ileal content of birds fed a negative control diet, the AGP salinomycin or different organic acids as alternatives to AGP, among the two fingerprint techniques (RFLP, CE-SSCP) used in this study to detect difference between dietary treatments, only RFLP was able to conduct to clear cluster of microflora samples according to treatment. Salinomycin treatment and the 4 organic acid treatments were clearly different from the negative control diet. Analysis of these same ileal samples with CE-SSCP did not lead to cluster. However this technique was able to detect disappearance of some bands with salinomycin. No difference of organic acid treatments with negative control diet was observed.

When studying microflora of different digestive contents (ileum, caeca, cloaca and fresh dropping) of birds fed a negative control diet, the AGP avilamycin or alternatives to AGP (prebiotic, probiotic, diet structure) with two fingerprint techniques, CE-SSCP and TTGE, although no cluster were observed according to dietary treatment, specific bands to treatment were detected with antibiotic and the alternatives to antibiotic.

As the changes observed with the three fingerprint techniques were not the same, it can be concluded that these techniques are complimentary.