Cecal drop reflects the chickens' cecal microbiome, fecal drop does not

J. Pauwels, B. Taminiau, G.P.J. Janssens, M. De Beenhouwer, L. Delhalle, G. Daube, F. Coopman

PII: S0167-7012(15)30039-7
DOI: doi: 10.1016/j.mimet.2015.08.006
Reference: MIMET 4711

To appear in: *Journal of Microbiological Methods*

Received date: 24 June 2015
Revised date: 6 August 2015
Accepted date: 6 August 2015

Please cite this article as: Pauwels, J., Taminiau, B., Janssens, G.P.J., De Beenhouwer, M., Delhalle, L., Daube, G., Coopman, F., Cecal drop reflects the chickens' cecal microbiome, fecal drop does not, *Journal of Microbiological Methods* (2015), doi: 10.1016/j.mimet.2015.08.006

REVISED

Cecal drop reflects the chickens' cecal microbiome, fecal drop does not

J. Pauwels[1], B. Taminiau[2], GPJ. Janssens[1], M. De Beenhouwer[3], L. Delhalle[2,4], G. Daube[2] and F. Coopman[5*]

[1] Laboratory of Animal Nutrition, Faculty of Veterinary Medicine, Ghent University, Heidestraat 19, B-9820, Merelbeke, Belgium

[2] FARAH, Department of food sciences, Faculty of Veterinary Medecine, Liège University, B43b, Quatier Vallée 2, avenue de Cureghem 10, B-4000 Liège, Belgium.

[3] Plant Conservation and Population Biology, Biology Department, KU Leuven, Kasteelpark Arenberg 31, B 3001 Heverlee, Belgium

[4] Quality Partner S.A., Rue Hayeneux 19, B-4040 Herstal, Belgium.

[5] Department of Applied Biosciences, Faculty of Biosciences Engineering, Ghent University, V. Vaerwijckweg 1, B-9000 Gent, Belgium

[*] Corresponding author: Frank.Coopman@UGent.be

Highlights

- No differences were found between cecal drop and cecal content in terms of both bacterial diversity and community composition.
- Bacterial diversity in fecal drop differed from that in cecal content.
- The changes in microbiota in fecal drop differed from the changes in microbiota in cecal content when diet and breed variations were introduced.
- Cecal drop can be used to map cecal microbiota which will reduce the sample size in longitudinal studies.

Abstract

Microbiota in the gastro-intestinal tract are closely related to both the intestinal and overall health of the host. Experimental chickens have always been euthanized in order to identify and quantify the bacteria in cecal content. In this study, quantification and identification of the microbial populations in cecal drop, cecal content and fecal drop samples from chickens showed that cecal drop contains a bacterial community that is very similar (concerning bacterial diversity, richness and species composition) to cecal content, as opposed to the bacterial community found in fecal drop. Cecal drop analysis thus allows for longitudinal experiments on chickens' cecal bacteria. The varying results in the analysis of fecal samples questions the method's reliability in reflecting the true cecal microbiota in chickens.

Keywords

Cecal drop, fecal drop, cecal content, cecal microbiota

Introduction

In the chickens' gastro intestinal tract (GIT) the number and variety of bacteria is highest in the ceca (10^{10} – 10^{11} cells/g) (Barnes et al., 1972; Bjerrum et al., 2006). The cecal microbiome plays an important role in fermentation processes and production of short chain fatty acids (SCFA) (Patterson and Burkholder, 2003; Rehman et al., 2007). Also, the cecum can host pathogenic and zoonotic bacteria that cause severe risks to human health (Herman et al., 2003; Zorman et al., 2006).

Chicken ceca are known to have a complex motility. Several times a day the ceca contract, pushing their content in two directions: towards the ileum and towards the cloaca, excreting a cecal drop (Herrick and Edgar, 1947; Clench, 1999; Janssen et al., 2009). The ceca fill again by use of peristaltic and antiperistaltic contractions at their entrances (Fenna and Boag, 1974). Cecal drops have been studied in chickens before and they were used to detect hazardous bacteria regarding food-safety such as *Campylobacter* or *Salmonella* (Herman et al., 2003; Okamura et al., 2008). An early study (Stern and Robach, 1992) compared three samples: cecal drop, fecal drop and cloacal swab and found cecal drop to be the most sensitive sample for the detection of *Campylobacter*. However, when identifying or quantifying the complete microbiota of the cecum, cecal drop has, to the best of our knowledge, never been used.

In studies with other animal species, the microbiota in one or more parts of the gastro-intestinal tract have been investigated through, for example, excreta, fecal or fistula samples (Harmoinen et al., 2001; De Filippo et al., 2010; Budding et al., 2014). In rabbits, bacteria in the caecotrophes were shown by denaturing gradient gel electrophoresis to be 71% similar to cecal microbiota (Rodriguez-Romero et al., 2009). So far, studies on the entire cecal microbiota of chickens have always been based on samples straight from the cecum, for

which chickens had to be euthanized (Bjerrum et al., 2006; Saengkerdsub et al., 2007; Danzeisen et al., 2011).

From a statistical point of view, the required sample size in longitudinal studies will decrease if cecal drop is used, this is because the same birds can be re-sampled for every point in time and differences between individuals will therefore be ruled out. Moreover, from an ethical point of view, the chickens will not need to be euthanized. The aim of this study is to compare the microbiome of three different samples: cecal content, cecal drop and fecal drop and to determine whether these samples can be used as a reference for cecal content and, if so, which of these drops serves as the most effective reference.

Experimental procedures

Experimental setup

Two hundred and forty male chicks, sixty from each of four different breeds (Cobb 500, Cobb-Sasso 175, Sasso and Sussex) were placed in pens of fifteen birds each, with breeds randomly designated to pens. The chicks received full vaccinations for Newcastle disease, infectious bronchitis, coccidiosis, Gumboro disease and Marek's disease.

Diets

Two groups of each breed were fed a standard commercial diet and the other two groups were fed an alternate diet containing mealworms (*Tenebrio molitor*), lucerne and ostrich pellets. Both diets were analyzed for dry matter, crude ash, ether extract, crude fibre, neutral detergent fibre (NDF), acid detergent fibre (ADF) and crude protein (Table 1). Metabolizable energy (ME) was calculated according to Wiseman (Wiseman, 1987). Dry matter and crude ash content were determined by drying the feed to a constant weight at 103°C and combustion at 550°C, respectively. Diethyl ether extract was analyzed using the Soxhlet method (ISO, 1973). Crude fibre was determined using the Association of Official Analytical methods (Association of Official Analytical methods (AOAC), 1995a; Association of official analytical chemists (AOAC), 1995b). To determine NDF and ADF, the methods of Van Soest *et al*. (Van Soest et al., 1991) were used. Crude protein (6.25 × Nitrogen) was determined using the Kjeldahl method (ISO 5983-1, 2005). Water and feed were provided ad libitum. To prevent diarrhea, the chicks fed the alternate diet received a mix of 1/3 alternate diet and 2/3 commercial diet between day 0 and 5. From day 6 to 10 they were fed a mix of 2/3 alternate diet and 1/3 commercial diet. From day 11 on, they were fed the alternate diet only.

Tabel 1: Nutrient composition of the test diets.

	Commercial diet	Alternate diet
Dry Matter (g/ kg OM)	902	911
Crude Ash (g/ kg DM)	56	72
Ether Extract (g/ kg DM)	73	43
Crude Fibre (g/ kg DM)	36	130
Acid Detergent Fibre (g/ kg DM)	13	18
Neutral Detergent Fibre (g/ kg DM)	67	68
Crude Protein (g/ kg DM)	215	187
Metabolizable Energy (MJ)	15	10

OM: Organic Matter, DM: Dry Matter. Metabolizable Energy was calculated according to Wiseman (Wiseman, 1987). All other nutrients were analyzed.

Sampling

Since the chickens were from four different breeds, their growth rates varied. The weekly bodyweight per pen was used to compose a Gompertz curve (GraphPad Prism 5, GraphPad software, USA). Based on the inflection point of this curve, a prediction could be made concerning the point in time that the chickens would achieve their maximum growth rate. In this way chickens were compared at the same physiological age. At the point of maximal growth for a particular breed-diet combination, the chickens from that combination were observed closely. The first cecal drop excreted was taken as a sample using sterile aliquots and stored in liquid nitrogen. Later (a maximum of 14 minutes), a sample of a freshly excreted fecal drop from the same chicken, was obtained in the same way. Fecal drop was collected from all but two chickens in the designated timeframe. Directly after the collection of both excretions, the chicken was euthanized with an intravenous injection of 1ml sodium-pentobarbital (Release®, 300mg/ml), the GIT was dissected and a sample of cecal content was taken using a sterile aliquot and stored in liquid nitrogen. At the end of the day, the samples were stored at -80°C.

DNA extraction

Bacterial DNA was isolated from each sample using the QIAamp DNA Stool minikit (Qiagen, Venlo, the Netherlands), following the manufacturer's recommendations. The DNA was eluted into DNAse/RNAse-free water and its concentration and purity were evaluated by optical density using the NanoDrop ND-1000 spectrophotometer (Isogen, St-Pieters-Leeuw, Belgium). DNA samples were stored at -20 °C until use in 16S rDNA amplicon pyrosequencing analysis.

16S rDNA gene library construction and pyrosequencing

16S PCR libraries were generated with the primers E9-29 and E514-530, specific for bacteria (Wang and Qian, 2009). The oligonucleotide design included 454 Life Sciences' A or B sequencing titanium adapters (Roche Diagnostics, Vilvoorde, Belgium) and multiplex identifiers (MIDs) fused to the 5' end of each primer. The amplification mix contained 5 U of FastStart high fidelity polymerase (Roche Diagnostics, Vilvoorde, Belgium), 1x enzyme reaction buffer, 200 µM dNTPs (Eurogentec, Liège, Belgium), 0.2 µM of each primer and 100 ng of genomic DNA in a volume of 100 µl. Thermocycling conditions consisted of a denaturation step at 94 °C for 15 min followed by 25 cycles of 94 °C for 40 s, 56 °C for 40 s, 72 °C for 1 min and a final elongation step of 7 min at 72 °C. These amplifications were performed on an Ep Master system gradient apparatus (Eppendorf, Hamburg, Germany). The PCR products were run on a 1% agarose gel electrophoresis and the DNA fragments were extracted and purified using the SV PCR purification kit (Promega Benelux, Leiden, the Netherlands). The quality and quantity of the products were assessed using a Picogreen dsDNA quantitation assay (Isogen, St-Pieters-Leeuw, Belgium). All libraries were run in the same titanium pyrosequencing reaction using Roche MIDs. All amplicons were sequenced using the Roche GS-Junior Genome Sequencer instrument (Roche, Vilvoorde, Belgium).

16S rDNA data processing

The 16S rDNA sequence reads were processed using the MOTHUR software package (Schloss et al., 2009). The quality of all the sequence reads was denoised using the Pyronoise algorithm implemented in MOTHUR and filtered according to the following criteria: minimal length of 425 bp, an exact match to the barcode and 1 mismatch allowed to the proximal primer. The sequences were checked for the presence of chimeric amplifications using Uchime (Edgar et al., 2011). The resultant read sets were compared to a reference dataset of aligned sequences of the corresponding region derived from the SILVA database of full-length rDNA sequences (http://www.arb-silva.de/) implemented in MOTHUR (Pruesse et al., 2007). The final reads were clustered into operational taxonomic units (OTUs) with the nearest neighbor algorithm using MOTHUR with a 0.03 distance unit cutoff. A taxonomic identity was attributed to each OTU by comparison with the SILVA database (80% homogeneity cutoff). As MOTHUR is not dedicated to taxonomic assignment beyond the genus level, all unique sequences for each OTU were compared to the SILVA dataset, version 111, using the BLASTN algorithm (Altschul et al., 1990). For each OTU, a consensus detailed taxonomic identification was given based upon the identity (less than 1% of mismatches with the aligned sequence) and the metadata associated with the most frequent hits (validated bacterial species or not).

Statistical analysis

Subsampled datasets were obtained and used to evaluate the richness and microbial diversity of the samples using MOTHUR. To capture the multidimensionality of biodiversity, various indices of diversity and community composition were calculated and compared. Rarefaction curves (Colwell and Coddington, 1994), microbial biodiversity (Simpson and non-parametric (NP) Shannon diversity index – (Chao, 2003)), richness estimators (Observed species richness

(S_{obs}) and $Chao_1$ estimator – (Chao and Bunge, 2002)) and bacterial evenness were calculated for each sample. Simpson and the NP Shannon index give an estimated index value for diversity. NP Shannon is used when undetected species are present in a sample. Simpson diversity was calculated to measure the probability that two individuals, randomly selected from a sample will belong to the same species. The $Chao_1$ estimator is used to estimate the richness of the detected species (OTUs in this case) in a sample and can be compared to the actual number of OTUs observed in samples. S_{obs} was determined as the number of OTUs present per sample. Evenness was determined to quantify the similarity between samples numerically (Colwell et al., 2012).

Analysis of molecular variance (AMOVA) was performed to compare the genetic diversity between two populations with the genetic diversity that would result from pooling both populations. Additionally, nonmetric multidimensional scaling (NMDS) was used to visualize possible differences in the bacterial communities. The ordination was run in PC Ord (5.0) using the Sørensen distance measure, with six starting dimensions, 40 iterations and an instability criterion of 10^{-5} (McCune and Mefford, 2006). Also UniFrac was used to calculate distance measures in bacterial communities between sample origins using phylogenetic information (Lozupone and Knight, 2005).

Because three samples were taken from each chicken, we included 'chicken' as a random factor in all models to account for pseudoreplication. Moreover, diet and breed could be expected to affect the bacteria in the cecum (Shakouri et al., 2009; Stanley et al., 2012). To analyze the effect of variation by both factors on the difference between the microbiota in cecal content and cecal drop on the one hand and cecal content and fecal drop on the other hand, different mixed models were run. In the first one, cecal content and cecal drop were considered whereas in the second one, only cecal content and fecal drop were taken into account. All first order and two-way interactions between the different variables were tested

in a full model. For the final models, the *F* and *P*-values of the explanatory variables were reported in accordance with Murtaugh (Murtaugh, 2014).

Biosample accession numbers

All the biosample sequences were deposited at NCBI (http://www.ncbi.nlm.nih.gov) and are available under the BioProject ID: PRJNA287778.

Results

The day of maximal growth, and therefore the day of sampling, was different for each breed-diet combination (Table 2).

Table 2: Day of maximal growth rate for four chicken breeds fed a commercial or an alternate diet.

Breed	Cobb		CobbSasso		Sasso		Sussex	
Diet	C	A	C	A	C	A	C	A
Age of sampling	43d	49d	45d	50d	53d	54d	57d	60d

C: commercial diet; A: alternate diet

Across all samples analyzed, a total number of 6667 OTU's were found, belonging to ten different phyla. *Firmicutes* appeared to be the most abundant phylum in the three samples with a higher level ($P < 0.01$) in fecal drop compared to both cecal content and drop. The second most abundant phylum was the one of the *Bacteroidetes* in cecal content and cecal drop and *Proteobacteria* in fecal drop. *Bacteroidetes* were less abundant ($P < 0.001$) in fecal drop compared to the other two samples. No differences in levels of *Proteobacteria* could be found between the three samples. *Actinobacteria* were low in abundance, but still their level was higher ($P < 0.001$) in the fecal samples compared to the cecal samples (Figure 1).

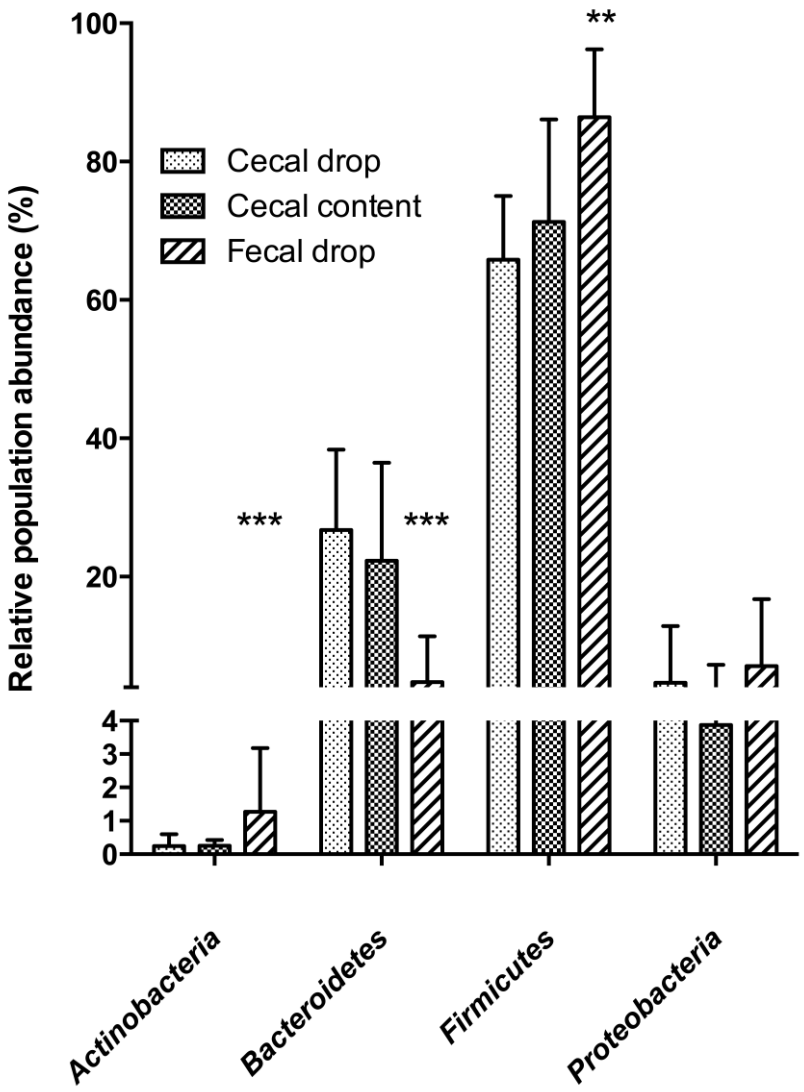

Figure 1: Abundance of the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* in the three samples: cecal drop, cecal content and fecal drop (with **: $P < 0.01$ and ***: $P < 0.001$). Overall results for the four chicken breeds and both diets, commercial and alternate (n = 14 for cecal drop and cecal content, n = 12 for fecal drop).

Four of the most abundant genera (*Alistipes*, *Bacteroides*, *Lachnospiraceae* and *Ruminococcaceae* unclassified genera) were more abundant ($P < 0.001$; $P < 0.01$; $P < 0.001$ and $P < 0.001$ respectively) for both cecal content and cecal drop compared to fecal drop. In contrast to four other abundant genera (*Enterococcus*, *Gallibacterium*, *Peptostreptococcaceae* unclassified genus and *Lactobacillus*) where the level was higher ($P < 0.01$; $P < 0.05$; $P < 0.001$ and $P < 0.001$ respectively) in fecal drop. The abundance estimates of these eight

genera did not differ between cecal content and cecal drop (Figure 2). The relative abundance of the most common genera (cut-off is 3%) in the three samples: cecal drop, cecal content and fecal drop are presented (Figure 3).

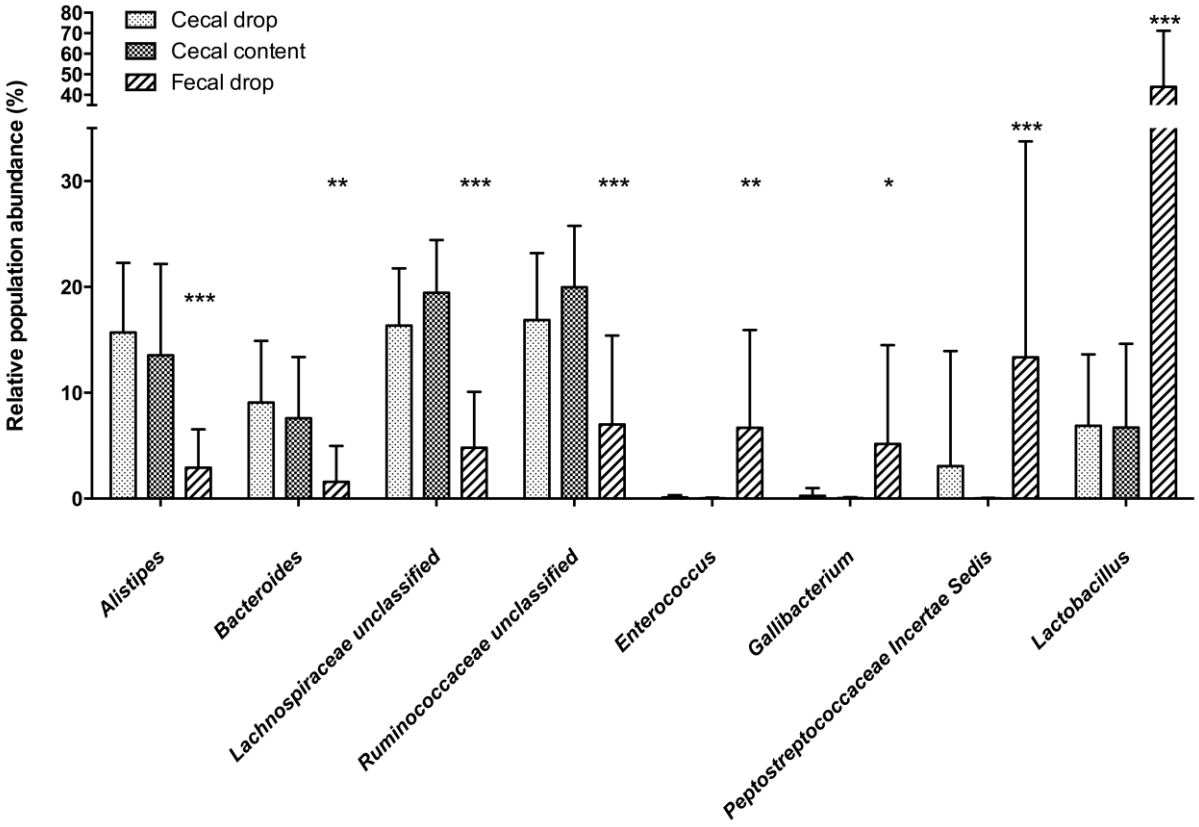

Figure 2: Abundance of bacterial genera in three different samples: cecal drop, cecal content and fecal drop (with *: $P < 0.05$; **: $P < 0.01$ and ***: $P < 0.001$). Overall results for the four chicken breeds and both diets, commercial and alternate (n = 14 for cecal drop and cecal content, n = 12 for fecal drop).

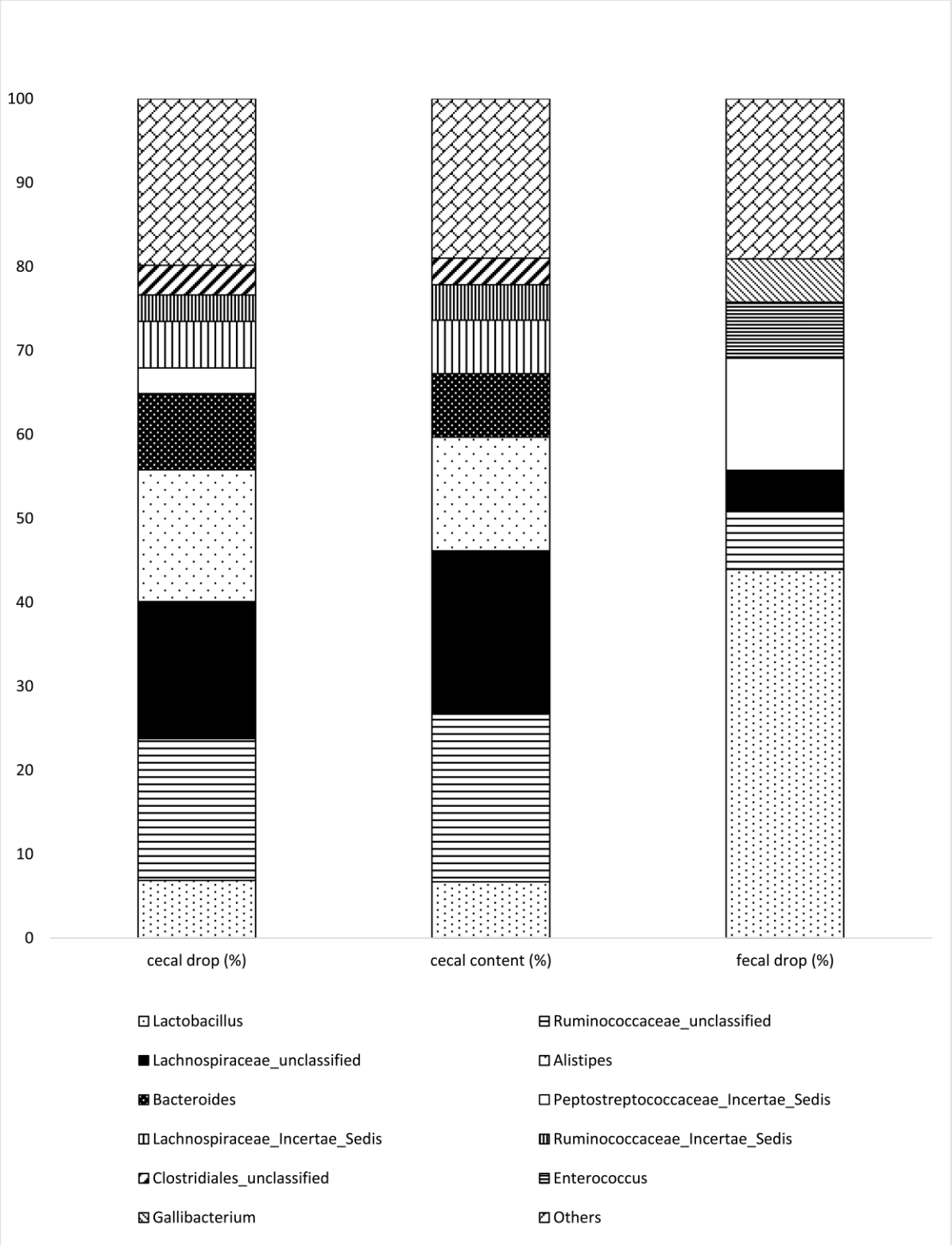

Figure 3: Relative abundance of most common bacterial genera in cecal drop, cecal content and fecal drop (in %). All genera with an abundance > 3% are presented (n = 14 for cecal drop and cecal content, n = 12 for fecal drop).

Diversity

Analysis of molecular variance (AMOVA) indicates a genetic diversity between the bacterial populations found in fecal drop and in cecal content ($P < 0.001$). The same applies for the comparison between fecal drop and cecal drop ($P < 0.001$). The genetic diversity within the bacterial populations found in cecal drop and cecal content did not differ from the genetic diversity when pooling both populations ($P = 0.917$).

Observed Species Richness (S_{obs}) and $Chao_1$ analysis showed a significantly ($P \leq 0.002$ for both) lower richness in fecal drop compared to cecal drop and cecal content (Table 3). Between cecal content and cecal drop, no significant difference in bacterial richness in species level could be found ($P = 0.902$ for S_{obs} and $P = 0.878$ for $Chao_1$).

Bacterial diversity in species level was tested by NP Shannon and Simpson analyses. Both analyses showed a lower ($P \leq 0.001$) diversity in fecal drop compared to cecal drop and cecal content (Table 3). Between cecal content and cecal drop, no significant difference in bacterial diversity in species level ($P = 0.805$ for NP Shannon and $P = 0.945$ for Simpson) could be found.

Table 3: Mean and standard deviation of the different diversity estimates for three samples: cecal drop, cecal content and fecal drop

	cecal drop	cecal content	fecal drop	Sig.
S_{obs}	260 ± 60 [a]	271 ± 46 [a]	138 ± 94 [b]	< 0.001
$Chao_1$	387 ± 91 [a]	407 ± 105 [a]	225 ± 133 [b]	< 0.001
Simpson	0.06 ± 0.07 [a]	0.04 ± 0.02 [a]	0.3 ± 0.3 [b]	< 0.001
NP Shannon	4 ± 0.7 [a]	4 ± 0.3 [a]	3 ± 1 [b]	< 0.001

Evenness	0.7 ± 0.1 [a]	0.8 ± 0.05 [a]	0.5 ± 0.2 [b]	0.002

Different superscripts (a and b) indicate significant differences using the post hoc Tukey test in the linear mixed model.

Community composition

The Weighted UniFrac of the three samples in species level indicated a similar population structure between cecal drop and cecal content (W-Score: 0.37, $P < 0.001$). The analysis for fecal drop compared to cecal drop and to cecal content showed a higher W-Score (0.85 and 0.89 respectively, $P < 0.001$ for both) indicating a different population structure between fecal drop and both cecal drop and cecal content.

The community composition was further compared with NMDS analysis over two axes since 96% of the variation could be explained by two axes (NMDS1 and NMDS2). For NMDS1 no difference ($P = 0.170$) could be found between cecal content and cecal drop, but both cecal content and cecal drop differed significantly ($P < 0.001$ for both) from fecal drop. The same situation was found for NMDS2, with no difference ($P = 0.497$) between both cecal content and cecal drop and a significant ($P < 0.001$ for both) difference between both cecal samples and fecal drop (Figure 4).

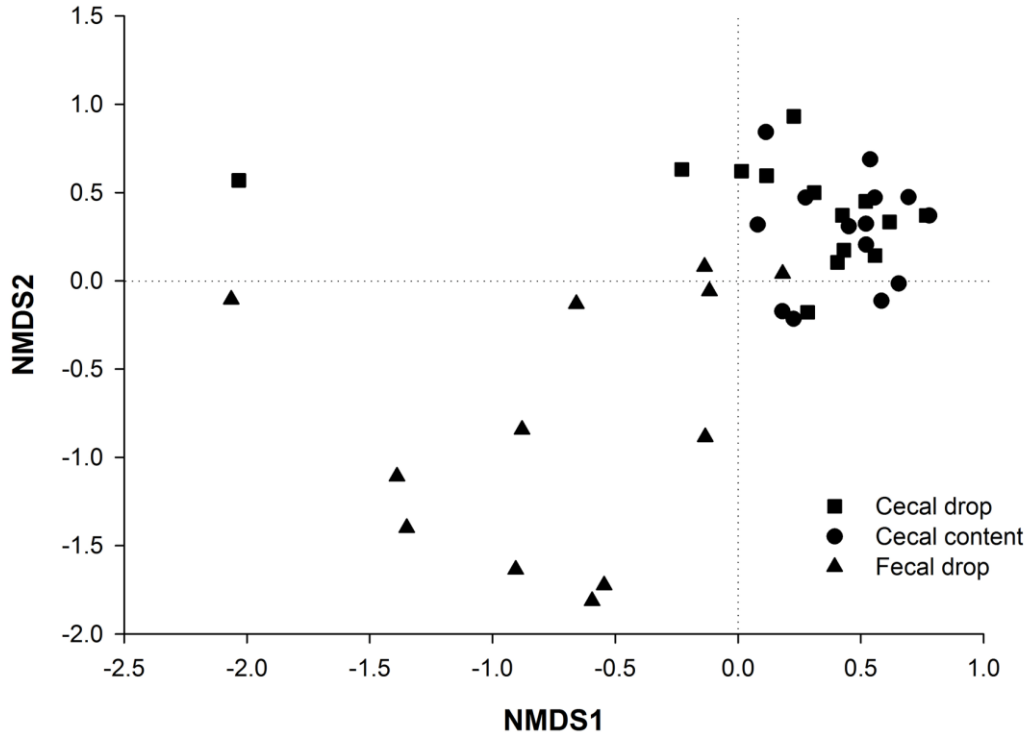

Figure 4: NMDS analysis for three samples: cecal drop, cecal content and fecal drop, over two axes (n = 14 for cecal drop and cecal content, n = 12 for fecal drop).

Variation by breed and diet

Linear mixed models indicated that the similarity between cecal content and cecal drop regarding diversity estimates and community composition was retained when variation was created in the cecal microflora by diet and/or breed. No significant difference in bacterial richness between cecal content and cecal drop was found, neither by S_{obs} nor by $Chao_1$-analysis (P = 0.641 and P = 0.544 respectively). For S_{obs}, no interaction between any of the factors or significant effect by breed or diet was found (P > 0.05). For $Chao_1$, a significant interaction between breed and diet was found (P = 0.015). In addition, Simpson, NP Shannon and Evenness analyses never showed a significant difference between cecal content and cecal

drop regarding bacterial diversity (P = 0.305; P = 0.280 and P = 0.218 respectively). The linear mixed models for Simpson, NP Shannon and Evenness, showed no interaction between any of the factors (P > 0.05). Regarding the community composition, no difference between cecal drop and cecal content could be found for NMDS1 or NMDS2 (P = 0.138 and P = 0.102 respectively). Both breed and diet affected the NMDS2 values (P < 0.001 and P = 0.001 respectively), no interactions were found (P > 0.05).

Linear mixed models, analyzing the data concerning cecal content and fecal drop, showed significant interactions between sample and diet for both bacterial richness analyses: S_{obs} and $Chao_1$ (P = 0.008 and P = 0.005). NP Shannon showed a significant interaction (P < 0.001) between sample and diet on bacterial diversity. Breed was not found to have an effect (P > 0.05) on S_{obs}, $Chao_1$ or NP Shannon estimates. For Simpson and Evenness analysis, an interaction between diet and sample (P = 0.004 and P = 0.001 respectively) and breed and sample (P = 0.026 and P = 0.039 respectively) was found regarding bacterial diversity. The means of all diversity and richness estimates indicated a greater diversity for cecal content compared to fecal drop and for the alternate diet compared to the commercial diet. Regarding the community composition, a significant interaction between diet and sample was found for both NMDS1 (P = 0.009) and NMDS2 (P = 0.002). For NMDS2, a significant effect of breed was found (P = 0.004) without interaction with sample or diet.

Discussion

In literature, the use of fecal samples as a reference for the gut microbiota in different species is still under discussion. Three different kinds of studies can be distinguished: the first group of studies consider fecal samples to be a reliable sample in quantifying and identifying the bacteria in the gut (Claesson et al., 2011), the second group limits the use of fecal samples to monitoring shifts in the microbiota of the gut (Mai et al., 2004; Lubbs et al., 2009) and the last group considers fecal samples to be of limited use as a reference for the gut microbiota (Eckburg et al., 2005; Mentula et al., 2005). In our study, fecal drop showed a bacterial diversity, richness and community composition that is low compared to cecal content. This suggests that a fecal sample is not reliable in mapping the complete cecal microbiome in chickens. In addition, the interactions between the factors, sample and diet, were significant for all diversity estimates as well as community estimates, which indicate a different effect of changing diet on the two samples, cecal content and fecal drop. This shows that fecal drop, under the circumstances tested, cannot be considered as a reliable sample to monitor shifts and changes in cecal content.

Cecal drop showed a very similar bacterial diversity and richness and a similar community composition when compared to cecal content. Even when variation by breed and diet was created, no differences could be found in the bacterial community or diversity patterns. These results make cecal drop the best alternative (as a sample unaffected by variation) to monitor the cecal microbiota. This alternative creates an advantage in longitudinal studies since, by use of cecal drop, the same birds can be re-sampled for every point in the time and no correction for individual differences will be required. This will significantly reduce the number of animals needed in trials. In addition, the chickens don't have to be euthanized at the end of the experiment, which will refine the method used in terms of animal welfare. As such, the use of cecal drop analysis will therefore improve two of the three R's (Reduction,

Refinement and Replacement) that increase humanity in experiments with animals (Russell and Burch, 1959). In the poultry industry, cecal drop analysis can be used to screen for pathogenic and/ or zoonotic bacteria in the cecum without the need for killing animals to attain cecal content. In addition, this sample will represent the cecal bacteria in a more reliable way than fecal drop analysis.

The alternate diet, based on the scavenger diet of rural chickens, showed an increase in bacterial diversity and a change in the community composition compared to a standard commercial diet, which is conventionally used in the industry. It is, however, not clear whether these changes in bacterial diversity and community composition, caused by the diet, also affect functions such as digestibility, immunity or gut health.

Cecal content was sampled by separating ileum and cecum and emptying the cecum into an aliquot by squeezing the content from the top of the cecum towards the opening. It must be considered that the sample might, for example, not have included (all of) the mucosa-associated bacteria. Also, by opening the ileocecal junction and squeezing the content out, contact with oxygen could not be avoided. However, this was only for a few seconds, aliquots were closed and stocked in liquid nitrogen immediately. The method of sampling might have resulted in differences in the microbiome in the cecum and the bacteria in the sample of cecal content.

When using cecal drop to map the cecal microbiota, numbers must be considered carefully since facultative anaerobic bacteria tend to overgrow the strict anaerobic bacteria. *Lactobacillus, Peptostreptococcaceae, Enterococcus* and *Gallibacterium* -all facultative anaerobic bacteria, except for some obligate anaerobic *Peptostreptococcaceae*- increased their concentrations when voided as a cecal drop compared to their concentrations in cecal content. However, except for the *Peptostreptococcaceae*, these increases did not change the overall

profile. This is in contradiction to the concentrations of the facultative anaerobic bacteria found in fecal drop, being four to seventy times higher compared to that found in cecal content. This could be explained by the longer storage of the feces in the cloaca and by the stickier content of the cecal drop compared to fecal drop, which makes it much more difficult for oxygen to penetrate (Lombardo et al., 1996; Clench, 1999). The concentrations of the strict anaerobic bacteria, *Ruminococcaceae, Lachnospiraceae*, *Alistipes*, *Bacteroides* and *Clostridiales* in cecal content compared to cecal drop were very similar. Though, lower concentrations were found in fecal drop for each of them, indicating that they were overgrown.

Conclusion

The bacterial diversity and community composition in fecal drop differs from cecal content for all analyses performed, indicating that fecal drop is an unreliable reference for mapping the cecal microbiota. In addition, the microbiota in fecal drop changed in a different manner compared to the microbiota in cecal content when variation was created by diet and breed. This indicates that fecal drop is not reliable in representing shifts in the cecal microbiome either. Regarding bacterial diversity and community composition, no differences could be found between cecal drop and cecal content, indicating that cecal drop analysis is a good reference for monitoring the microbiota in the cecum. This will reduce the sample size in longitudinal studies considerably and alleviates the necessity to correct for inter-individual differences.

Acknowledgements

The authors sincerely thank Herman De Rycke and Ruben Van Gansbeke for their technical assistance. This study was funded by the VLIR-UOS. Vanessa Van Schoor is acknowledged sincerely for proofreading this manuscript.

References

Barnes, E.M., Mead, G.C., Barnum, D.A., and Harry, E.G. (1972) The intestinal flora of the chicken in the period 2 to 6 weeks of age, with particular reference to the anaerobic bacteria. *Br. Poult. Sci.* **13.**:

Bjerrum, L., Engberg, R.M., Leser, T.D., Jensen, B.B., Finster, K., and Pedersen, K. (2006) Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. *Poult. Sci.* **85**: 1151–1164.

Budding, A.E., Grasman, M.E., Eck, A., Bogaards, J. a., Vandenbroucke-Grauls, C.M.J.E., Van Bodegraven, A. a., and Savelkoul, P.H.M. (2014) Rectal swabs for analysis of the intestinal microbiota. *PLoS One* **9**: 5–12.

Claesson, M.J., Cusack, S., O'Sullivan, O., Greene-Diniz, R., de Weerd, H., Flannery, E., et al. (2011) Composition, variability, and temporal stability of the intestinal microbiota of the elderly. *Proc. Natl. Acad. Sci. U. S. A.* **108**: 4586–4591.

Clench, M.H. (1999) The avian cecum: Update and motility review. *J. Exp. Zool.* **283**: 441–447.

Colwell, R.K., Chao, A., Gotelli, N.J., Lin, S., Mao, C.X., Chazdon, R.L., and Longino, J.T. (2012) Models and estimators linking individual-based and sample-based rarefaction, extrapolation, and comparison of assemblages. *J. Plant Ecol.* **5**: 3–21.

Danzeisen, J.L., Kim, H.B., Isaacson, R.E., Tu, Z.J., and Johnson, T.J. (2011) Modulations of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment. *PLoS One* **6.**:

Dunkley, K.D., Dunkley, C.S., Njongmeta, N.L., Callaway, T.R., Hume, M.E., Kubena, L.F., et al. (2007) Comparison of in vitro fermentation and molecular microbial profiles of high-fiber feed substrates incubated with chicken cecal inocula. *Poult. Sci.* **86**: 801–810.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., et al. (2005) Diversity of the human intestinal microbial flora. *Science* **308**: 1635–1638.

Fenna, L. and Boag, D.A. (1974) Filling and emptying of the galliform caecum. *Can. J. Zool.* **52**: 537–540.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J.B., Massart, S., et al. (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc. Natl. Acad. Sci. U. S. A.* **107**: 14691–14696.

Harmoinen, J. a, Mättö, J.M., Rinkinen, M.L., Wilsson-Rahmberg, M., and Westermarck, E. (2001) Permanent jejunal fistula: promising method for obtaining small intestinal chyme without disturbing intestinal function. *Comp. Med.* **51**: 252–256.

Herman, L., Heyndrickx, M., Grijspeerdt, K., Vandekerchove, D., Rollier, I., and De Zutter, L. (2003) Routes for Campylobacter contamination of poultry meat: epidemiological study from hatchery to slaughterhouse. *Epidemiol. Infect.* **131**: 1169–1180.

Herrick, C. a. and Edgar, S. a. (1947) Some Relationships between Cecal Function and Coccidiosis of Chickens. *Poult. Sci.* **26**: 105–107.

Janssen, P.W.M., Lentle, R.G., Hulls, C., Ravindran, V., and Amerah, A.M. (2009) Spatiotemporal mapping of the motility of the isolated chicken caecum. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **179**: 593–604.

Lombardo, M.P., Thorpe, P.A., Cichewicz, R., Henshaw, M., Millard, C., Steen, C., and Zeller, T.K. (1996) Communities of cloacal bacteria in tree swallow families. *Condor* **98**: 167–172.

Lozupone, C. and Knight, R. (2005) UniFrac : a New Phylogenetic Method for Comparing Microbial Communities UniFrac : a New Phylogenetic Method for Comparing Microbial Communities. *Appl. Environ. Microbiol.* **71**: 8228–8235.

Lubbs, D.C., Vester, B.M., Fastinger, N.D., and Swanson, K.S. (2009) Dietary protein concentration affects intestinal microbiota of adult cats: A study using DGGE and qPCR to evaluate differences in microbial populations in the feline gastrointestinal tract. *J. Anim. Physiol. Anim. Nutr. (Berl).* **93**: 113–121.

Mai, V., Katki, H. a, Harmsen, H., Gallaher, D., Schatzkin, A., Baer, D.J., and Clevidence, B. (2004) Effects of a controlled diet and black tea drinking on the fecal microflora composition and the fecal bile acid profile of human volunteers in a double-blinded randomized feeding study. *J. Nutr.* **134**: 473–478.

McCune, B. and Mefford, M.J. (2006) PC-ORD: Multivariate Analysis of Ecological Data.

Mentula, S., Harmoinen, J., Heikkilä, M., Westermarck, E., Rautio, M., Huovinen, P., and Könönen, E. (2005) Comparison between cultured small-intestinal and fecal microbiotas in beagle dogs. *Appl. Environ. Microbiol.* **71**: 4169–4175.

Miller, M.R. (2015) Cecal Fermentation in Mallards in Relation to Diet Linked. *Condor* **78**: 107–111.

Moss, R. (1973) The digestion and intake of winter foods by wild ptarmigan in Alaska. *Condor* **75**: 293–300.

Murtaugh, P. (2014) In defence of P values. *Ecology* **95**: 611–617.

Okamura, M., Ohba, Y., Kikuchi, S., Suzuki, A., Tachizaki, H., Takehara, K., et al. (2008) Loop-mediated isothermal amplification for the rapid, sensitive, and specific detection of the O9 group of Salmonella in chickens. *Vet. Microbiol.* **132**: 197–204.

Patterson, J. a and Burkholder, K.M. (2003) Application of prebiotics and probiotics in poultry production. *Poult. Sci.* **82**: 627–631.

Rehman, H.U., Vahjen, W., Awad, W. a, and Zentek, J. (2007) Indigenous bacteria and bacterial metabolic products in the gastrointestinal tract of broiler chickens. *Arch. Anim. Nutr.* **61**: 319–335.

Rodriguez-Romero, N., Abecia, L., Balcells, J., Martinez, B., and Fondevila, M. (2009) Comparison of bacterial profile from caecal content and caecotrophes in growing rabbits fed on two levels of indigestible fibre. In, *XXXIX Jornadas de Estudio, XIII Jornadas sobre Producción Animal, Zaragoza, España, 12 y 13 de mayo de 2009*., pp. 784–786.

Russell, W.M.S. and Burch, R.L. (1959) The principles of Humane Experimental Technique 2nd ed. UFAW, London.

Saengkerdsub, S., Anderson, R.C., Wilkinson, H.H., Kim, W.K., Nisbet, D.J., and Ricke, S.C. (2007) Identification and quantification of methanogenic archaea in adult chicken ceca. *Appl. Environ. Microbiol.* **73**: 353–356.

Shakouri, M.D., Iji, P. a., Mikkelsen, L.L., and Cowieson, a. J. (2009) Intestinal function and gut microflora of broiler chickens as influenced by cereal grains and microbial enzyme supplementation. *J. Anim. Physiol. Anim. Nutr. (Berl).* **93**: 647–658.

Van Soest, P.J., Robertson, J.B., and Lewis, B. a (1991) Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **74**: 3583–3597.

Stanley, D., Denman, S.E., Hughes, R.J., Geier, M.S., Crowley, T.M., Chen, H., et al. (2012) Intestinal microbiota associated with differential feed conversion efficiency in chickens. *Appl. Microbiol. Biotechnol.* **96**: 1361–1369.

Stern, N.J. and Robach, M.C. (1992) Nondestructive Sampling of Broilers for Campylobacter-Spp. *Poult. Sci.* **71**: 0–3.

Wiseman, J. (1987) Feeding of non-ruminant livestock.

Zorman, T., Heyndrickx, M., Uzunović-Kamberović, S., and Smole Možina, S. (2006) Genotyping of Campylobacter coli and C. jejuni from retail chicken meat and humans with campylobacteriosis in Slovenia and Bosnia and Herzegovina. *Int. J. Food Microbiol.* **110**: 24–33.