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Development of cecal-predominant microbiota in broilers during a complete rearing using denaturing gradient gel electrophoresis

J. E. Blajman[A], *M. V. Zbrun*[A,B], *M. L. Signorini*[B,C], *J. A. Zimmermann*[A], *E. Rossler*[A], *A. P. Berisvil*[A], *A. Romero Scharpen*[A], *D. M. Astesana*[A], *L. P. Soto*[A,B] and *L. S. Frizzo*[A,B,D]

[A]Laboratory of Food Analysis, Institute of Veterinary Science of the Litoral (ICiVet-Litoral), National University of the Litoral (UNL)/National Council of Scientific and Technical Research (CONICET), Kreder 2805 (S3080HOF), Esperanza, Santa Fe, Argentina.
[B]Department of Public Health, Faculty of Veterinary Science, National University of the Litoral (UNL), Kreder 2805 (S3080HOF), Esperanza, Santa Fe, Argentina.
[C]National Institute of Agricultural Technology EEA Rafaela, National Council of Scientific and Technical Research (CONICET), Ruta 34 Km 227 (2300), Rafaela, Santa Fe, Argentina.
[D]Corresponding author. Email: lfrizzo@fcv.unl.edu.ar

Abstract. Understanding of the intestinal microbiota is crucial to enhance intestinal health and performance parameters in animals. A more exhaustive research of the intestinal microbiota of broilers could be of interest to implement appropriate intervention measures. The aim of the present study was to investigate the development of the predominant cecal microbiota in broilers that were fed a *Lactobacillus salivarius* DSPV 001P strain during a complete rearing using denaturing gradient gel electrophoresis (DGGE). Bacterial DNA from cecal samples of 24 broilers at different ages were amplified by PCR and analysed by DGGE. A total of 35 DGGE products were excised and sequenced. Distinctive differences in bacterial communities were observed in the caecum as broilers age. At early stages, identified bacteria within the caecum of broilers were predominantly *Clostridium*-related species. Also, some sequences had the closest match to the genus *Escherichia*/*Shigella*. Furthermore, the caecum was a reservoir rich in uncultured bacteria. The major difference observed in our study was an increase of potentially beneficial *Lactobacillus* at Day 45. These results may be attributed to modulation of the microbiota by the probiotic supplementation. The obtained data could be relevant for future studies related to the influence of the microbiota resulting from probiotic supplementation on the performance and the immunological parameters of broilers.

Additional keywords: bacterial communities, intestinal health, *Lactobacillus salivarius*, probiotic.

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Introduction

It is worth remarking that bacterial community in the gastrointestinal tract (GIT) of broilers could be introduced from the surrounding environment at the pre-hatching stage (Klasing 1998), in the incubator or hatcher, and at the post-hatching stage (Pedroso *et al.* 2006). Bacteria subsequently colonise the GIT until a complex and diverse bacteria population develops. Changes in the composition of the animal's microbiota can have beneficial or detrimental effects on digestion, nutrient absorption, intestinal morphology, pathogenesis of intestinal disease, and immune responses of the animal host (Santos *et al*. 2007). In commercial broiler production, broilers raised under intensive conditions are exposed to various intrinsic stressors every day. A combination of high stocking density, heat stress and a short time interval between flock placements is more likely to result in a microbiota imbalance and consequently a high susceptibility to diseases (Kabir 2009; Sasaki *et al.* 2014).

The broilers' microbiota has largely been studied by culture-based methods (Rolfe 2000). Although other parts of the GIT of broilers might also be important sites for microbiota–host interactions, the most densely populated microbial community within the broiler gut is found in the caecum (Sergeant *et al.* 2014). The microbiota of the caecum is very diverse and 1 g of caecal content may contain 10^{11} bacteria (Mead 1997). However, the use of conventional cultured-based methods has some major limitations. Some authors have estimated that only 10–60% of the total bacteria in the caecum were detected by culture (Barnes *et al*. 1972; Salanitro *et al*. 1974). Bacterial species that are not cultivable because of unknown growth conditions, stress imposed by cultivation procedures, or obligate interactions with the host or other bacteria, are missing (Gérard *et al*. 2008). Furthermore, cultivation and biochemical techniques have resulted in the misclassification of some bacteria (Tellez *et al*. 2006). Therefore, molecular approaches are being used to investigate the bacterial ecosystem of animals, those

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aforementioned approaches revealing a more diverse bacterial community (Zhu *et al*. 2002; Collado and Sanz 2007; Petersson *et al*. 2009; Torok *et al*. 2011; Choi *et al*. 2014). Among them, denaturing gradient gel electrophoresis (DGGE) is a fingerprinting method based on electrophoresis of PCR-amplified 16S rDNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturing agents. DGGE is capable of detecting differences between DNA fragments of the same size but with different sequences; besides, it is sensitive enough to detect bacteria that constitute up to 1% or more of the total bacterial community (Muyzer *et al*. 1993; Murray *et al*. 1996). Although this molecular technique has its restrictions, including the possibility that DNA extraction and amplification might be biased in favour of certain bacteria and sequences, it nevertheless provides an overview of the microbial diversity present in a particular sample (Zhu *et al*. 2002; Martínez *et al*. 2012).

A more exhaustive research of the intestinal microbiota of broilers could not only be of interest to basic microbiological ecology but may also be of practical importance to implement appropriate intervention measures. The aim of the present study was to investigate the development of the cecal-predominant microbiota in broilers that were fed a *Lactobacillus salivarius* DSPV 001P strain with *in vitro* probiotic properties (Blajman *et al*. 2015) during a complete rearing using DGGE.

Material and methods

Bacterial growth and animal sampling

Lactobacillus salivarius DSPV 001P, a strain of avian origin with *in vitro* probiotic properties, was cultured in a fermenter (Figmay, Córdoba, Argentina) in MRS broth and incubated for 18 h at 37°C. Then bacterial cells were harvested by centrifugation at 4800*g* for 10 min at 4°C, supernatant was removed, and cell pellets were freeze-dried with skim milk as cryoprotective agent in a lyophiliser (Martin Christ, Osterode am Harz, Germany). Ninety-six newly hatched Cobb broilers were used in the trial. There were three replicates with 32 broilers per replicate. The flock was placed in a broiler house with reused litter. Feed devoid of antibiotics or anticoccidials and water were provided *ad libitum*. This experiment lasted for 45 days. The strain was administered to the diet during the first 16 days at a dose of at least 1×10^{10} colony forming units (cfu)/broiler during 9 days and at least 1×10^{9} cfu/broiler during the remaining 7 days. Every 2 weeks, six broilers (two per replicate) were killed by cervical dislocation by a person with appropriate qualifications. All procedures used in this study were approved by the Ethics and Security Committee of the Faculty of Veterinary Science, National University of the Litoral and consistent with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, Federation of Animal Sciences Societies (FASS 1999). The caecum of broilers were removed aseptically, and ~200 mg of content and mucosa were collected into a tube containing 1 mL of sterile phosphate-buffered saline pH 7.4, and stored at −20°C until DNA extraction.

DNA extraction

Genomic bacterial DNA was extracted from caecum samples utilising a DNA Purification Kit (Promega, Madison, WI, USA) and following the manufacturer's instructions for Gram-positive bacteria. The amount of DNA was quantified and adjusted to 25–50 ng/µL using Take3 device and Gen5 software for Windows version 2.01 in a multimode microplate reader (Synergy HT, multi-mode microplate reader, BioTek Instruments, Inc., Winooski, VT, USA).

PCR amplification

Polymerase chain reaction amplifications of total bacterial community DNA were performed using primer pair HDA1-GC (5′-**CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G**AC TCC TAC GGG AGG CAG CAG T-3′; the GC-clamp is in boldface) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′). These primers amplify the V3 region of the 16S rDNA genes (position 339–539 in *Escherichia coli*), resulting in fragments of 200 bp (Gong *et al*. 2008). The PCR (50-mL reaction mixtures) amplification program consisted of preheating at 94°C for 5 min and 35 cycles of denaturing (94°C, 30 s), annealing (60°C, 1 min) and extension (72°C, 30 s). To eliminate artefact DGGE bands, the PCR was ended with a final 30-min extension step at 72°C to reduce the formation of heteroduplex PCR products (Janse *et al*. 2004). Also, the PCR products were checked by electrophoresis on a 2% agarose gel containing 0.01% (v/v) GEL RED (Genbiotech, Buenos Aires, Buenos Aires, Argentina) and viewed by ultraviolet light (DyNA Light UV Transilluminator, LabNet, UV light source wavelength 302 nm).

DGGE ladder

A DGGE ladder containing sequences of GIT bacterial strains was prepared from individual pure cultures (wild-type *Enterococcus faecium*, wild-type *L. salivarius* DSPV 001P, wild-type *Salmonella enteritidis* 421 and wild-type *Campylobacter jejuni* C173). Genomic bacterial DNA from the pure cultures was extracted using the DNA Purification Kit (Promega), and DNA samples were stored at −20°C. Polymerase chain reaction products were obtained using the primers HDA1-GC and HDA2, and a PCR program as described previously. The PCR products were mixed in equal amounts to obtain the DGGE ladder, whereas the ladders in DGGE analysis were put to normalise the gel patterns and; this normalisation enables the comparison of different DGGE gels (Temmerman *et al*. 2003).

DGGE analyses

A total of six caecal samples from each age group of broilers were run on DGGE. The DGGE analysis of PCR amplicons was performed using the Bio-Rad DCode Universal Detection System (Bio-Rad, Mississauga, ON, Canada). The amplicons were separated in 8% (wt/vol) polyacrylamide (acrylamide/bisacrylamide 35.7 : 0.8) gel containing a 40–55% gradient of urea and formamide increasing in the direction of electrophoresis [a 100% denaturing solution consisting of 7 M urea (Sigma-Aldrich Chemie, Steinheim, Germany) and 40% deionised formamide (Sigma-Aldrich Chemie)]. Amplified samples were mixed with an equal volume of 2 × loading buffer [0.05% (wt/vol) bromophenol blue, 0.05% (wt/vol) xylene cyanol, and 70% (vol/vol) glycerol] and 20 µL were placed in each sample well. The electrophoresis was conducted in 1 × TAE buffer [20 mM

Tris (pH 7.4), 10 mM sodium acetate, and 0.5 M EDTA] under 130 V at 60°C for 4 h. Gels were stained with *SYBR* Safe and viewed by Safe Image Blue-Light.

Examination of DGGE gels

The TIFF files of gel images were analysed with BioNumerics software version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). Bands were manually assigned in the software. Cluster analysis was conducted using the Dice's similarity coefficient for band matching with 1% position tolerance and the unweighted pair group method with arithmetic means to generate the dendrogram. The degree of similarity was represented by a similarity coefficient. Cophenetic correlation coefficients were calculated and represented on the root of each cluster in the dendrogram. This parameter was used to express the consistency of a cluster and represents the correlation between distance values calculated during tree building and the observed distance.

Sequencing of DGGE products

Representative bands were excised from the gel by a sterile scalpel, which was transferred into 50 µL of sterile distilled water and incubated for 48 h at 4°C. After centrifugation, 4 µL of the supernatant was taken as a template for reamplification of the bands using the same primers and conditions for PCR as described earlier. Before sequencing, the amplicons were purified using a kit Wizard SV Gel and PCR Clean-Up System (Promega). Sequencing was performed with the Standard-Sequencing System (MACROGEN, Seoul, South Korea). The resulting DNA sequence information was analysed by use of BLAST program (Altschul *et al*. 1990).

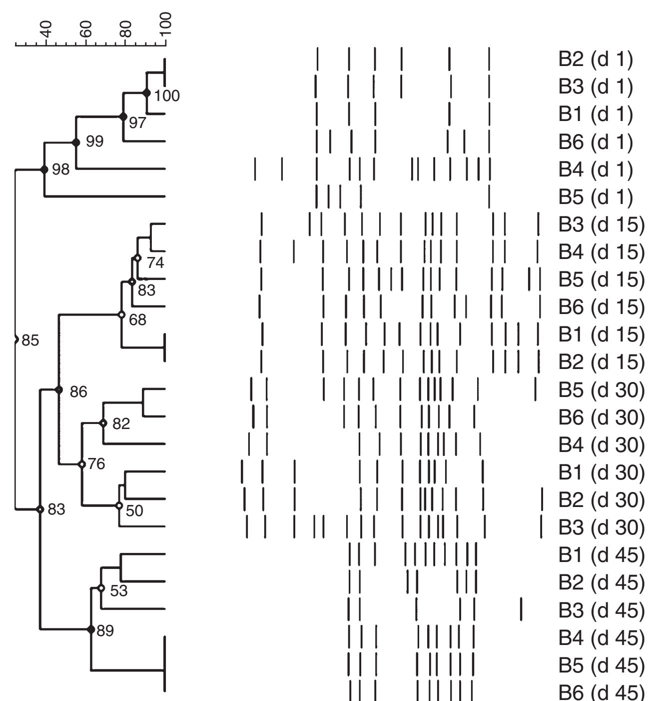

Fig. 1. Cluster analysis of denaturing gradient gel electrophoresis profiles of caecal microbiota of broilers fed *Lactobacillus salivarius* DSPV 001P strain with *in vitro* probiotic properties. The dendrogram was band-based and was constructed using unweighted pair group method with arithmetic means in BioNumerics software. Cophenetic correlation coefficients are represented on the root of each cluster in the dendrogram. Similarity between samples is indicated by the percentage coefficient bar located above the dendrogram.

Results

DGGE of the PCR products obtained with universal primers from cecal samples of broilers at four different ages (1 day, 15 days, 30 days, and 45 days) resulted in diverse profiles. Cecal samples showed a large number of bands that were distributed along the entire gradient. More numbers of bands were resolved in the middle than in the top and the bottom of the gel. The number of discernible bands per sample increased from Day 1 to Day 30 and then decreased, showing temporal succession in the cecal microbiota. It was noted that several bands appeared and then disappeared after each sampling period and were substituted by other bands (Fig. 1).

A 40% similarity of DGGE bacterial profiles from 1-day-old broilers was found. Cecal microbiota from 15-day-old broilers had similar DGGE profiles with 80% similarity coefficient. Samples from 30-day-old broilers had 60% similarity. A similar relationship in the DGGE bacterial profiles was also observed in samples from 45-day-old broilers. Cophenetic correlation values are shown in the dendrogram. The cophenetic value for the whole dendrogram (85%) indicates that the dendrogram did not distort the original structure in the input data (Fig. 1).

DGGE products were sequenced and assigned to species or genus using BLAST in the GenBank database. The band corresponding to *L. salivarius* DSPV 001P strain was detected on DGGE banding patterns at Day 45 (Table 1). A total of 35 bands (Fig. 2) were analysed from the cecal samples of broilers, and sequences had 83–100% similarity to 16S rDNA database sequence (Table 1). Broilers at the same age had bands in similar positions in all samples tested. The intensity of these bands was variable, reflecting the possible presence of similar microorganisms at difference abundance in the samples analysed (Cressman *et al*. 2010) (Fig. 2).

Distinctive differences in bacterial communities were observed in the caecum as broilers aged. The comparison of DNA fragments with GenBank sequences evidenced the presence of strains belonging to the genus *Clostridium*, *Enterococcus*, *Lactobacillus*, *Shigella*, and *Escherichia* at Day 1 (Table 1). *Clostridiales* was the most prevalent order in broilers' caecum at Day 15, with a high proportion of members from genus *Clostridium*. Interestingly, some sequences could also be classified to the genus *Alistipes*, *Anaerostipes*, *Blautia*, *Hespellia*, *Robinsoniella*, *Ruminococcus*, and *Robinsoniella*. Beneficial bacteria such as *Lactobacillus* was not detected at Day 15 (Table 1). At Day 30, caecum microbiota was more diversified and it was mainly dominated by *Clostridium*, *Lactobacillus*, *Streptococcus*, and uncultured bacteria (Table 1). The caecum microbiota profiling of broilers at Day 45 consisted of *Clostridium*, *Lactobacillus*, *Escherichia*, and uncultured bacteria. Comparatively, a higher population of *Lactobacillus* was

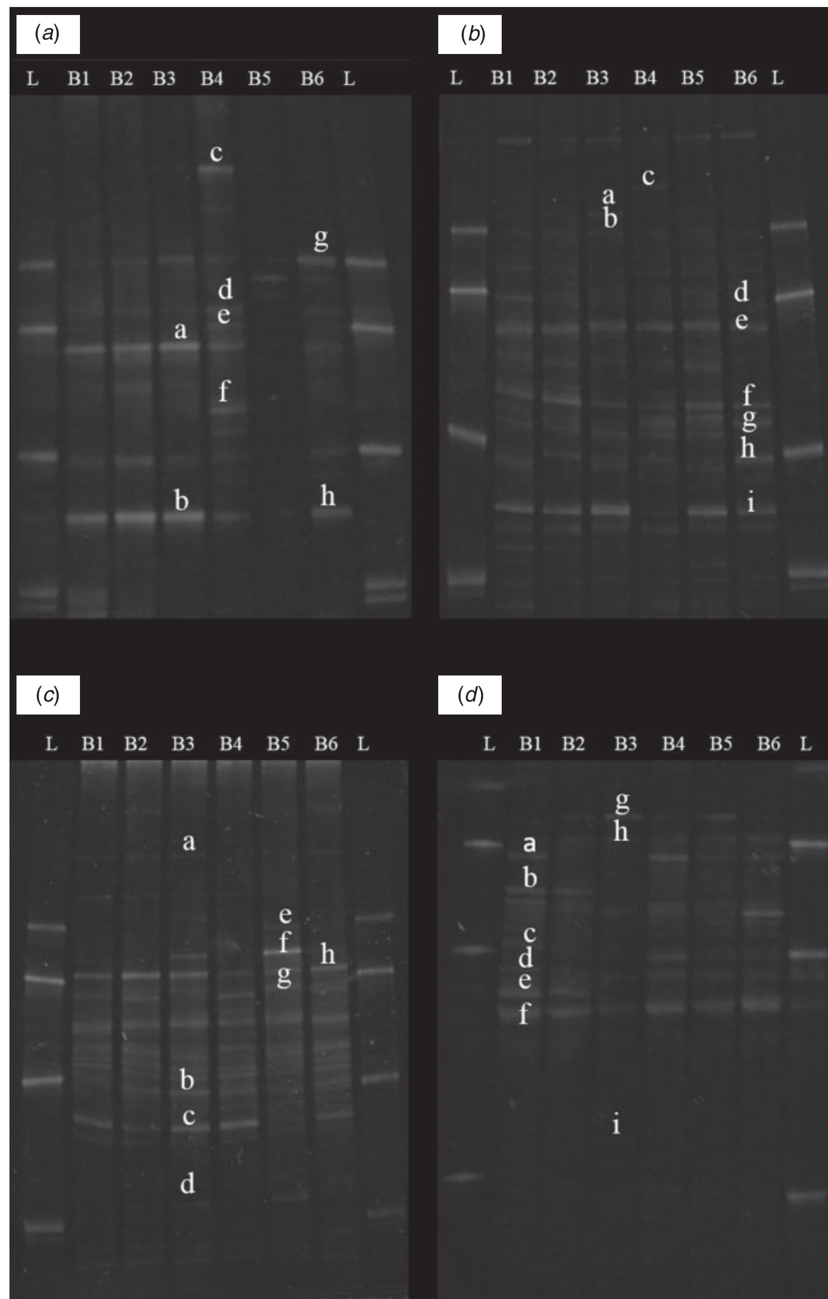

Fig. 2. (*a*) Denaturing gradient gel electrophoresis (DGGE) of PCR products of caecal samples from six broilers at Day 1. (*b*) DGGE of PCR products of caecal samples from six broilers at Day 15. (*c*) DGGE of PCR products of caecal samples from six broilers at Day 30. (*d*) DGGE of PCR products of caecal samples from six broilers at Day 45. (L) DGGE ladder (from top to bottom of the gel: wild-type *Enterococcus faecium*, wild-type *Lactobacillus salivarius* DSPV 001P, wild-type *Salmonella enteritidis* 421 and wild-type *Campylobacter jejuni* C173).

observed in the caecum of broilers at Day 45. In total, 8 of 35 sequenced DNA fragments from cecal samples were related to sequences of uncultured bacteria. Ten sequenced DNA fragments were related to sequences of cultured bacteria. The remaining 18 sequences shared equal percentage of identity with uncultured and cultured bacteria (Table 1).

Discussion

Viable counting on selective media recovers less than 20% of bacteria (Vaughan *et al*. 2000). However, the use of molecular techniques enables the uncultured bacteria to be studied without selective pressures inherent to traditional cultured-based methods. The present study examined development of cecal-predominant

Table 1. Putative identity of cecal bacteria from broilers

Band	Closest sequence relative	Identity (%)	NCBI Accession number
	1-day-old broilers		
a	*Enterococcus* sp.	98	JQ739629.1
b	Uncultured bacterium, *E. coli*, *Shigella* sp.	99	KF109509.1/KT005237.1/KR148992.1
c	*Enterococcus faecium*	99	KR265159.1
d	Uncultured bacterium, *Escherichia coli*	99	KF109509.1/ KT005237.1
e	Uncultured *Lactobacillus* sp.	95	GQ179737.1
f	Uncultured bacterium, *Clostridium* sp.	96	JX223097.1
g	*Enterococcus* sp.	98	KJ394444.1
h	Uncultured bacterium, *E. coli*, *Shigella* sp.	99	KJ421448.1/CP010344.1/KM051098.1
	15-day-old broilers		
a	Uncultured bacterium, *Clostridium* sp., *Hespellia* sp.	91	EU771998.1/ KJ722502.1/KC854374.1
b	Uncultured bacterium, uncultured *Robinsoniella* sp., *Robinsoniella peoriensis*	99	KT599233.1/KP102830.1/JX424580.1
c	Uncultured bacterium, uncultured *Clostridium* sp.	97	HG810865.1/ KP106011.1
d	Uncultured bacterium	83	EU771779.1
e	Uncultured bacterium, uncultured *Clostridium* sp., uncultured *Blautia* sp.	98	AY990703.1/KP108904.1/KP102198.1
f	Uncultured bacterium, uncultured *Vallitalea* sp., *Clostridium* sp.	97	JX223097.1/ KF758641.1/KJ722502.1
g	Uncultured *Lachnospiraceae* bacterium, uncultured *Clostridium* sp., uncultured *Anaerostipes* sp., uncultured *Ruminococcus* sp.	99	KP108786.1/ KP108904.1/KP109148.1/ KP106621.1
h	Uncultured bacterium, *Clostridium* sp.	97	KP780129.1/ KJ722502.1
i	Uncultured bacterium, uncultured *Clostridium* sp.	97	KM367755.1/ KP108904.1
	30-day-old broilers		
a	Uncultured bacterium	91	HG810869.1
b	Uncultured bacterium	99	KM367501.1
c	Uncultured *Lachnospiraceae* bacterium, uncultured *Clostridium* sp.	97	KP102507./AY675969.1
d	Uncultured bacterium	100	HG967644.1
e	Uncultured bacterium, *Clostridium* sp.	84	KC806031.1/HG326494.1
f	*Lactobacillus* sp., *L. crispatus*, *L. jensenii*, *L acidophilus*, *L. helveticus*, *L. gallinarum*	100	KR232863.1/KC561106.1/DQ317562.1/ KP987308.1/KT368991.1/LC071810.1
g	Uncultured *Streptococcus* sp., *Streptococcus* sp.	97	KP104503.1/ KR232855.1
h	Uncultured bacterium	95	KT775118.1
	45-day-old broilers		
a	Uncultured *Lactobacillus* sp.	100	HQ794794.1
b	Uncultured bacterium, uncultured *Alistipes* sp.	98	GU104253.1/KP105298.1
c	Uncultured bacterium, uncultured *Robinsoniella* sp.	100	HG967643.1/KP102830.1
d	Uncultured *Lachnospiraceae* bacterium	89	KP104134.1
e	Uncultured bacterium	96	GQ178922.1
f	Uncultured *Lachnospiraceae* bacterium, *Clostridium* sp.	98	KR086556.1/KJ722502.1
g	*L. acidophilus*, *L. helveticus*, *L. gallinarum*, *L. amylovorus*	100	KP987308.1/KT368991.1/LC071810.1/ KT185019.1
h	*Lactobacillus salivarius*	100	KP979479.1
i	Uncultured *Escherichia* sp.	99	DQ856964.1

microbiota in broilers that were fed a *L. salivarius* DSPV 001P strain during a complete rearing using DGGE. This technique is capable of providing a fingerprint of bacterial community in an environmental sample after a direct DNA extraction. In our study, broilers of the same age, reared under the same conditions, presented different banding patterns, suggesting that host-specific factors are important in the establishment of broiler bacterial community. On the one hand, the possibility to identify the bacterial species by sequencing the DGGE products is an important advantage of the method. But on the other hand, DGGE analysis of PCR amplicons is not always suitable for the identification of all species. The sequences from DGGE products to be compared in the database were relatively small 16S rDNA fragments (200 bp). In our investigation it represented a factor which impeded a reliable identification of species as some sequences shared equal percentage of identity with different bacteria. Another possibility is the identification of bacteria by comparison of the PCR amplicons migration distances in DGGE gels with those of reference strains present in the DGGE ladder, which is easier than the sequencing of the bands, but it does

not guarantee an unequivocal identification of bacteria (Ercolini *et al*. 2001). Cases of comigration of PCR amplicons were observed from different species in the DGGE gels (Meroth *et al*. 2003; Abecia *et al*. 2007). Though different in sequences, some species might have identical melting behaviour and therefore they migrate at the same distance in DGGE gels. Our results showed bands in the same position as the reference strain *L. salivarius* DSPV 001P in broiler samples of all ages. Nevertheless, sequencing was coincident with *L. salivarius* only in 45-day-old broilers. Moreover, it was not possible to determine if the sequenced strain was the administered strain *L. salivarius* DSPV 001P or an indigenous one. For that reason, DGGE cannot be used as a unique tool to track probiotic strains throughout the broilers' production period, but it could be effective in corroborating the occurrence of certain microbial species in a lyophilised probiotic preparation (Fasoli *et al*. 2003). Another disadvantage of using DGGE is the feasibility of having multiple copies of the 16S rRNA and thus multiple bands displayed for only one species, which overestimates the community diversity and richness detected by DGGE (Nübel *et al*. 1996). In our study, we pinpointed different bands as the same bacteria that could correspond to a single PCR amplicon. Considering our information, a possible support of other techniques to DGGE should be evaluated. It would be interesting to complement the data generated with techniques that are independent of amplification bias and with methods that allow the enumeration of different phylogenetic groups in the GIT of broilers (Feng *et al*. 2010; Kačániová *et al*. 2013; Lunedo *et al*. 2014).

A total of 35 bands (DGGE products) were analysed from cecal samples of broilers. The microbiota in 1-day-old broilers appeared to be less developed and amplicons showed between 5–13 bands. However, Van der Wielen *et al*. (2002) reported that intestinal samples of 1-day-old broilers had no bands in DGGE gels except for the crop where a low number of bands were detected. This does not imply that bacteria were not present in caecum of 1-day-old broilers, but the bacterial numbers were probably below the detection limit. Differences in environment, age, hygiene level, diet, type of broiler, geography and climate may be a factor affecting cecal microbiota (Yin *et al.* 2010). In our country a common practice in broiler production is to employ reused litter, which harbors more nutrients, moisture and bacteria of intestinal origin than fresh litter. Therefore, the reused litter may explain some of the observed differences (Lu *et al*. 2003; Cressman *et al*. 2010). According to the general consensus, caecum microbiota becomes more complex as the broilers aged (Danzeisen *et al.* 2011). Based on our study, the number of profile bands increased from Day 1 to Day 30 and then decreased reflecting temporal succession in cecal microbiota, which can be attributed to a stable microbiota in GIT of mature broilers (Gong *et al.* 2002). Only a few sequences could be identified to the species level; however, the majority could be only classified to the genus, family, or order level. Bacteria identified within the caecum of broilers were predominantly potentially pathogenic *Clostridium*-related species. This is important because the caecum is the main site of *C. perfringens* colonisation, the major causative agent in necrotic enteritis (Stanley *et al.* 2012). Also, some sequences had the closest match to the genus *Escherichia*/*Shigella*, which is of particular interest due to the emergence of pathogenic *E. coli* strains with the ability to colonise both humans and broilers (Mora *et al.* 2010). Furthermore, the caecum was a reservoir rich in uncultured bacteria. It is interesting to note that our results are in agreement with previous studies: Zhou *et al*. (2007) reported that most of clones out of 13 sequenced were closely related to uncultured bacteria. In caecum investigated by Mohd Shaufi *et al*. (2015), from the phylum Firmicutes, Clostridia was the most dominant class in the caecum of broilers at different ages, with the members from order *Clostridiales* being most abundant. Choi *et al*. (2014) found that strict anaerobes such as *Alistipes*, unclassified *Ruminococcaceae*, and unclassified *Lachnospiraceae* were dominant in broiler caecum. Consistent with our current observations, Lu *et al*. (2003) reported that *Clostridium*, *Eubacterium*, and *Ruminococcus* dominated the bacterial community of broiler caecum at all ages. 16S rRNA gene sequences identified as *Lactobacillus*, *Escherichia*, and *Streptococcus* were also observed. Previous reports (Mohd Shaufi *et al.* 2015) found that the population of *Lactobacillus* was low in broiler caecum at all intervals. Therefore, the major difference observed in our study is an increase of potentially beneficial *Lactobacillus* detected in broiler caecum at Day 45. These results may be attributed to modulation of the microbiota by the probiotic supplementation. When the population of beneficial bacteria such as *Lactobacillus* is comparatively lower than the potentially pathogenic bacteria such as *Clostridium*, there is a need for manipulation to improve the intestinal health. Our findings suggest that an early probiotic administration may have a direct impact on the development of cecal microbiota in broilers.

Conclusion

DGGE was an appropriate analysis to define the predominant composition of broiler cecal microbiota in regional breeding conditions and under the supplementation of a *L. salivarius* DSPV 001P strain with *in vitro* probiotic properties during a complete rearing. We have shown that succession of cecal microbiota was affected by broilers age. At early stages, identified bacteria within the caecum of broilers were predominantly *Clostridium*-related species. However, we observed an increase of potentially beneficial *Lactobacillus* at Day 45. Future *in vivo* studies related to the influence of the microbiota resulting from probiotic supplementation on the performance and the immunological parameters of broilers should be conducted.

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