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PCR-DGGE Analysis of Caecal Microflora of Natustat™-Supplemented Turkeys Challenged with *Histomonas meleagridis*

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Abstract: Histomoniasis is a disease of turkeys on litter or range caused by the fragile protozoan *Histomonas meleagridis*, a parasite of worms, primarily spread in faeces, in *Heterakis gallinarum* (caecal worm) eggs or in *Eisenia foetita* (earthworms). Symptoms include poor feed conversion ratio (FCR), decreased body weight (BW), diarrhea, caecal and liver lesions, darkening of the facial regions and sometimes death. Nitarsone can be used as an aid in the prevention of histomoniasis. Natustat™ (Alltech, Inc., Nicholasville, KY), a proprietary plant derived product, is a natural alternative for the prevention of histomoniasis disease in poultry. In this trial, Natustat™, was used at 1.925kg/Tonne and compared with nitarsone at 0.1875kg/Tonne in male Hybrid turkey diets to 42 d of age on histomonad infected litter from broiler breeders. Infected and uninfected, non-supplemented control groups were also included. On d 28, 35 and 42 of the trial, 16 birds from each group were euthanized. Caecal contents were removed, lyophilized and pooled by group for each sampling day. These samples were subjected to a molecular culture-independent methodology; polymerase chain reaction combined with denaturing gradient gel electrophoresis (PCR-DGGE), to identify shifts in caecal microbial populations between the different dietary supplemented groups. The similarity index, Sorenson's pairwise similarity coefficient was used to compare caecal PCR-DGGE profiles to each other. In addition sequence analysis was performed on PCR-DGGE bands of interest. Resultant sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) sequence analysis package online at the National Centre for Biotechnology Information (NCBI) homepage http://www.ncbi.nlm.nih.gov. Notable shifts in the PCR-DGGE profiles were observed between the controls and the supplemented groups. It was concluded that PCR-DGGE profiles could be used to monitor bacterial constituents of caecal contents, indicating that Natustat™ has the ability to manipulate the composition of the intestinal microflora.

Key words: PCR-DGGE, Natustat™, *Histomonas meleagridis*, caecal, microflora

Introduction

Histomoniasis (also known as Blackhead, Histomoniasis or Infectious Enterohepatitis) is caused by the flagellated protozoan *Histomonas meleagridis*. Infection can cause severe disease with symptoms including poor feed conversion ratio (FCR), reduced body weight (BW), diarrhea, caecal and liver lesions, darkening of the facial regions and sometimes death. For more than 30 years, histomoniasis had been well controlled with dimetridazole. After the ban of dimetridazole in the EU as a treatment and as a feed additive, the disease was controlled reasonably well by nifursol, also employed as a feed additive. The ban of nifursol in 2003 has lead to numerous outbreaks of the disease throughout the EU. Currently, nitarsone (Histostat®, Alpharma, Inc., Fort Lee, NJ, USA) is approved by the FDA as an aid in the prevention of histomoniasis. However, in Europe due to increasing restrictions on antibiotic therapeutics, poultry producers may be forced to use alternatives.

Natustat™ (Alltech, Inc., Nicholasville, KY), a proprietary plant derived product, is a natural alternative for the control of histomoniasis disease in poultry (Duffy *et al*., 2004; 2005). Decreases in caecal and liver lesion scores, increases in body weight and improved feed conversion ratios have been observed in Histomonas challenged turkeys and broilers supplemented with Natustat™ (Duffy *et al*., 2004; 2005).

The caecal microflora of poultry has been investigated extensively in the past by use of culture-based methodologies (Barnes and Impey, 1972; Barnes, 1972; Barnes, 1979, Mead and Adams, 1975; Salanitro *et al*., 1974). Limitations associated with conventional culturing methods include low sensitivities (Dutta *et al*., 2001), inability to detect unculturable bacteria and unknown species, slow turnabout time and poor reproducibility (Huijsdens *et al*., 2002). Only recently, have molecular approaches been employed to investigate the bacterial ecology of the chicken intestine (Apajalahti *et al*., 2001; Gong *et al*., 2002; Knarreborg *et al*., 2002; Lu *et al*., 2003; Netherwood *et al*., 1999; Zhu *et al*., 2002). Indeed, molecular techniques have revealed that the composition of the microbiota varies with different diets, and with the application of feed additives

(Apajalahti *et al.*, 2001, Knarreborg *et al.*, 2002; Zhu *et al.*, 2002) or probiotics (Netherwood *et al.*, 1999).

The culture-independent technique, polymerase chain reaction combined with denaturing gradient gel electrophoresis (PCR-DGGE), is a molecular method used to study bacterial communities and population dynamics in microbial ecology (Donskey *et al.*, 2003; Li *et al.*, 2003; Satokari *et al.*, 2001; Schwiertz *et al.*, 2003). PCR-DGGE is an extremely useful tool in the area of poultry gastrointestinal microbial ecology. One group have demonstrated the usefulness of PCR-DGGE in the analysis of chicken digestive microflora (Hume *et al.*, 2003). Their study examined changes in different intestinal bacterial groups in chickens as they aged, as they were withheld feed and upon antibiotic administration. PCR-DGGE was successful in illustrating that all of these parameters had an effect on bacterial populations and thus the efficacy of this molecular technique to monitor changes and differences in gastrointestinal bacterial communities in chickens. In addition, PCR-DGGE was also effective in showing that the application of probiotics in the feed of broilers considerably lowers the *Clostridium* spp. population, lowering the risk of spreading this species in the housing through faecal contamination (Decroos *et al.*, 2004). Furthermore, PCR-DGGE has also been employed to monitor human fecal *Bifidobacterium* populations in a prebiotic and probiotic feeding trial (Satokari *et al.*, 2001).

The purpose of this study was to apply PCR-DGGE to observe changes in caecal microbial populations due to dietary supplementation with Natustat™ or nitarsone in turkeys challenged with *Histomonas meleagridis*. In this method, DNA is first extracted from cells of all species in the community of interest. Variable regions, usually of the 16S rRNA gene, are amplified using primers homologous to conserved regions of the gene. Indeed, specific groups of microorganisms may be focussed upon when group-specific regions are targeted, e.g., lactic acid bacteria (Walter *et al.*, 2001). This technology allows the sequence-specific separation of a mixture of partial 16S rRNA gene amplicons of the same length according to their sequence composition, thus facilitating profiling of microbial communities in polyacrylamide gels containing a denaturing gradient (Muyzer *et al.*, 1993). In this study, shifts in caecal microbial populations due to treatment with Natustat™ or nitarsone were examined using both universal and lactic acid bacteria (LAB)-specific primers.

Materials and Methods

Histomoniasis disease challenge trial: A histomoniasis disease challenge trial was conducted in Virginia Diversified Research Corporation, Harrisonburg, VA 22801, USA. A total of 1,120, day old male (tom) Hybrid turkey poults were placed, 35 per pen in 32 pens (8 replicate pens/treatment). The four treatment groups included 1) non-challenged, non-supplemented control (CON); 2) histomonad challenged, non-supplemented control (hCON); 3) histomonad challenged, nitarsone (hNIT; Histostat®, Alpharma, Inc) supplemented at 0.1875 kg/tonne; and 4) histomonad challenged, Natustat™ (hNAT; Alltech, Inc.), supplemented at 1.925 kg/tonne. The challenge used in this study was via a natural *Histomonas meleagridis* infected litter introduced on day 7 (Duffy *et al.*, 2004; 2005). On d 28, 35 and 42 of the trial, 16 birds from each group were randomly selected and euthanized. Caecal contents were removed, lyophilized and pooled by group for each sampling day. Caecal contents were weighed and filter sterilized (0.22 µm) maltodextrin was added to produce a final concentration of 10% (w/w). Samples were lyophilized and stored at -80°C.

Preparation of genomic DNA from caecal contents: A number of methods of DNA preparation from lyophilized caecal contents were assessed for optimal DNA recovery. Preparation of DNA using the Sigma DNA extraction kit (Sigma-Aldrich, St. Louis, Missouri, USA) according to manufacturer's instructions proved most efficient.

Amplification of the DNA target sequences for DGGE: The V3 (position 339 to 539 in the *E. coli* gene) (Muyzer *et al.*, 1993) and LAB-specific regions (position 352-697) (Tannock *et al.*, 2004) of the 16S rRNA gene were amplified, in separate reactions, from caecal contents. Primers were obtained from Sigma-Genosys, Cambridge, UK.

PCR reactions (100 µL) contained 500 ng genomic DNA, 10X $(NH_4)_2SO_4$ reaction buffer, $MgCl_2$ (3 mM), primers (500 ng each) and deoxynucleotide triphosphates (dNTPs) (500 µM each). Taq DNA polymerase, Bio-X-Act Taq (Bioline™, London, UK), which has highly efficient 3'-5' exonuclease proof-reading activity, was utilized. The PCR amplification program for the amplification of the V3 region consisted of 94°C for 2 minutes and 30 cycles of 93°C for 30 sec, 64°C for 30 sec, 72°C for 30 sec, followed by 10 minutes at 72°C. The optimized program for LAB-specific amplification was 94°C for 2 mins; 35 cycles of 94°C for 30 secs, 62°C for 1 min and 68°C for 1 min, and finally 68°C for 15 mins.

Analysis of amplified PCR products was carried out on a 1% (w/v) agarose gel using 1X Tris acetate EDTA (TAE) running buffer, pH 8.3. To remove unincorporated nucleotides and primers, PCR products were purified using the High Pure PCR product purification kit (Roche, Basel, Switzerland). Single stranded DNA remaining following PCR was degraded using mung bean nuclease (Promega, Madison, Wisconsin, USA). The reaction mixture contained 15 µL purified PCR product, 10X mung bean nuclease buffer, 5 U mung bean

Table 1: Sorenson's pairwise similarity coefficients (Cs) calculated for V3 PCR-DGGE profiles

(a) PCR-DGGE profile similarity analysis for samples taken on different days for various groups.

Group	Cs (%)			
	CON	hCON	hNIT	hNAT
D28	100	100	100	100
D35	93.3	87.3	90.5	87.3
D42	89.4	83.6	82.5	84.9

(b) PCR-DGGE profile similarity analysis for various groups at different time points.

Group	Cs (%)		
	D28	D35	D42
CON	100	100	100
hCON	91.3	72.3	63.6
hNIT	61.9	69.4	54.9
hNAT	57.1	65.3	52.3

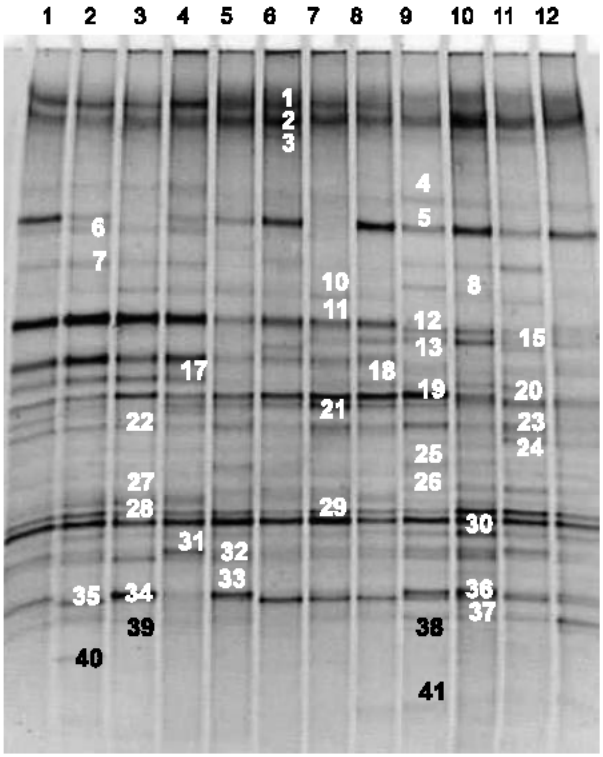

Fig. 1: V3 PCR-DGGE analysis of lyophilized caecal samples from Virginia Diversified Research, corporation trial. Lane 1; group 1 d28, Lane 2; group 1 d35, Lane 3; group 1 d42, Lane 4; group 2 d28, Lane 5; group 2 d35, Lane 6; group 2 d42, Lane 7; group 3 d28, Lane 8; group 3 d35, Lane 9; group 3 d42, Lane 10; group 4 d28, Lane 11; group 4 d35, Lane 12; group 4 d42.

nuclease, in a final volume of 30 µL. The mixture was incubated at 37°C for 30 mins and subsequently placed on ice until DGGE analysis.

Denaturing gradient gel electrophoresis (DGGE): DGGE (Fischer and Lerman, 1983) was performed using a D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Hercules, California, USA). Using horizontal PCR-DGGE, the optimal separation for sequence specific PCR amplicons was obtained in 8% (w/v) polyacrylamide (acrylamide/bisacrylamide 37.5:1). It was determined that gels containing a 30-70% linear denaturant gradient was optimal for universal PCR-DGGE and 45-55% denaturing gradient for LAB-specific DGGE analysis. The 100% denaturing solution consisted of 40% (v/v) formamide and 7.0 M urea. DGGE loading dye (15 mL) [2X loading dye contained 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, 70% (v/v) glycerol in sterile HPLC grade H_2O] was added to purified PCR products (15mL) prior to application to the gel. A stacking gel (7.5 mL) consisting of 8% acrylamide, 0% denaturing solution was also applied to the gels. Electrophoresis was conducted in 1X TAE buffer at a constant voltage of 130 V at 60°C for 5 hours 30 mins for universal V3 DGGE and at 55 V at 60°C for 16 hours for separation of LAB-specific amplicons. Gels were silver stained using a SilverSNAP® Stain Kit II (Pierce Biotechnology Inc., Rockford, Illinois, USA), and viewed using a Vilber Lourmat imaging system (Cedex, France). The Sorenson's pairwise similarity coefficient (Cs) was calculated using the Bio ID Gel Documentation system (Vilber Lourmat, Cedex, France).

Elution, subcloning and sequencing of DNA from DGGE gel: Stained bands, present in the PCR-DGGE profiles of pooled caecal contents from the different supplemented and non-supplemented groups, were excised and DNA was eluted from the polyacrylamide gel using a previously described method (Rölleke *et al.*, 1996). Eluted DNA was subcloned into a plasmid shuttle vector (pCR 2.1™) (Invitrogen, Carlsbad, California, USA) and *E. coli* strain INVαF' competent cells (Invitrogen, Carlsbad, California, USA) were transformed. Ten clones for each band were sequenced to test for co-migration of DNA fragments from different species. Sequencing of insert DNA was carried out by Oswel (Southampton, UK), using M13 forward and reverse sequencing primers. Analysis of sequence data and homology comparisons was performed using the Basic Local Alignment Search Tool (BLAST), online at the National Centre for Biotechnology Information (NCBI) homepage (<http://www. ncbi. nlm. nih.gov>).

Results

PCR-DGGE analysis: PCR amplification of the V3 region in caecal samples generated a 200 bp product.

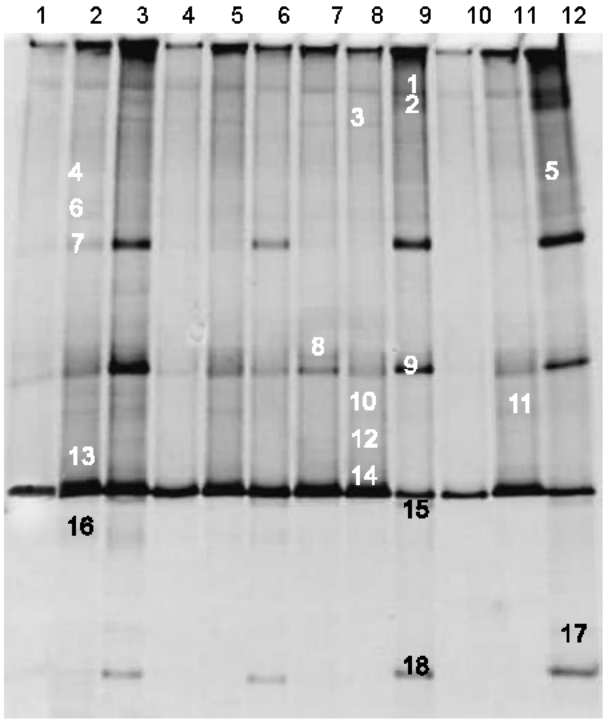

Fig. 2: Lactic acid bacteria-specific PCR-DGGE analysis of lyophilized caecal samples from Virginia Diversified Research corporation trial. Lane 1; group 1 d28, Lane 2; group 1 d35, Lane 3; group 1 d42, Lane 4; group 2 d28, Lane 5; group 2 d35, Lane 6; group 2 d42, Lane 7; group 3 d28, Lane 8; group 3 d35, Lane 9; group 3 d42, Lane 10; group 4 d28, Lane 11; group 4 d35, Lane 12; group 4 d42.

Amplicons of 380 bp were produced in LAB-specific PCR. The V3 PCR-DGGE gel is illustrated in Fig. 1 and the LAB-specific PCR-DGGE gel is presented in Fig. 2. This technique clearly demonstrates the variation that existed in bacterial constituents between the caecal samples on different days and between various supplement groups.

Profile similarity analysis: The Sorenson's pairwise similarity coefficient (Cs) is a similarity index used to compare species composition of different ecosystems. A Cs value of 100% indicates that DGGE profiles are identical while completely different profiles result in a Cs value of 0%. Similarity indices were calculated to compare all PCR-DGGE profiles to each other. It was determined that Cs values remained relatively constant between groups on the different days (with values close to 100%). However, when this coefficient was used to compare profiles from the different supplementation groups on a specific day, profiles were shown to differ, resulting in lower Cs values (e.g., on day 28, comparing the CON group to hCON, hNIT and hNAT, Cs values were 91.3%, 61.9% and 57.1%, respectively) (Table 1).

Sequence analysis: Elution of DNA from bands labelled in Fig. 1 (1-41) and Fig. 2 (1-18) was performed and the DNA was subsequently subcloned and sequenced. Multiple clones from each band were sequenced and each clone was noted to produce an identical sequence indicating that co-migration of DNA fragments from different strains did not occur. Sequences were analyzed using BLAST. Sequence homology matches for each band are presented in Table 2 and 3.

Discussion

The study presented demonstrates the application of PCR-DGGE in the detection of shifts in caecal microbial populations of turkeys due to dietary supplementation with Natustat™ or the chemical, nitarsone. While PCR-DGGE has been applied in the analysis of shifts in chicken gastrointestinal microflora (Hume *et al.*, 2003), no previous report exists in the literature of this technique being utilised in the examination of gastrointestinal microbial populations in turkeys.

Caecal contents samples from birds were subjected to PCR-DGGE, to identify shifts in caecal microbial populations between the supplemented groups and controls on three different sampling days. Subsequently, the similarity index, (Cs value) was calculated and used to compare all PCR-DGGE profiles to each other. Employing this coefficient, it was determined that little temporal shift within the groups was apparent when the entire bacterial population was analyzed, i.e., PCR-DGGE using the V3 primer set (Table 1a). However, when the CON group was compared to the hCON, hNIT and the hNAT groups on different days, lower Cs values were observed (Table 1b). This result suggests that the supplementation of the turkeys' diets in this study, resulted in a shift in the bacterial population. This was shown to be particularly true for Natustat™, as the hNAT profile was consistently least similar to the CON group on each sampling day, exhibiting a Cs value of 52.3 on day 42 of the trial.

A number of bands (41) were excised from the V3 PCR-DGGE gel and were subjected to sequence and BLAST analysis (Fig. 1). It was noted, that more than 58% of fragments displayed high sequence homology to unculturable but viable bacterial species (Table 2). This is not unusual, as other investigators have found similar results. Barnes, (1979); Mead, (1989) and Salanitro *et al.*, (1974); have estimated that only 10 to 60% of the total bacteria in the caecum of chickens were detected by culture. Thi Ngoc Lan *et al.* (2002), identified 90% of bacteria present in the caecum of chickens as non-cultivable. The culturable species identified in our study included those from the genera *Bacteroides*, *Clostridium*, *Lactobacillus*, *Ruminococcus*, *Bifidobacterium*, *Fusobacterium*, *Proteobacterium* and

Table 2: Sequence analysis of PCR-DGGE-generated bands from V3 PCR-DGGE of caecal samples from Histomonas challenge trial

Band No.	NCBI BLAST matches	NCBI Accession number	% homology
	Closest relative		
1	*Megamonas hypermegale* isolate W2	AY729961	96
2	Uncultured bacterium	AJ863542	92
3	Uncultured *Clostridium* sp.	AY305312	95
4	*Lactobacillus aviaries*	AB001837	97
5	Uncultured antarctic soil bacterium clone bh6.8	AF419206	92
6	Unidentified bacterium clone CJJN26	AY654982	93
7	*Ruminococcus flavefaciens* strain AR46	AF104837	96
8	*Lactobacillus crispatus* clone FX157-2	AY335503	96
9	*Pasteurella dagmatis* cp07.08	AY827873	98
10	*Ruminococcus* sp. CO41	AB064892	98
11	Unidentified bacterium clone CCCM55	AY654956	96
12	*Lactobacillus salivarius* strain RA2105	AY389804	99
13	Uncultured alpha proteobacterium clone 5-7-8	AF544953	97
14	Uncultured bacterium adhufec40	AF132270	98
15	*Lactobacillus murinus*	AY324630	97
16	Uncultured bacterium clone ABLCf89	AF499909	98
17	Uncultured gamma *Proteobacterium*	AF114507	95
18	Uncultured bacterium clone ME28	AY916231	98
19	Swine fecal bacterium FPC109	AF445199	98
20	*Lactobacillus ultunensis*	AY253660	99
21	Uncultured bacterium	AJ862535	98
22	*Microscilla marina*	M58793	97
23	Uncultured alpha *Proteobacterium* clone 5-7-8	AF544953	95
24	*Lactobacillus helveticus* strain NCIMB 11971	AY369116	98
25	Uncultured chicken cecal bacterium	AB075651	97
26	Uncultured bacterium clone abc28g08.x1	AY668379	94
27	Uncultured *Clostridium* sp. clone 2C12	AY685919	96
28	Unidentified bacterium	AY654954	97
29	Uncultured chicken cecal bacterium	AB075629	99
30	*Bifidobacterium animalis*	AB125919	98
31	*Fusobacterium* sp. CO5	AB064908	95
32	*Bacteroides* sp. CB40	AB064919	97
33	Uncultured bacterium adhufec40	AF132270	99
34	*Bacteroides* sp. CB4	AB064919	97
35	Uncultured bacterium ckncm298-B3-17	AF376202	98
36	Uncultured alpha *Proteobacterium*	AF544953	95
37	Uncultured bacterium clone FB34-22	AY527806	98
38	Uncultured bacterium ckncm305-B2-7	AF376209	98
39	Uncultured *Bacteroides* sp. clone TNAv1-8	AY597130	97
40	Uncultured chicken cecal bacterium	AB075620	94
41	*Capnocytophaga* sp. ChDC OS43	AF543293	97

Only highest matches are presented

Capnocytophaga. These results concur with those from another study, investigating the diversity of intestinal bacterial community of the broiler chicken (Lu *et al*., 2003). This group reported the presence of the majority of these genera (i.e., *Lactobacillus*, *Clostridium*, *Proteobacterium*, *Flavobacterium*, *Bacteroides* spp.) when they analyzed partial 16S rRNA gene sequences from the caecum of chickens. While the bacterial genera *Ruminococcus* spp. was first identified in the rumen of cattle, it appears that bacteria displaying high sequence homology to *Ruminococcus* spp were present in caecal contents of turkeys in this trial (i.e., bands 7 and 10). Indeed, while this may seem surprising, Thi Ngoc Lan *et al.* (2002) and Lu *et al.* (2003), similarly identified sequences exhibiting high sequence homology to *Ruminococcus* spp. in chicks using 16S rDNA clone libraries.

Fig. 1 and 2 illustrate that certain *Lactobacillus* and

Table 3: Sequence analysis of PCR-DGGE-generated bands from lactic acid bacteria specific-PCR-DGGE of cecal samples from Histomonas challenge trial

Bank No.	NCBI BLAST matches	NCBI Accession number	% homology
	Closest relative		
1	*Lactobacillus pontis*	AJ422033	97
2	Uncultured *Lactobacillus* sp.	AF335884	99
3	Uncultured bacterium clone abc23e08.x1	AY668158	99
4	*Lactobacillus crispatus* clone FX30-2	AY335500	99
5	*Lactobacillus fermentum* MD-9	AY373589	98
6	*Lactobacillus sp.* RA2053	AY445123	100
7	*Lactobacillus aviaries*	AB001837	98
8	Uncultured bacterium	AJ308392	97
9	*Lactobacillus gasseri* strain KC29	AF243157	99
10	*Lactobacillus* sp. T059	AY391826	99
11	*Lactobacillus suntoryeus* strain LH5	AY675251	95
12	Uncultured *Lactobacillus* sp. clone LabS14	AF335913	97
13	*Lactobacillus salivarius subsp. salicinius*	AB125911	95
14	*Lactobacillus acidophilus* strain GGT14	AF375937	99
15	*Lactobacillus salivarius* strain RA2115	AY389803	98
16	Uncultured *Lactobacillus* sp. clone D3-3-1	AY676003	98
17	*Lactobacillus mucosae* strain RA2071	AY445125	97
18	Uncultured *Lactobacillus* sp. clone LabS14	AF335913	97

Only highest matches are presented

Bifidobacterium spp. are present in caecal contents from the various turkeys supplemented with Natustat™ and nitarsone in addition to the control groups, i.e., *L. murinus, L. mucosae, L. ultunensis, L. helveticus, L. crispatus, L. gasseri, L. suntoryeus, L. acidophilus, L. pontis, L. ferementum, L. aviaries,* uncultured lactobacilli and *B. animalis*. Additionally, a number of these species appear to be present in the Natustat™-treatment group only, i.e., *L. helveticus*, *L. murinus* and *L. suntoryeus*. The *B. animalis* band (band 30, Fig. 1) appears to be much more dense in the Natustat™-treatment group while the band exhibiting high sequence homology to *L. mucosae* is present only in the Natustat™-treatment group and the CON group. These are probiotic species (Gardiner *et al.*, 2004; Leblanc *et al.*, 2004; Tzortzis *et al.*, 2004; Mainville *et al.*, 2005) and this may suggest that Natustat™ stimulated the growth of these species in the caeca of turkeys. Their presence may lend to the success of Natustat™ in the control of histomoniasis (Duffy *et al.*, 2005).

Of interest, in LAB-specific PCR-DGGE (Fig. 2), is the presence of certain bands appearing most intense on day 42 in each treatment group (Bands 7, 9, 18). These display high sequence homology to *Lactobacillus aviaries*, *L. gasseri* and an uncultured *Lactobacillus* sp., respectively. This indicates that the concentration of these species increased in the caecal contents of turkeys in a time dependent manner, irrespective of the treatment or challenge given.

This study concludes that PCR-DGGE can be applied successfully to monitor shifts in bacterial constituents of turkey caecal contents, due to differences in feed supplementation. In addition, it is effective in illustrating that products such as Natustat™ have the ability to manipulate the composition of the intestinal microflora. Natustat™ supplementation has been reported to decrease caecal and liver lesion scores, increase body weight and improve feed conversion ratios in turkeys (Duffy *et al.*, 2005). Indeed the shifts noted in caecal bacterial constituents due to differences in feed supplementation may be responsible for the beneficial effects in the parameters measured.

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