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ANIMAL RESEARCH PAPER

The use of multiple restriction enzymes in terminal restriction fragment length polymorphism analysis and identification of performance-related caecal bacterial groups in growing broiler chickens

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SUMMARY

Four restriction enzymes (AluI, HhaI, MspI and RsaI), either individually or in combination, were used in terminal restriction fragment length polymorphism (T-RFLP) analysis to: (i) characterize the chicken intestinal bacterial community; and (ii) tentatively identify intestinal bacterial groups related with increased performance parameters in broiler chickens. Balanced commercial diets free of any feed antibiotics were offered to broilers assigned randomly to one of the five dietary treatments: control (C) (commercial diet with no additive), inulin (I), fructose caramel, propyl propane thiosulphonate (PTS-O)-45 and PTS-O-90. Except for the inulin-supplemented diet, multivariate statistical analysis of T-RFLP profiles based on individual enzymes or their combinations showed that the caecal bacterial community composition was significantly different among diets, particularly between the control and the supplemented diets. Individual RsaI and the combination AluI + RsaI proved to be the most useful to discriminate between dietary treatments. *Clostridiaceae* 1, *Lachnospiraceae, Ruminococcaceae* and *Micrococcaceae* were tentatively identified as those families most likely to be implicated in defining the caecal microbiota composition of growing broiler chickens, and also as those most closely related with differences in productive parameters.

INTRODUCTION

Sequences of intestinal origin assigned to 915 species within 655 genera and representing 13 existing bacterial phyla have been reported recently in broilers (Wei *et al*. 2013). The cell density of the gastrointestinal microbiota in poultry ranges from 10^7 to 10^{11} bacteria/g gut content (Apajalahti *et al*. 2004) and has a significant impact on the growth and health of the birds by affecting gut function. The microbiota resident in the gastrointestinal tract is dynamic (Greiner & Bäckhed 2011) and plays key roles in the normal physiological functions such as nutritional, immunological and protective functions, including the general well-being and health of the host animals (Delzenne & Cani 2011; Frick & Autenrieth 2013). Accordingly, understanding of the mechanisms implicated and how changes in the gut microbiota composition may alter the host's nutrient and energy utilization efficiency and health is currently an important issue for intensive animal production industries.

Earlier studies have predominantly used culture-dependent approaches for identifying the composition of poultry gut microbiota (Barnes 1979; Mead 1989). Such methods are laborious and incomplete, and, therefore, not suitable for extensive monitoring of the intestinal microbiota including unknown or uncultured species (Apajalahti *et al*. 2003). In fact, only a small fraction of bacteria can be studied because the growth requirements of most bacteria are still unknown and therefore cannot be reproduced under laboratory conditions. Recent molecular studies targeting the bacterial 16S *r*RNA gene have yielded a more detailed insight into the intestinal microbial

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community composition of poultry (Nava *et al*. 2009; Pourabedin *et al*. 2014; Stanley *et al*. 2014). Caeca contain the majority of the bacteria residing in the intestinal tract of poultry (Rehman *et al*. 2007) and caecal bacteria community structure has been found to be more diverse and complicated than previously reported using culture-based methods. Thus, it has been estimated that just 0·10 of the identified bacterial 16S *r*RNA gene sequences among 640 or more species from 140 genera represent previously known bacterial species, the remaining sequences belonging to new species or even new genera (Apajalahti *et al*. 2004).

Terminal restriction fragment length polymorphism (T-RFLP) analysis has been used previously for examining the chicken intestinal microbiota (Gong *et al*. 2002; Torok *et al*. 2008; Geier *et al*. 2009). The method relies on variations in the position of restriction sites among sequences, and the determination of the length of fluorescently labelled Terminal restriction fragments (T-RFs) by high-resolution gel electrophoresis on automated DNA sequencers. T-RF lengths can be predicted from the known sequences. Thus, the T-RFLP method can theoretically identify the specific organisms in a community based on their T-RF lengths. However, there are many instances where the same T-RF length is predicted for multiple species of bacteria. Therefore, the use of multiple restriction enzymes in the analysis is required if it is intended to increase the specificity of phylogenetic inferences derived from T-RFLP profiles (Dunbar *et al*. 2001).

For several decades, the use of additives such as antibiotics and chemotherapeutics in prophylactic doses has been a way to improve animal welfare and to obtain economic benefits in terms of improved animal performance and reduced medication costs. However, worldwide concern about the development of antimicrobial resistance and about transference of antibiotic resistance genes from animal to human microbiota led to banning the use of antibiotics as growth promoters in the European Union in January 2006 (EC Regulation 1831/2003, European Union 2003). As a consequence, there is a worldwide attempt to reduce the use of antibiotic growth promoters in animal production and to encourage the search for new and safer alternatives. Although not the only way to improve performance, some alternatives include the use of feed additives such as acidifiers, enzymes, prebiotics and probiotics in order to promote animal health by modulating the intestinal microbial community (Gaggìa *et al*. 2010). In the present work, additives were used that had been identified previously as able to increase productive parameters in broilers, and which were also found to modulate the intestinal microbiota composition in broilers (Peinado *et al*. 2013*a*, *b*). Other additives, such as exo-enzymes, are known to modulate bacterial composition as a consequence of improved feed digestibility (Boros *et al*. 1998; Torok *et al*. 2008). However, in most cases, it is difficult to establish whether the changes observed in the microbiota composition were either a cause or a consequence of differences in nutrient digestibility and/or absorption within the intestine. Therefore, ideally the best model would be that in which, at least theoretically, the main effect of dietary manipulation was directed towards the modulation of the microbiota composition, and productive parameters were affected as a consequence of microbiota modulation. For this reason, in the present work additives were chosen which, due to their chemical composition, were likely to affect primarily the intestinal microbiota composition (Ruiz *et al*. 2010; Peinado *et al*. 2013*a*, *b*).

Accordingly, the present work investigated the use of multiple restriction enzymes in T-RFLP analysis to: (i) characterize the chicken intestinal bacterial community; and (ii) tentatively identify intestinal bacterial groups related with increased performance parameters in broilers. The method was applied to the analysis of the composition of caecal microbiota of birds fed control or supplemented diets. The identification of the most relevant bacterial groups linked to broiler productivity is very relevant in order to better understand the relationship between the intestinal microbiota composition and bird physiology. Also, if some of these bacteria could be linked to other parameters such as immune responses, the competitive exclusion of pathogens or production differences, they could be developed as poultry probiotics, which has obvious productive and welfare implications.

MATERIALS AND METHODS

Birds, diets and housing

The additives used in the present study were: (i) inulin, (ii) a di-D-fructose dianhydrides-enriched caramel (FC) and (iii) propyl propane thiosulphonate (PTS-O). Details on the chemical nature of FC and the garlic derivative PTS-O have been reported

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previously (Ortiz Mellet & García Fernández 2010; Ruiz *et al*. 2010). Inulin (92·8%) was from Farmusal (Granada, Spain).

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Spanish Council for Scientific Research (CSIC, Spain), and the animals were cared for in accordance with the Spanish Ministry of Agriculture guidelines RD 1201/2005 (Ministerio de la Presidencia 2005). A total of 240 males, 1-day-old broiler chickens of the Cobb strain were used. Birds were weighed on arrival and raised in wire-floored batteries. Chickens were assigned randomly to one of the five dietary treatments: Control (commercial diet with no additive), inulin (commercial diet supplemented with 20 g inulin/kg diet), FC (commercial diet supplemented with 20 g FC/kg diet), PTS-O-45 (commercial diet supplemented with 45 mg PTS-O/kg diet) and PTS-O-90 (commercial diet supplemented with 90 mg PTS-O/kg diet). Treatments had eight replicates of six birds each. Cages were provided with convenient heating and the birds received a lighting regime of 23 h light: 1 h darkness. Balanced commercial diets (Table 1) free of any feed antibiotics and formulated to match the requirements for growing birds of this age and genotype were used (Peinado *et al*. 2013*a*). Chickens were fed *ad libitum* for 21 days. Productive parameters (feed intake, growth, feed/gain ratio, digestibility and energy utilization) were determined at the end of the experiment as described previously (Peinado *et al*. 2013*a*, *b*). At 21 days of age, a total of 25 birds (five from each dietary treatment) were selected randomly and killed by intra-thoracic injection of the euthanasia solution T61 (0·2 ml per bird) (Laboratorios Intervet SA, Salamanca, Spain). Samples (*c*. 2 g) were collected from the caeca, which were opened with sterile scissors and contents placed in the sterile vials, frozen at –80 °C and freeze-dried (Ruiz & Rubio 2009).

Table 1. *Ingredients and composition (g/kg) of the experimental diet*

Ingredient	Amount in experimental diet (g/kg)
Maize	462
Soy flour	310
Wheat	150
Vit + min mix*	30
Animal fat	20
Calcium carbonate	16·0
Calcium phosphate	3·1
Sodium chloride	4·5
Chromium oxide	2·0
Methionine	2·2
Lysine	0·2
Calculated composition	
Metabolizable energy (MJ/kg)	12·17
Crude protein	193·0
Crude fibre	33·7
Fat	44·6
Calcium	7·1
Phosphorous	6·2
Methionine + cysteine	8·6
Lysine	12·0

*The mineral–vitamin mix contained (per 30 kg): vitamin A, 7 500 000 IU; vitamin D_3, 1 500 000 IU; vitamin E, 25 g; vitamin B_2, 2 g; thyamin B_{12}, 10 mg; vitamin B_6, 67 mg; calcium pantothenate, 7·5 g; nicotinic acid, 10 g; folic acid, 25 mg; vitamin K_3, 1 g; choline chloride, 250 g; Fe, 4 g; Cu, 750 mg; Co, 50 ng; Zn, 38 g; Mn, 42 g; I, 680 mg; Se, 45 mg; Coccidiostate (Nistatin + Nicarbacin), 0·50 kg; BHT, 250 mg.

Terminal restriction fragment length polymorphism analysis

Lyophilized caecal samples were transferred into 2 ml bead beater vials containing three beads of Zirconia and mixed by physical disruption for 1 min in a Mini-Bead Beater (BioSpec Products, Bartlesville, UK) to obtain a small-size particle and facilitate subsequent DNA extraction. Total DNA was extracted from 40 mg of the caecal samples using a QIAamp DNA stool kit (Qiagen, West Sussex, UK), following the manufacturer's instructions and as described previously (Ruiz & Rubio 2009; Peinado *et al*. 2013*a*, *b*).

Conditions for DNA amplification and digestion were standardized as follows: a 1497-base pair (bp) fragment of the 16S *r*RNA gene was amplified using a 6-carboxyfluorescein-labelled forward primer [27F-FAM (5′–6-FAM-AGAGTTTGATCMTGGCTCAG-3′)] and a reverse primer [1541R (5′-AAGGAGGTGATCCAGCCGCA-3′)] (Huang *et al*. 2009). The same fragment was amplified using a hexachloro-fluorescein-labelled forward primer [27F-HEX (5′-HEX-AGAGTTTGATCMTGGCTCAG-3′)] and a reverse primer [1541R (5′-AAGGAGGTGATCCAGCCGCA-3′)]. Polymerase chain reactions (PCRs) were done in 50 μl volumes containing 1 × PCR buffer (Dominion-MBL, Córdoba, Spain), 1·5 mM magnesium chloride ($MgCl_2$), 0·2 μM of each primer

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(Roche, Mannheim, Germany), 0·2 mM of each deoxynucleoside triphosphate (Dominion-MBL, Córdoba, Spain), 1·0 U Taq DNA polymerase (Dominion-MBL, Córdoba, Spain) and 50 ng of total nucleic acid template. All PCRs were done in triplicate and run in a $P \times 2$ Thermal Cycler (Thermo Electron Corporation, Waltham, MA, USA) with the following amplification conditions: 1 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 57 °C and 45 s at 72 °C; and 10 min at 72 °C (Højberg *et al*. 2005). The PCR products of the replicates were pooled and 25 μl of each product was verified by electrophoresis on 1% agarose gels. The fluorescent-labelled 1497 bp product was purified with the kit NucleoSpin™ Extract II (Macherey-Nagel, Düren, Germany) and eluted in a final volume of 25 μl of Milli-Q water (Millipore, Billerica, MA, USA). The DNA concentration of the resultant PCR product was quantified with a NanoDrop ND-100 Spectrophotometer. The PCR products (80 ng) labelled with FAM were digested for 4 h at 37 °C with 10 U of one of the restriction enzymes AluI or HhaI (New England Biolabs, Ipswich, MA, USA) in 20 μl reaction mixtures in the recommended enzyme buffer. Also, PCR products (80 ng) labelled with HEX were digested 4 h at 37 °C with 10 U of one of the restriction enzymes MspI or RsaI (New England Biolabs, Ipswich, MA, USA) in 20 μl reaction mixtures in the recommended enzyme buffer. After digestion, samples were stored at –20 °C.

For each sample, the products obtained after digestion with AluI enzyme (labelled with FAM) and those obtained after digestion with MspI enzyme (labelled with HEX) were pooled, purified with the MinElute Reaction Cleanup kit (QIAGEN, West Sussex, UK), and eluted in a final volume of 15 μl of Milli-Q water. The same process was followed with the products obtained after digestion with HhaI enzyme (labelled with FAM) and those obtained after the digestion with RsaI enzyme (labelled with HEX). This purification step was introduced to desalinate the digests and increase the detection of T-RF peaks. Direct exposure of labelled DNA to light was avoided in order to minimize the loss of fluorescence intensity.

Fragment analysis was performed on an ABI 3130xl capillary electrophoresis analyser using a 50 cm long capillary array and POP-7™ polymer (Applied Biosystems, Alcobendas, Spain). Briefly, a sample volume of 2 μl containing *c*. 10 ng of DNA (5 ng of each restriction reaction) was mixed with 0·1 μl of GeneScanTM LIZ-600® Size Standard (Applied Biosystems) and 10·4 μl of Hi–Di formamide (Applied Biosystems). GeneScanTM LIZ-600 was used because it is a relatively wide-range standard, and although a standard with an even wider range exists (LIZ1200), it is quite difficult to use: the equipment's electropherogram parameters have to be adjusted specifically, and the manufacturers do not advise its use in some cases. Therefore, although all bands were taken into account for the calculation of the relative proportion of each band, only bands with sizes ranging between 20 and 600 bp were used for assigning a band to a defined group(s) of bacteria. Samples were then denaturalized by heating at 85 °C for 2 min and chilled on ice for at least 5 min: DNA fragments were injected at 1·6 kV for 100 s and run at 15 kV for 1 h using the G5 dye set. Electropherograms were visualized and further analysed with the GeneMapper software version 4.0 (Applied Biosystems). The peak detection limit was set at 75 relative fluorescent units (RFU) and common peaks were excluded from the analysis.

Sample data consisted of size (base pairs) and peak area for each TRF. To standardize the DNA loaded on the capillary, the sum of all the TRF peak areas in the pattern was used to normalize the peak detection threshold in each sample. For each enzyme, following the method of Kaplan *et al*. (2001), a new threshold value was obtained by multiplying a pattern's relative DNA ratio (the ratio of total peak area in the pattern to the total peak area in the sample with the smallest total peak area) by the smallest peak area detected by the Genemapper software at the default 75 unit limit. For each sample, peaks with a lower area were deleted from the dataset. The resulting fragments were treated as operational taxonomic units (OTUs), representing particular bacterial species or taxonomically related groups.

Terminal restriction fragment length polymorphism phylogenetic assignment tool

The T-RFLP phylogenetic assignment tool (PAT) can be used to obtain phylogenetic assignments based on data from a number of restriction enzyme digests. To infer the potential bacterial composition in the samples, *in silico* restriction profiles were obtained from the Ribosomal Database Project II website (http://rdp.cme.msu.edu/index.jsp). *In silico* databases were constructed from 16S *r*RNA reads available from the Ribosomal Database Project with the 27F and 1541R primer pair and AluI, MspI, HhaI or RsaI

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restriction enzymes or their combinations. The resulting custom database output files contained 23 256 sequences. Each database was uploaded along with the T-RFLP data onto the T-RFLP PAT interface (https://secure.limnology.wisc.edu/trflp/index.jsp). Data were analysed using default settings as outlined by Kent *et al*. (2003).

Statistical analysis

The OTUs obtained from the caeca of the 25 broiler chickens were analysed using multivariate statistical techniques (PRIMER 6 and PERMANOVA1, PRIMER-E, Ltd., Plymouth, UK). These analyses were used to examine similarities in chicken gut microbial communities, identify OTUs accounting for differences observed in microbial communities, and the relationship between OTUs or bacterial groups and productive parameters. Bray–Curtis measures of similarity (Bray & Curtis 1957) were calculated to examine similarities between gut microbial communities of birds from the T-RFLP-generated (OTU) data matrices, following standardization, and square root transformation. The Bray–Curtis similarity coefficient (Bray & Curtis 1957) is a reliable measure for biological data on community structure, and is not affected by joint absences that are commonly found in microbial data (Clarke 1993).

Analysis of similarity (ANOSIM) (Clarke 1993) was used to test whether gut microbial communities were significantly different between treatments. The *R* statistic value describes the extent of similarity in the ANOSIM, with values close to unity indicating that the groups are entirely separate and a zero value indicating that there is no difference among groups. Analysis of similarity percentages (SIMPER) (Barnes 1979) was performed to determine the overall average similarity in caecal microbial community compositions among birds fed the same diet, and to determine groups driving significant differences in bacterial community compositions between dietary treatments (Barnes 1979; Park *et al*. 2006). Hierarchical cluster analysis (CLUSTER) (Barnes 1979) using the group average cluster mode on Bray–Curtis similarity data was done to show changes in gut microbial communities associated with diet. Principal component analysis (PCA) was used to study the relationship between OTUs or bacterial groups and productive parameters, that is, the most important OTUs or bacterial groups which correlated with productive variables (Torok *et al*. 2008). It has been reported that PCA is not appropriate where data contain many 'zeros' or where observations (species) exceed the total number of samples (Clarke & Warwick 2001), as is usually the case for T-RFLP data. To avoid this limitation, SIMPER analysis was used prior to PCA to select those OTUs responsible for larger dissimilarities, and only those OTUs were used for the PCA analysis.

RESULTS

Productive parameters

Results on productive parameters of birds fed on control diets or diets supplemented with FC or PTS-O have been reported previously (Peinado *et al*. 2013*a*, *b*). Briefly, the final body weight (BW) of birds fed on the FC diet was higher ($P < 0{\cdot}01$) than controls or inulin-fed birds. The feed/gain ratios of birds fed diets PTS-O-45 or PTS-O-90 were significantly ($P < 0{\cdot}05$) lower (better) than controls. Energy, N, fat, dietary fibre and non-starch polysaccharides faecal digestibilities were greater ($P < 0{\cdot}05$) than controls in chickens fed on diets containing FC or PTS-O.

Terminal restriction fragment length polymorphism analysis

The T-RFLP analysis of 16S *r*RNA genes using universal primers for bacteria indicated complex communities in all samples. Analysis of electropherograms revealed that the four enzymes used in the present study generated a different number of T-RFs (55, 39, 43 and 44 for AluI, Hha I, MspI and RsaI respectively with sizes ranging from 20 to 945 bp; and 38, 26, 42 and 33 for AluI, Hha I, MspI and RsaI respectively with sizes ranging from 20 to 600 bp). Due to the internal standard used, only peaks ranging in size from 20 to 600 bp (that is 139 OTUs) could be used to infer the potential bacterial composition of the samples. Accordingly, T-RFs smaller than 20 bp or bigger than 600 bp were discarded in the analysis due to lack of reliability. Among the OTUs obtained with the AluI enzyme, 0·44 were compatible with different identified bacteria, whereas 0·19 and 0·37 were classified into the uncultured and unidentified categories, respectively. Likewise, the proportions obtained with the other enzymes were, respectively, 0·45, 0·39 and 0·16 for HhaI; 0·60, 0·36 and 0·04 for MspI; and 0·41, 0·37 and 0·22 for RsaI (data not shown).

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Multivariate statistical analysis showed that the bacterial community composition was affected significantly ($P < 0{\cdot}05$) by diet, and this was found both with individual enzymes and with their combinations (Table 2). For individual enzymes, the use of RsaI gave place to significant ($P < 0{\cdot}05$) differences among dietary treatments. For enzyme combinations, all combinations of two, three or four enzymes resulted in at least one significant ($P < 0{\cdot}05$) difference. Caecal bacterial populations of birds fed on diets FC and both PTS-O were different from controls for most enzyme combinations, but no significant diet-associated differences in bacterial community composition were detected between Control and Inulin-fed chickens. This is in line with the cluster analysis (Fig. 1), which showed that Control and Inulin-fed birds tended to group together; this was particularly evident when AluI + RsaI or the combination of all four enzymes was used.

As for the global *R* value for differences among diets (Table 3), values were from 0·012 (MspI alone) to 0·321 (RsaI alone). Among combinations of enzymes, AluI + RsaI gave the highest *R* value. This is likely in connection with the number of assignations found for the individual enzymes or their combinations. About double number of phyla assignations were found with AluI or RsaI as compared with HhaI or MspI in all five treatments (see Dataset S1 in the Supplementary Materials: go to http://journals.cambridge.org/AGS). This would imply that digestions with AluI, RsaI, or their combinations, would be more powerful to discriminate differences in microbiota composition. Also, the number of assignations in the caecal contents of chickens fed on diets FC and PTS-O-90 were usually higher than those in Control and Inulin groups, being *Actinobacteria, Firmicutes* and *Proteobacteria* the most represented phyla (Dataset S1 and Fig. S1 in the Supplementary Materials: go to http://journals.cambridge.org/AGS). Within families, *Dermabacteraceae, Intrasporangiaceae, Microbacteriaceae, Micrococcaceae* and *Streptomycetaceae* were the most abundant *Actinobacteria; Clostridiaceae 1, Lachnospiraceae* and *Ruminococcaceae* were the most abundant *Firmicutes*; and *Burkholderiaceae, Rhodocyclaceae* and *Xanthomonadaceae* were the most abundant *Proteobacteria* (Dataset S2 in the Supplementary Materials: go to http://journals.cambridge.org/AGS).

The OTUs contributing to the top 0·50 of dissimilarity in bacterial community composition between diets were identified within the caeca using the four restriction enzymes individually or in combination (Tables 4 and 5). A total of 20 OTUs of sizes between 20 and 600 bp, out of the 139 total OTUs obtained, were identified as good discriminators between diets, most of them common in the individual and combined analysis.

As indicated above, among combinations of enzymes, AluI + RsaI gave the higher *R* value (Table 3). The *in silico* database constructed from 16S *r*RNA reads available at the Ribosomal Database Project with the 27F and 1541R primer pair and AluI + RsaI restriction enzymes was uploaded along with the current T-RFLP data onto the T-RFLP PAT interface (https://secure.limnology.wisc.edu/trflp/index.jsp) in order to infer the potential bacterial composition in the samples. Genera and families contributing to the top 0·90 of dissimilarity in bacterial community composition between diets were identified within the caeca using the combination of AluI + RsaI. It was found that genera corresponding to families *Clostridiaceae 1, Streptomycetaceae, Lachnospiraceae, Ruminococcaceae, Micrococcaceae* and *Coriobacteriaceae* were those responsible for most of the dissimilarity among treatments (Table 6).

The PCA analysis was finally done with the OTU distribution for each individual enzyme combined with productive parameters (final BW, feed/gain ratio, feed intake, and energy, N, fat, fibre and non-starch polysaccharides faecal digestibilities). The OTUs with higher scores with respect to productive parameters with each enzyme were obtained and compared with the SIMPER analysis of OTUs contributing to the larger dissimilarity. A number of OTUs which were coincident in both analyses were selected. Those OTUs were 464–465 RsaI, 184–186 HhaI, 452–455 RsaI, 199–203 MspI, 284–286 MspI, 235–239 AluI, 185 RsaI, 479–480 MspI, 74–78 MspI, 470–473 AluI, 253–255 AluI and 253–255 MspI. Therefore, these OTUs were identified as those which contributed most to dissimilarity among treatments, and at the same time with higher scores with productive parameters. Accordingly, the assignations for each of them were obtained by using the corresponding database, and the families with higher number of OTUs were obtained. Only families present in the broiler intestinal microbiota were considered. Those families were identified according to those included in chicken databases (http://chickenmicrobiome.xbase.ac.uk/index.php), and also those obtained by pyrosequencing in studies by the current group of authors (data not shown). Those

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Table 2. *One-way ANOSIM of caecal microbial communities as analysed with the four restriction enzymes or their combinations*

	Control	Inulin	FC	PTS-O-45	PTS-O-90
AluI					
Control	0	0·5352	0·0845	0·4606	0·2481
Inulin		0	0·0445*	0·3609	0·512
FC			0	0·2456	0·8126
PTS-O-45				0	0·7045
PTS-O-90					0
MspI					
Control	0	0·2776	0·0598	0·3391	0·5099
Inulin		0	0·6585	0·8737	0·9445
FC			0	0·4897	0·3376
PTS-O-45				0	0·4252
PTS-O-90					0
HhaI					
Control	0	0·4818	0·0664	0·312	0·0077*
Inulin		0	0·0895	0·4389	0·0562
FC			0	0·69	0·8112
PTS-O-45				0	0·4226
PTS-O-90					0
RsaI					
Control	0	0·1968	0·0072*	0·0092*	0·025*
Inulin		0	0·0157*	0·1477	0·3141
FC			0	0·6367	0·4033
PTS-O-45				0	0·5683
PTS-O-90					0
AluI + MspI					
Control	0	0·4233	0·0206*	0·4727	0·3768
Inulin		0	0·0759	0·4226	0·7623
FC			0	0·1745	0·7759
PTSO-45				0	0·6971
PTSO-90					0
AluI + HhaI					
Control	0	0·424	0·0633	0·3123	0·0073*
Inulin		0	0·0859	0·4452	0·0515
FC			0	0·4202	0·9404
PTS-O-45				0	0·3837
PTS-O-90					0
AluI + RsaI					
Control	0	0·17	0·0062*	0·0244*	0·0238*
Inulin		0	0·0074*	0·3057	0·256
FC			0	0·2638	0·6185
PTS-O-45				0	0·6417
PTS-O-90					0
MspI + HhaI					
Control	0	0·4914	0·0633	0·3481	0·0073*
Inulin		0	0·1969	0·503	0·0583
FC			0	0·499	0·8911
PTS-O-45				0	0·342
PTS-O-90					0
MspI + RsaI					
Control	0	0·0922	0·0062*	0·0248*	0·0238*

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Table 2. (*Cont.*)

	Control	Inulin	FC	PTS-O-45	PTS-O-90
Inulin		0	0·033*	0·2301	0·3538
FC			0	0·5681	0·3031
PTS-O-45				0	0·6941
PTS-O-90					0
HhaI + RsaI					
Control	0	0·3938	0·0062*	0·0244*	0·0073*
Inulin		0	0·1023	0·414	0·0741
FC			0	0·6933	0·7564
PTS-O-45				0	0·4044
PTS-O-90					0
AluI + MspI + HhaI					
Control	0	0·4369	0·0643	0·3215	0·0082*
Inulin		0	0·0861	0·6253	0·0608
FC			0	0·3722	0·9599
PTS-O-45				0	0·5667
PTS-O-90					0
AluI + MspI + RsaI					
Control	0	0·2239	0·0062*	0·0503	0·0238*
Inulin		0	0·0157*	0·3781	0·3718
FC			0	0·2409	0·571
PTS-O-45				0	0·7824
PTS-O-90					0
MspI + HhaI + RsaI					
Control	0	0·3046	0·0062*	0·0322*	0·0073*
Inulin		0	0·0925	0·4054	0·0741
FC			0	0·4513	0·7909
PTS-O-45				0	0·5319
PTS-O-90					0
AluI + MspI + HhaI + RsaI					
Control	0	0·322	0·0078*	0·0318*	0·0072*
Inulin		0	0·0426*	0·5769	0·0449*
FC			0	0·2325	0·8013
PTS-O-45				0	0·6216
PTS-O-90					0

* *P*-values <0·05 were considered significant.

families were *Ruminococcaceae* (eight different OTUs), *Lachnospiraceae* (six different OTUs), *Caldilineaceae* (four different OTUs), *Clostridiaceae 1* (four different OTUs), *Comamonadaceae* (four different OTUs), *Micrococcaceae* (four different OTUs) and *Thermomicrobiaceae* (four different OTUs). Interestingly, among those, *Clostridiaceae 1, Lachnospiraceae, Ruminococcaceae* and *Micrococcaceae* were coincident with the results of the SIMPER analysis of OTUs obtained with the AluI + RsaI combination (Table 6, see above). Accordingly, it can be concluded that those families are among those most likely implicated in defining the microbiota composition of growing broiler chickens, and also those more closely related with differences in productive parameters.

DISCUSSION

Microbial profiling techniques based on T-RFLP analysis have been developed to characterize a bacterial community in a single assay (Chee *et al.* 2010). Although T-RFLP is not a technique for the absolute characterization and identification of microbial communities, it enables a 'snap-shot' view of the complex bacterial population at any

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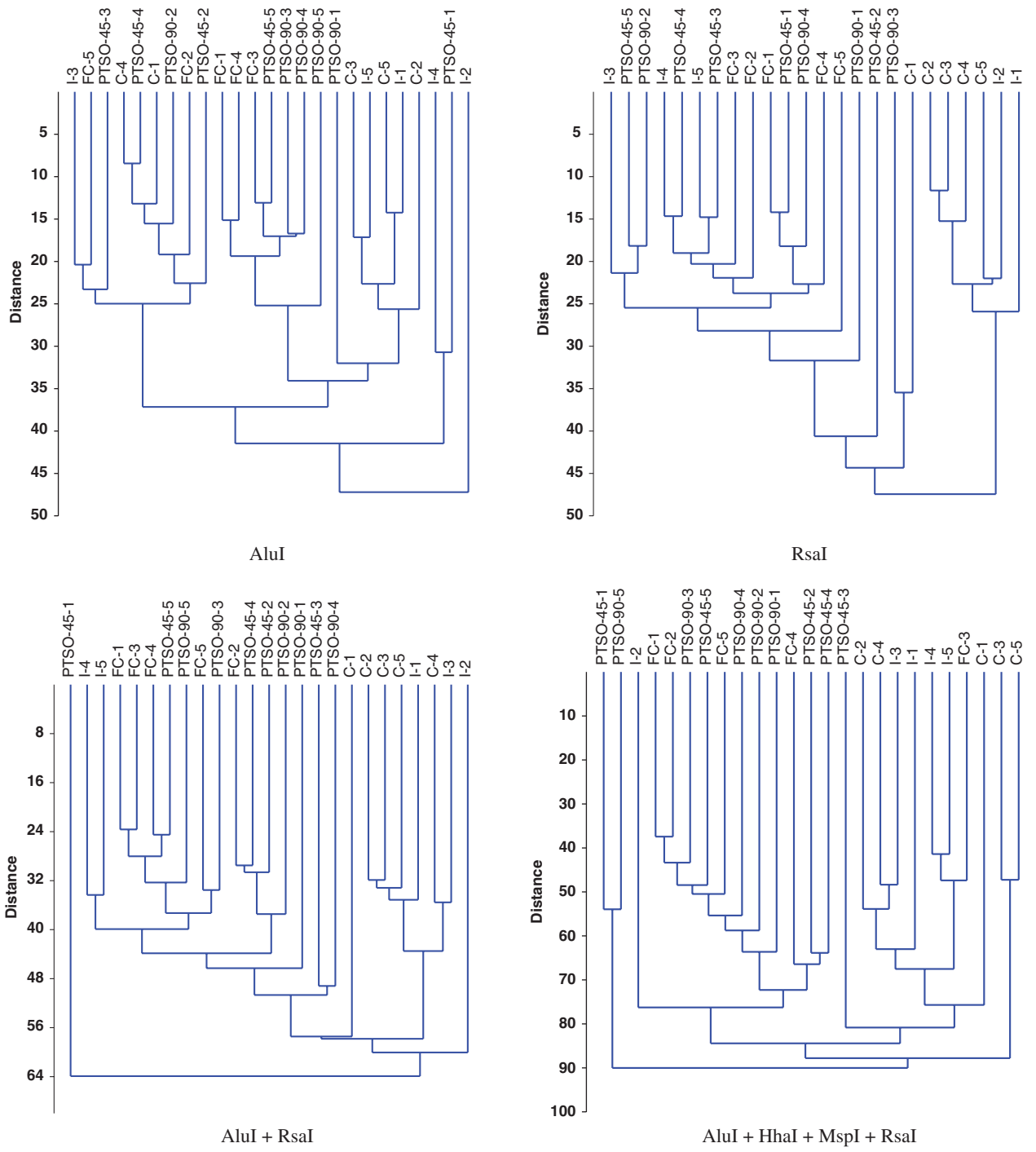

Fig. 1. Cluster analysis based on assignations in the different phyla for individual enzymes or their combinations C1–C5, control (commercial diet with no additive); I1–I5, inulin (commercial diet supplemented with 20 g inulin/kg diet); FC1–FC5, fructose caramel (commercial diet supplemented with 20 g FC/kg diet); PTS-O-45-1-PTS-O-45-5, PTS-O-45 (commercial diet supplemented with 45 mg of propyl propane thiosulphonate/kg diet); PTS-O-90-1-PTS-O-90-5 and PTS-O-90 (commercial diet supplemented with 90 mg of propyl propane thiosulphonate/kg diet).

particular time, making it ideal for comparative analysis. In addition, using T-RFLP analysis in combination with multivariate statistical techniques, OTUs representing particular bacterial species or taxonomically related groups characteristic of dietary treatments can be identified and related to bird performance parameters (Torok *et al*. 2008). The present work investigated the use

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Table 3. *P-values and* R *statistic calculated from one-way ANOSIM of caecal microbial communities as analysed with the four restriction enzymes or their combinations*

	P-value	*R* statistic*
AluI	0·213	0·052
MspI	0·386	0·012
HhaI	0·058	0·146
RsaI	0·011	0·321
AluI + MspI	0·190	0·048
AluI + HhaI	0·043	0·141
AluI + RsaI	0·001	0·255
MspI + HhaI	0·052	0·130
MspI + RsaI	0·002	0·233
HhaI + RsaI	0·004	0·239
AluI + MspI + HhaI	0·047	0·125
AluI + MspI + RsaI	0·002	0·212
MspI + HhaI + RsaI	0·004	0·228
AluI + MspI + HhaI + RsaI	0·003	0·225

*The *R* statistic and *P*-values are shown for comparisons between restriction enzymes or their combinations.

Table 4. *OTU contribution to the dissimilarity in caecal microbial communities associated with diet as determined with four different restriction enzymes individually*

out	Average dissimilarity	Individual contribution (%)	Cumulative contribution (%)
AluI			
495–497	7·007	13·56	13·56
213–214	5·711	11·05	24·61
235–239	3·646	7·055	31·66
470–473	3·036	5·875	37·54
253–255	2·917	5·644	43·18
297–298	2·837	5·489	48·67
HhaI			
184–186	6·925	15·55	15·55
22–25	6·603	14·83	30·38
389–391	5·441	12·22	42·6
576–577	3·702	8·314	50·92
MspI			
562–563	5·101	10·39	10·39
308–311	4·617	9·401	19·79
199–203	3·075	6·261	26·05
67–69	3·044	6·197	32·25
241–243	2·821	5·744	37·99
284–286	2·641	5·377	43·37
479–480	2·115	4·307	47·67
RsaI			
464–465	12·16	22·3	22·3
460–463	10·74	19·68	41·98
452–455	4·389	8·046	50·02

of four multiple restriction enzymes, namely AluI, HhaI, MspI and RsaI, in T-RFLP analysis to: (i) characterize the chicken intestinal bacterial community; and (ii) tentatively identify intestinal bacterial groups related with increased performance parameters in broilers.

Most studies of intestinal microbiota composition by T-RFLP are based on the use of only one restriction endonuclease (Chee *et al*. 2010; Jozefiak *et al*. 2010; Viveros *et al*. 2011). However, single T-RFs obtained with one enzyme may represent more than one organism, so the identification has been, in most cases, only at higher taxonomic level (family or even order). The use of multiple restriction enzymes in the analysis is required if it is intended to increase the specificity of phylogenetic inferences derived from T-RFLP profiles (Dunbar *et al*. 2001). The combination of fingerprints obtained with the four restriction endonuclease digests in the current work enabled a more precise, yet still tentative identification of the fragments. Analysis of electropherograms revealed that the four enzymes used (AluI, HhaI, MspI and RsaI) generated a different number of T-RFs, with a total of 139T-RFs (OTUs) of sizes between 20 and 600 bp. On the other hand, similarity in microbiota composition within the caeca of birds on a given diet has been reported to be low (Torok *et al*. 2008), ranging from 0·42 to 0·48, and this variation could have been masked if samples had been pooled. However, the high-throughput nature of T-RFLP makes it very appropriate for the individual analysis of a large number of samples per treatment. The present study used 25 individually treated caecal samples corresponding to chickens which were distributed in five treatments with five birds per treatment.

Significant differences between treatments, as determined by *P*-values, were greater for RsaI, followed by HhaI, AluI and MspI. Analysis of similarity (*R* statistic) also revealed that RsaI gave place to the higher dissimilarity, followed by HhaI, AluI and MspI. As for combinations of two enzymes, AluI + RsaI resulted in the lowest *P*- values and highest *R* statistic. Combinations of three or even four enzymes did not result in better *P*- or *R* values compared with the combinations of two enzymes. That would suggest that RsaI would be the best choice for studying microbiota composition differences among dietary treatments in chickens, at least at the caecal level, if only one

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Table 5. *OTU contribution to the dissimilarity in caecal microbial communities associated with diet as determined with the combination of four different restriction enzymes*

out	Average dissimilarity	Individual contribution (%)	Cumulative contribution (%)
464–465 RsaI	3·205	6·111	6·111
460–463 RsaI	2·71	5·167	11·28
562–563 MspI	1·842	3·511	14·79
495–497 AluI	1·677	3·197	17·99
308–311 MspI	1·58	3·012	21
184–186 HhaI	1·504	2·867	23·86
213–214 AluI	1·478	2·817	26·68
22–25 HhaI	1·31	2·497	29·18
452–455 RsaI	1·163	2·217	31·4
199–203 MspI	1·11	2·115	33·51
67–69 MspI	1·07	2·04	35·55
389–391 HhaI	1·04	1·983	37·53
241–243 MspI	0·998	1·902	39·44
284–286 MspI	0·976	1·86	41·3
235–239 AluI	0·851	1·622	42·92
185 RsaI	0·848	1·616	44·53
449–450 RsaI	0·839	1·599	46·13
479–480 MspI	0·814	1·552	47·68
389–392 RsaI	0·756	1·442	49·13
74–78 MspI	0·754	1·438	50·56

enzyme was to be used. AluI + RsaI, MspI + RsaI or HhaI + RsaI would be the best combinations of two enzymes, and would improve discrimination between treatments. Results confirmed those conclusions, as ANOSIM significant differences among dietary treatments were best found with RsaI alone or the combinations AluI + RsaI, MspI + RsaI and HhaI + RsaI. This is confirmed by the fact that AluI, RsaI, AluI + RsaI and MspI + RsaI were those enzymes or combinations which gave the highest number of assignations in the different phyla and families for individual enzymes or their combinations (Dataset S1 and S2 in the Supplementary Material at http://journals.cambridge.org/AGS). However, despite variations in the specific ability of different enzyme combinations to show significant effects of the diet in the present experimental conditions, it does not necessarily mean that those most specific are also the most representative for this particular ecosystem. Inulin supplementation did not result in significant differences in microbiota composition, compared with controls, with any of the enzymes or their combinations. This is in agreement with the cluster diagrams, where the samples coming from chickens fed on the inulin diet grouped mostly with those coming from the control birds. Compared with controls, differences in productive parameters were also more significant for FC and PTS-O fed birds than they were for inulin fed birds (Peinado *et al*. 2013*a*, *b*). Indeed, data on the influence of dietary inulin on the caecal microbiota composition of broilers are scarce and contradictory. In fact, Rehman *et al*. (2008) found that inulin altered the caecal microbial metabolic activity without any major impact on the composition of intestinal bacterial communities measured by denaturing gradient gel electrophoresis. However, the present results confirmed previous reports on the effectiveness of PTS-O and FC to modulate intestinal microbiota composition in broilers (Peinado *et al*. 2013*a*, *b*).

The microbiota resident in the gastrointestinal tract plays key roles in the normal nutritional, physiological, immunological and protective functions of the host (Vispo & Karasov 1997; Frick & Autenrieth 2013), and also in the efficiency of nutrient uptake and utilization (Frick & Autenrieth 2013). It is possible that the growth of beneficial bacteria or the suppression of detrimental bacterial species, or both, may be at least partly responsible for the improved productivity in broiler chickens fed on diets supplemented with exogenous enzymes or antibiotics (Torok *et al*. 2008, 2011*a*, *b*). With the four enzymes used in the present study, either individually or in combination, 20 OTUs

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Table 6. *Genus (including groups* Clostridium XIVa *and* Clostridium IV*) and family contribution to the dissimilarity in caecal microbial communities associated with diet as determined with the combination of AluI + RsaI*

	Average dissimilarity	Individual contribution (%)	Cumulative contribution (%)
Genus			
Clostridium sensu stricto	18·82	39·62	39·62
Streptomyces	7·74	16·3	55·92
Clostridium XlVa	3·55	7·47	63·38
Blautia	2·25	4·73	68·11
Arthrobacter	1·80	3·78	71·89
Acetivibrio	1·32	2·77	74·66
Ruminococcus	1·15	2·41	7·07
Desulfivibrio	0·94	1·98	79·05
Euzebya	0·93	1·96	81·02
Butyrivibrio	0·80	1·69	82·71
Robinsoniella	0·80	1·68	84·39
Cryptobacterium	0·66	1·39	85·78
Bifidobacterium	0·65	1·36	87·14
Corynebacterium	0·64	1·35	88·49
Butyricicoccus	0·60	1·26	89·76
Clostridium IV	0·59	1·24	91·00
Family			
Clostridiaceae 1	19·2	43·49	43·49
Streptomycetaceae	7·83	17·74	61·22
Lachnospiraceae	7·00	15·85	77·07
Ruminococcaceae	2·50	5·66	82·73
Micrococcaceae	1·84	4·18	86·91
Coriobacteriaceae	1·38	3·11	90·02

were identified which were responsible for 0·50 of the dissimilarity among treatments. The identity of OTUs can be predicted from *in silico* PCR amplification and restriction of 16S *r*RNA sequences found in a public database (http://mica.ibest.uidaho.edu). However, it is known that a limitation of T-RFLP analysis is the inability to reliably assign OTUs to phylogenetic groups because one single OTU may be related to a number of different bacterial groups. This limitation can be in part addressed if a combination of two or more restriction enzymes are used because coincidence of OTUs restricts the possibilities of assignation to a given bacterial group (Dataset S1 and S2 in the Supplementary Material: go to http://journals.cambridge.org/AGS). Also, a novel strategy has been developed (Widmer *et al.* 2006) based on the targeted isolation and cloning of T-RFs of interest. The determination of the bacterial sequence information of interest will then be obtained, and this may allow the development of dietary strategies to induce desirable changes in the chickens' gut microbiota for enhancement of growth and productivity.

Individual OTUs contributing to larger dissimilarity by SIMPER were then analysed by PCA to identify those mainly related to productive parameters, and assignations were then obtained from PAT. On the other hand, genus and family assignations were also obtained for the OTUs coming from the combination AluI + RsaI. Families *Clostridiaceae 1, Lachnospiraceae, Ruminococcaceae* and *Micrococcaceae* were coincident in the analysis of individual OTUs and the combination AluI + RsaI, which suggests that those families are among those most likely implicated in defining the microbiota composition of growing broiler chickens, and also those more closely related with differences in productive parameters. As for the genera contributing most to dissimilarity, these were *Clostridium sensu stricto* spp. (family *Clostridiaceae* 1), *Streptomyces* spp. (family *Streptomycetaceae*), *Clostridium XlVa* spp. (family *Lachnospiraceae*), *Blautia* spp. (family *Lachnospiraceae*), *Arthrobacter* spp. (family *Micrococcaceae*), *Acetivibrio* spp. (family *Ruminococcaceae*) and *Ruminococcus* spp. (family *Ruminococcaceae*). All these genera belong

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to phyla *Actinobacteria* and *Firmicutes* which, together with *Proteobacteria*, were those most represented in the caecal bacterial contents (dataset S1 and Fig. S1 in the Supplementary material: go to http://journals.cambridge.org/AGS) (Lu *et al*. 2003*a*). These results are in line with differences in the $\log_{10}$ number of copies in the intestinal content of birds fed on diets containing PTS-O and FC which were previously (Peinado *et al*. 2013*a*, *b*) found by using qPCR for *Lactobacilli* spp. (family *Lactobacillae*, phylum *Firmicutes*), *C. coccoides* (now *Blautia* spp)-*E. rectale* (family *Lachnospiraceae*, phylum *Firmicutes), C. leptum* (family *Ruminococcaceae*, phylum *Firmicutes*), *Enterobacteria* and *Escherichia – Shigella* (family *Enterobacteriaceae*, phylum *Proteobacteria*). However, it is important to underline that the statistical analysis performed here only gives information on the statistical relationships between variables, but it cannot be concluded from it that a given bacterial group(s) is (are) responsible for differences in any given functional effect.

It is also important to bear in mind at this point that several gut bacteria isolated from chickens have already been shown to have various important biochemical properties. Thus, *Clostridium perfringens, Enterococcus faecium, Streptococcus bovis* and *Bacteroides* spp. were shown to have polysaccharide-degrading activity against the non-starch polysaccharides found in grain (Beckmann *et al*. 2006). Therefore, not only the bacterial species, but also the bacterial genes present within the gut are likely to be implicated in the explanation of putative effects on functional parameters such as immune function, digestibility of nutrients, etc. In any case, all the species referred above have been previously demonstrated to exhibit potentially beneficial activities. Thus, *Streptomyces* spp. have been included at least in three patents recommended as feed supplements for broilers due to their carbohydrase (patent no. 99-073458/07 Finnfeeds Int. Ltd.), or antibiotic activity against potential pathogens (patent no. 99-325920/27 Biotech Resources, and patent no. 2001-327455/34 Pharmacia & Upjohn Co.). *Clostridium XIVa* spp. include, among other species, the previously classified *Clostridium coccoides* (now *Blautia* spp.) which is considered a butyrate-producing bacterium; butyrate promotes gut health through cell proliferation and colonic cell turnover (Gong *et al*. 2002). Increased *C. coccoides* counts in the crop content and improved performance were found in chickens fed diets supplemented with FC (Peinado *et al*. 2013*b*). *Arthrobacter* spp. has been described to be involved in the degradation of wood and cycling of nitrogen and sulphur (Thayer 1976; Lu *et al*. 2003*b*). *Acetivibrio* spp., in particular *A. cellulolyticus*, have been distinguished by their ability to degrade cellulose (Patel *et al*. 1980). Finally, *Ruminococcaceae* have been previously identified as some of the potential performance-related phylotypes (Widmer *et al*. 2006), was included into the group of higher rate of colonization groups in broilers (Yin *et al*. 2010), and its bacteriocin albusin B has been shown to improve BW, modulate lipid metabolism, activate systemic antioxidant defence, elevate the caecal *Lactobacillus* spp. counts and affect the immune system function in broilers (Wang *et al*. 2011; Chen *et al*. 2013).

In conclusion, the study of complex microbial communities by T-RFLP using multiple restriction enzymes does increase the specificity of phylogenetic inferences derived from T-RFLP profiles. The method described in the present paper allows the use of multiple restriction enzymes in a time and cost saving way. Except for the inulin-supplemented diet, multivariate statistical analysis showed that the caecal bacterial community composition was significantly different among diets, as shown by T-RFLP analysis based on individual enzymes or their combinations. Individual RsaI and the combination AluI + RsaI proved to be the most useful to discriminate between dietary treatments. *Clostridiaceae 1, Lachnospiraceae, Ruminococcaceae* and *Micrococcaceae* were tentatively identified as those families most likely implicated in defining the caecal microbiota composition of growing broiler chickens, and also as those most closely related with differences in productive parameters. Pyrosequencing analysis is currently in course for the sequence identification of bacterial groups linked with performance parameters.

SUPPLEMENTARY MATERIAL

The supplementary material for this paper can be found at http://journals.cambridge.org/AGS

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