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Effect of time and diet change on the bacterial community structure throughout the gastrointestinal tract and in faeces of the northern brown bandicoot, *Isoodon macrourus*

Patricia J. O'Hara[A,C], *Athol V. Klieve*[A,B], *Peter J. Murray*[A], *Anita J. Maguire*[B], *Diane Ouwerkerk*[B] *and Karen Harper*[B]

[A]Wildlife Science Unit, Faculty of Science, The University of Queensland, Gatton Campus, Qld 4343, Australia.
[B]Agri-Science Queensland, EcoScience Precinct, Dutton Park, Qld 4102, Australia.
[C]Corresponding author. Email: patricia.ohara@uq.edu.au

Abstract. A significant gap, in not only peramelid nutritional physiology but marsupial nutrition as a whole, is the lack of information relating to microorganisms of the gastrointestinal tract. This research is a preliminary investigation that will provide a baseline for comparisons among peramelids. The high degree of 16S rRNA gene clones identified in this research that are closely related to culturable bacteria suggests that additional research will enable a more complete description of the gastrointestinal bacteria of *I. macrourus*. Most identifiable clones belonged to *Clostridium* and *Ruminococcus*. This research has confirmed that the hindgut of *I. macrourus*, the caecum, proximal colon and distal colon, are the main sites for microbial activity.

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Introduction

The northern brown bandicoot, *Isoodon macrourus*, is a small to medium-sized (500–3100 g), nocturnal, omnivorous Australian marsupial (Gott 1996; Hume 1999; Gordon 2004). The natural diet of *I. macrourus* consists of invertebrates and occasionally vertebrates, mainly small lizards and birds, with some plant material (leaves, stems, roots, seeds), fungi and fruit (Sandars 1952; Harrison 1963; Gott 1996; Norman 1998). Plant material is consumed in greater proportions during winter and autumn (Gott 1996).

As with all gastrointestinal systems, the bandicoot gut contains a complex microbial ecosystem that aids nutrition and digestive health. However, in common with many non-domesticated species, nothing is known of the complexity, variability or any of the bacterial species that may be present. Previous research has reported that the caecum is the main site for microbial activity in *I. macrourus* (McClelland *et al*. 1999).

The aim of this study was to observe the bacterial community profile along the gastrointestinal tract of *I. macrourus* and determine how much this varied along the gut and between individuals, whether limited dietary changes impacted on that structure and what were the dominant bacterial species present.

Materials and methods

Studies with captive bandicoots

Eight non-reproductive adult female *I. macrourus* were obtained from a captive colony at The University of Queensland Gatton Campus (27°32′27.85″S, 152°20′29.48″E). The bandicoots were fed three diets *ad libitum* for a period of 21 days: an insectivorous ($n = 3$), herbivorous ($n = 2$) or omnivorous ($n = 3$) diet.

The insectivorous diet consisted of mealworm larvae, which mimics the natural preference for larval coleopterans (Gott 1996). The herbivorous diet comprised carrot (35%), commercial bird seed (25%), peeled banana (25%), apple (5%), mushroom (5%) and mung bean sprouts (5%). These components are common in zoological diets for captive peramelids (Jackson 2003). The omnivorous diet contained equal proportions of both the insectivorous and herbivorous diets. Mealworms were cultured in bran and maintained in a cold room at 5°C to maximise storage life. Mealworms were fasted for 24 h before inclusion in the experimental diet to prevent ingestion of plant material by bandicoots. The herbivorous experimental diet was coarsely blended to minimise preferential selection of individual dietary components. Mealworms were not blended.

Bandicoots were offered 50–100 g day^{-1} of the insectivorous diet, 60–170 g day^{-1} of the herbivorous diet and 50–140 g day^{-1} of the omnivorous diet on an as-fed basis. The daily provision was sufficient to ensure feeding *ad libitum*. Fresh water was always available *ad libitum* and the experimental diet was provided during the final hour before dark (~1800 hours) each day.

Samples for bacterial profiling were collected from captive *I. macrourus*. The animals were euthanased by cardiac injection of 3 mL Lethabarb® (sodium pentobarbital, Virbac Australian Pty Ltd, Milperra, NSW, Australia) following isoflurane® (Abbott

Animal Health, Illinois, USA) anaesthetic. Gastrointestinal contents of each tract segment (small intestine, caecum, proximal colon and distal colon) were removed by washing the segment with distilled water. Tract contents were frozen at −20°C until analysis. Following thawing, samples were stirred and 1-mL aliquots placed into duplicate 1.3-mL microtubes.

Collection of faeces from free-living bandicoots

Faecal material was collected during two trapping periods (five nights' duration each) on a private property near Gatton, Queensland (27°32′57.92″S, 152°15′35.11″E). Seven traplines, consisting of seven traps spaced 10 m apart, were established on the property. Traplines were ~100 m apart. Rectangular, treadle-activated, wire cage traps (180 × 180 × 550 mm, Brisbane Wire Works, Geebung, Qld) were used to capture free-living *I. macrourus*. Plastic sheeting was secured underneath the trap with plastic cable ties to minimise environmental contamination of faeces voided by trapped bandicoots. Traps were baited with a single piece of bread spread with plum jam.

Live weight, sex and reproductive status were recorded for each *I. macrourus*. A Hauptner® metal ear tag (Sieper Group, Sydney, NSW, Australia) was inserted in the ear of each animal for identification if the bandicoot was recaptured during that and the subsequent trapping period. All bandicoots were released at point of capture. Within 1 h of collection of faecal material the samples were frozen at −20°C until analysis. Upon thawing, samples were homogenised before transferring ~200 mg into three 1.3-mL microtubes, where possible, to allow for repeat and future analyses.

DNA extraction

DNA was extracted from thawed samples with a QIAamp DNA Stool Mini Kit (QIAGEN Pty Ltd, Doncaster, Vic., Australia). Extracted genomic DNA was stored at −20°C. The manufacturer's extraction process was modified as follows: 0.5 g of 0.1-mm glass beads were used for physical disruption of bacterial DNA by bead beating (Yu and Forster 2005); 700 µL of Amp Stool Lysis buffer was added to gastrointestinal samples (750 µL of Amp Stool Lysis buffer was added to faecal samples) for lysing of bacterial cells, vortexed for 30 s, then repeated, following the addition of an inhibitEX tablet, the solution was vortexed for 1 min, then incubated at room temperature for 2 min; and DNA was eluted with 50 µL of Amp Elution buffer and incubated at room temperature for 2 min. This step was repeated.

DNA quantification

DNA from gastrointestinal contents and faeces was quantified with a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Scoresby, Vic., Australia) then standardised to concentrations of 1 ng μL^{-1}. If initial polymerase chain reaction (PCR) results were negative, indicating the presence of inhibitors in the DNA sample, sample DNA was diluted to 1 : 10 (template : distilled water).

PCR amplification

Primers 341F-GC (with GC clamp attached, shown in italics) (5′ *CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G*CC TAC GGG AGG CAG CAG 3′) and 534R (5′ ATT ACC GCG GCT GCT GG 3′) (Lane 1991) were used to obtain amplicons of the variable region 3 (V3) of the 16S rRNA genes (Muyzer *et al*. 1993). The reaction mixture (49 µL) for the V3 PCR contained 33.75 µL distilled water; 5.00 µL 10x PCR buffer; 4.00 µL $MgCl_2$ (25 mM); 4.00 µL deoxynucleoside triphosphate (dNTP); 1.00 µL 341F-GC (12.5 pmol μL^{-1}); 1.00 µL 534R (12.5 pmol μL^{-1}); and 0.25 µL Thermo Red Hot Taq DNA polymerase (5U μL^{-1}) (Roche Applied Science, Sydney, NSW, Australia).

One microlitre of DNA template was added to the above reaction mixture. Positive and negative controls were included in each PCR run. The positive control was sourced from previous research with DNA from rumen contents and was added to the reaction mixture. The negative control contained the reaction mixture and 1 µL of distilled water instead of the DNA template.

PCR amplifications were performed using a C1000™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The thermal profile for the V3 PCR was: an initial heat activation phase of 95°C for 5 min, followed by five cycles of 30 s denaturation at 95°C, then 10 s annealing at 65°C and 30 s elongation at 72°C. This was followed by 19 cycles of touchdown PCR of 95°C for 30 s, 65°C for 10 s (with a decrement of 0.5°C per cycle) and 72°C for 30 s. This was followed by 11 cycles of 95°C for 30 s, 55°C for 10 s and 72°C for 30 s and a final extension step of 72°C for 5 min. PCR amplification products were stored at −20°C.

Agarose gel electrophoresis

DNA and amplicons were verified using electrophoresis on agarose gels in sodium boric acid electrophoresis buffer. The presence and quality of genomic DNA was verified on a 1% agarose gel containing SYBR®Safe DNA gel stain (Invitrogen Australia Pty Ltd, Mulgrave, Vic., Australia) in SB buffer using a 1-Kb ladder (GeneRulerTM 1kb DNA ladder, Fermentas Life Sciences, Burlington, Canada). The ladder contained 12 bands, each containing an increasing multiple of a 1018 base pair (bp) DNA fragment. The presence of amplicons was verified by electrophoresis on a 2% agarose gel containing SYBR®Safe DNA gel stain in SB buffer using Hyperladder™ IV (Invitrogen Australia Pty Ltd, Mulgrave, Vic., Australia). The ladder contained regularly spaced DNA fragments of the following sizes: 1000, 800, 700, 600, 500, 400, 300, 200 and 100 bp. A result was considered positive when a band was present at ~200 bp in length. Gels were electrophoresed for 45 min at a constant voltage of 95 V using a Wide Mini-Sub Cell GT system (Bio-Rad Laboratories, Hercules, CA, USA). DNA bands were visualised and captured using a Gel Doc system (Bio-Rad Laboratories, Hercules, CA, USA).

Denaturing gradient gel electrophoresis (DGGE)

DGGE was used to generate bacterial profiles of the amplified V3 PCR products from DNA extracted from digesta samples from the small intestine, the caecum, and the proximal and distal colons of *I. macrourus*. Parallel DGGE was performed using D-Code™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) according to Muyzer *et al.*

(1993). Regions of the 16S rRNA genes, amplified by PCR, were passed by electrophoresis through a gel, measuring 160 mm × 160 mm × 1 mm in size, with an increasing denaturant gradient (30–60%). Gels were polymerised on PAG film (GelBond®, Lonza, MC, USA). Denaturants consisted of 40% acrylamide/bisacrylamide (24 mL), 20x TRIS-acetate-EDTA (TAE) buffer (3 mL), formamide and urea (Kocherginskaya *et al*. 2005). For the 60% denaturant, 28.8 mL of formamide and 30.24 g of urea were used. Half the quantities of formamide and urea were used for the 30% denaturant. A DGGE standardisation ladder, to allow within-gel comparisons of bacterial profiles, was produced in-house from a culture collection using the following bacteria in descending order on the gel: *Prevotella ruminocola brevis*, *Butyrivibrio fibrisolvens*, *Escherichia coli*, *Ruminococcus albus*, three *Selenomonas ruminantium* clones and two *Bifidobacterium pseudolongum* clones, as previously detailed by Klieve *et al*. (2007).

To ensure equal DNA loading from the samples onto DGGE gels, the quantity of PCR product loaded onto the gel varied and was between 3 and 20 µL depending on DNA concentration. Following electrophoresis, the gels were stained with silver nitrate to visualise the DNA bands by the method of Kocherginskaya *et al*. (2005). Gels were then scanned using an Epson Perfection V700 Photo scanner (Epson Australia Pty Ltd, Sydney, NSW, Australia).

Purification of PCR products before clone library

A faecal sample collected from a trapped free-living *I. macrourus* was selected for preparation of a clone library of the V3 amplicons. The sample was selected because the bacterial profile represented most bacterial species present in other profiles from faecal samples and was significantly similar to more than half of the faecal samples collected from free-living *I. macrourus* (from the August 2007 collection). Impurities were removed from PCR products from the selected *I. macrourus* faecal sample through a series of wash and spin steps using the QIAquick PCR Purification Kit (QIAGEN Pty Ltd, Doncaster, Vic., Australia) as per the manufacturer's instructions.

Clone library preparation

Cloning was undertaken with the TOPO TA Cloning® Kit for Sequencing (Invitrogen Australia Pty Ltd, Mulgrave, Vic., Australia) as per the manufacturer's instructions. The cloning reaction (6 µL in total volume) contained 1 µL of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$), 1 µL of vector and 4 µL of purified PCR product. The pCR®4-TOPO® plasmid vector (supplied by manufacturer) was linearised (by restriction enzymes) leaving a single overhanging 3′ thymidine (T) for cloning. The cloning reaction was performed at room temperature for 15 min then placed on ice before transformation.

One Shot® TOP10 *E. coli* was transformed according to the manufacturer's (Invitrogen Australia Pty Ltd, Mulgrave, Vic., Australia) instructions. To improve the transformation of *E. coli*, SOC medium (Super Optimal Broth with added glucose) was used. The SOC medium provided a nutrient source for the transformed *E. coli*. Aliquots of each transformation (50 µL neat, 30 µL plus 20 µL SOC medium, 20 µL plus 30 µL SOC medium and 10 µL plus 40 µL SOC medium) were spread onto prewarmed selective Luria Bertani medium (LB) plates that contained 50 µg mL^{-1} of ampicillin and incubated overnight at 37°C.

From the 10 µL plus 40 µL SOC medium transformation reaction, 30 ampicillin-resistant colonies were randomly selected using sterile, disposable loops. The residual bacteria on the disposable loops were transferred to tubes containing 5 mL LB and ampicillin. The tubes were incubated at 37°C for 12–16 h shaking at 100 rpm. Broth culture (4 mL) was centrifuged to obtain a pellet for plasmid DNA extraction. A sample of broth culture (850 µL) was mixed with 150 µL sterile glycerol in Nunc® CryoTubes® (Thermo Fisher Scientific Inc., Scoresby, Vic., Australia) for long-term storage of clones and frozen at −80°C.

Plasmid DNA extraction of the pelleted cells was performed using a DNA QIAprep SPIN Miniprep kit, as per the manufacturer's instructions (QIAGEN Pty Ltd, Doncaster, Vic., Australia). V3 PCR was performed on transformed competent cells to ensure the bacterial DNA was incorporated. PCR products were examined with DGGE. Cloned bacterial bands were aligned with the original complete faecal bacterial profile to determine the corresponding banding position in the bacterial profile.

DNA sequencing

The V3 region from cloned plasmids was sequenced using T3 (5′ATT AAC CCT CAC TAA AGG GA 3′) and T7 (5′TAA TAC GAC TCA CTA TAG GG 3′) primers with the ABI Prism™ BigDye Terminator Cycle Sequencing Ready Reaction Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA) as per the manufacturer's instructions. Sequencing separation was performed on an ABI 3730xl automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing of cloned plasmids was conducted by staff at the Animal Genetics Laboratory, The University of Queensland Gatton Campus.

DNA sequence assembly and alignment

Sequence data from the clones was manually edited using Sequencher® (Gene Codes Corporation, Ann Arbor, MI, USA). The assembled 16S rRNA gene sequences from the clones were compared with sequences in the GenBank® database using the BLAST® (Basic Local Alignment Search Tool) database search program (Altschul *et al*. 1990). Identity matches of ≥97% sequence similarity were regarded as belonging to the same species and an identity match of ≥94% the same genus (Bond *et al*. 1995). Sequences from this study and reference sequences identified in GenBank® were aligned using ClustalX ver. 2.1 (Larkin *et al*. 2007).

Phylogenetic analysis

PAUP* ver. 4.0 (Phylogenetic Analysis Using Parsimony) (Swofford 1998) was used to construct the phylogenetic trees for this study. Phylogenetic trees were generated using maximum parsimony (P), maximum likelihood (L) and distance matrix (D) analyses. Before constructing the trees, a series of likelihood ratio tests were performed using jModelTest 0.1.1 (Guindon and Gascuel 2003; Posada 2008) to determine the most suitable nucleotide substitution model to use for the L and D analyses. The model selected was a Transversion Model with equal base

frequencies (TVMef) and with rate variation amongst the sites (TVMef+G) for the 193 bases of 16S rRNA gene sequences used to produce the trees. Bootstrapping is a repeated sampling technique that provides information on the robustness of a phylogenetic tree, particularly the interior branches (Berry and Gascuel 1996; Nei *et al*. 1998). For the construction of the parsimony and distance trees, 1000 bootstrap replicates were undertaken whilst 100 bootstrap replicates were undertaken for the likelihood tree.

Statistical analyses

The DNA bands on the DGGE gel images were examined with BioNumerics ver. 6 (Applied Maths, Ghent, Belgium). BioNumerics applied the unweighted pair-group method with arithmetic averages (UPGMA) ordination in combination with curve-based (Pearson) clustering algorithms to the bacterial profiles. Pearson correlation coefficients were used to examine band intensities. Samples that clustered ≥80% were considered to be not significantly different (Tolosa 2006).

Bacterial diversity measures (the number of bands present within each profile, Simpson and Shannon diversity indices and evenness estimates) (Spellerberg 2008) were obtained from densitometric curve data for selected DGGE profiles and the means and standard deviations were calculated.

Results

Bacterial profiles from gastrointestinal tracts of captive I. macrourus

Initial analysis examined changes in bacterial community profiles along the lower gastrointestinal tract, from small intestine to distal colon, and similarities between intestinal segments on the same or different diets.

The dendrogram for examining bacterial band intensities (Pearson correlation) (Fig. 1) grouped the hindgut segments (proximal and distal colon) of all *I. macrourus* together by diet. In contrast, the bacterial band intensities for the small intestine did not cluster with their corresponding hindgut samples irrespective of diet. All of the hindgut clusters were above 80% with the exception of one *I. macrourus* fed the herbivorous diet and another bandicoot fed the omnivorous diet. For the bandicoot fed the herbivorous diet, the bacteria profiles in the proximal colon and distal colon clustered together at 82%, with the caecal bacterial profile grouping with the proximal colon and distal colon at 66%. For the bandicoot fed the omnivorous diet, the caecal bacterial profile clustered with the bacterial profiles for the proximal colon and distal colon at 66% (Fig. 1).

Common bacteria were found across all segments of the gastrointestinal tract. The insectivorous and omnivorous diets showed more similarity to each other with common bacterial bands present. The small intestine bacterial profiles were distinctly different from the hindgut segments in all *I. macrourus*, irrespective of experimental diets. The small intestine appears to have a less diverse bacterial profile than the hindgut in all *I. macrourus* (Table 1).

The total number of bands present in the small intestine of *I. macrourus* was consistently less than the number of bands present in the proximal colon in all experimental diets (Table 1). The Shannon Index indicated high species evenness and richness as values were close to 3.5. Low values for the Simpson Index support high bacterial species diversity in the various segments of the gastrointestinal tract of three *I. macrourus* (Table 1). All evenness values were ≥0.91, which suggests that there were similar proportions of all species present within the samples.

Bacterial profiles from the small intestine for bandicoots fed the insectivorous and omnivorous diets showed more similarity to each other than did those from *I. macrourus* fed the herbivorous diet (Fig. 2). Several bacterial bands were common across all diets. There was no significant trend for the bacterial profiles in the small intestine of *I. macrourus* fed the three experimental diets.

Caecal bacterial profiles show that several bacterial bands are common across all diets, although they varied in intensity (Fig. 3). The Pearson correlation dendrogram supports this result, with the two *I. macrourus* fed herbivorous diets

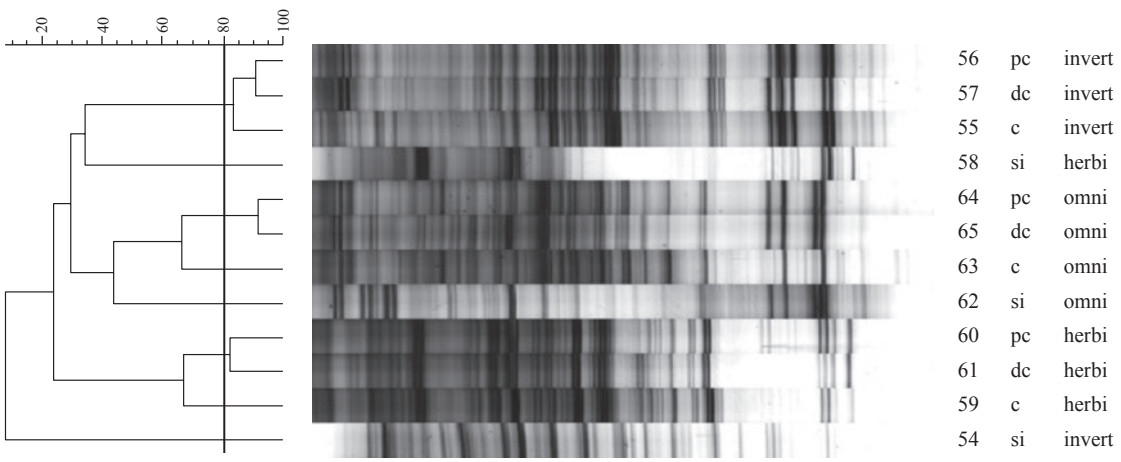

Fig. 1. Cluster analysis (Pearson correlation) of DGGE profiles of bacteria present in various segments of the gastrointestinal tract of three *I. macrourus* fed three experimental diets (two-digit numbers = sample ID, herbi = herbivorous diet, invert = insectivorous diet, omni = omnivorous diet, si = small intestine, c = caecum, pc = proximal colon, dc = distal colon).

Table 1. Species richness (total number of bands), Shannon and Simpson diversity indices and species evenness for samples obtained from different gastrointestinal tract segments of captive *I. macrourus* fed three experimental diets
invert = insectivorous diet, herbi = herbivorous diet, omni = omnivorous diet, SI = small intestine, C = caecum, PC = proximal colon, DC = distal colon

Sample ID	Diet	Segment	No. of bands	Shannon Index	Evenness	Simpson Index
54	Invert	SI	35	3.37	0.948	25.8
55	Invert	C	38	3.42	0.940	25.8
56	Invert	PC	45	3.60	0.946	31.1
57	Invert	DC	42	3.55	0.949	29.9
58	Herbi	SI	23	2.87	0.914	13.6
59	Herbi	C	36	3.42	0.956	27.0
60	Herbi	PC	39	3.46	0.944	26.5
61	Herbi	DC	38	3.50	0.962	29.1
62	Omni	SI	37	3.35	0.929	24.2
63	Omni	C	39	3.45	0.942	25.4
64	Omni	PC	36	3.35	0.936	23.2
65	Omni	DC	34	3.30	0.936	21.4
	mean ± s.d.		36.8 ± 5.31	3.39 ± 0.18	0.94 ± 0.01	25.2 ± 4.60
	SI mean ± s.d.		31.7 ± 7.57	3.20 ± 0.29	0.93 ± 0.02	21.2 ± 6.62
	C mean ± s.d.		37.7 ± 1.53	3.43 ± 0.02	0.95 ± 0.01	26.1 ± 0.83
	PC mean ± s.d.		40.0 ± 4.58	3.47 ± 0.12	0.94 ± 0.01	27.0 ± 3.97
	DC mean ± s.d.		38.0 ± 4.00	3.45 ± 0.13	0.95 ± 0.01	26.8 ± 4.69

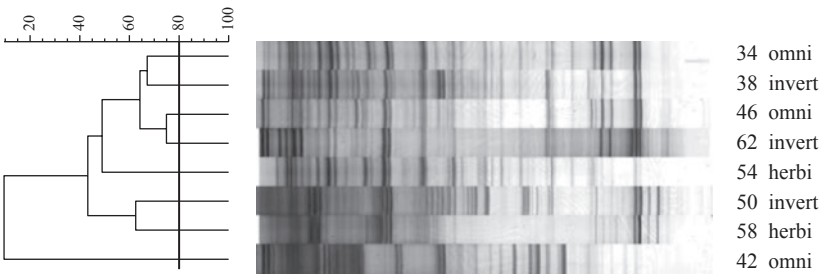

Fig. 2. Cluster analysis (Pearson correlation) of DGGE profiles of bacteria present in the small intestine of *I. macrourus* fed three experimental diets (two-digit numbers = sample ID, herbi = herbivorous diet, invert = insectivorous diet, omni = omnivorous diet).

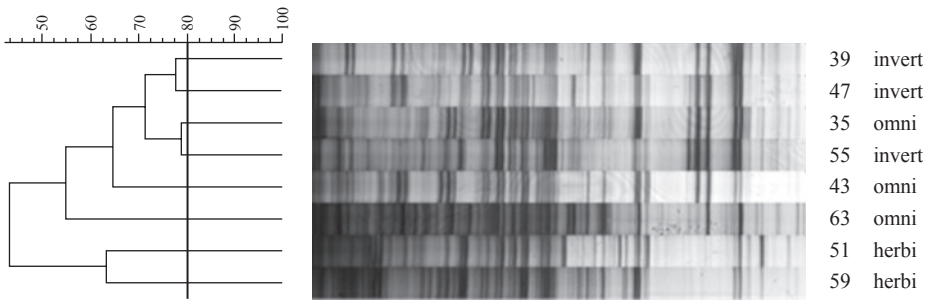

Fig. 3. Cluster analysis (Pearson correlation) of DGGE profiles of bacteria present in the caecum of *I. macrourus* fed three experimental diets (two-digit numbers = sample ID, herbi = herbivorous diet, invert = insectivorous diet, omni = omnivorous diet).

clustering separately from the bandicoots fed the insectivorous and omnivorous diets. However, the two *I. macrourus* fed the herbivorous diet show little similarity to each other as they clustered at ~63%.

Analysis of the bacterial profiles from proximal colon samples showed numerous common bands regardless of diet (Fig. 4). *I. macrourus* fed the herbivorous diet appeared to be more similar to each other than to either profiles from the

omnivorous or insectivorous diets as the bandicoots fed the herbivorous diet grouped separately at ~68% similarity. Proximal colon samples were mostly grouped by diet but only two *I. macrourus* on the insectivorous diet (Samples 48 and 56) clustered above 80%, indicating significant similarity.

The bacterial profiles of the distal colon showed more similarity between *I. macrourus* fed omnivorous and insectivorous diets than with bandicoots fed the herbivorous diet (Fig. 5). Whilst bacterial profiles from bandicoots fed the herbivorous diet clustered together, there was limited clustering above 80% (Fig. 5).

Bacterial profiles from faeces of free-living I. macrourus

Analysis of the bacterial bands across all faecal samples collected from free-living *I. macrourus* during April 2007 revealed several common bands (Fig. 6). An August 2007 faecal sample collected from *I. macrourus* was included to determine whether there was similarity of bacterial profiles between seasons.

There was no significant clustering of the profiles of bacteria in faecal samples collected from free-living *I. macrourus* captured in April 2007 based on the Pearson correlation dendrogram (Fig. 6). However, profiles of faecal samples collected from the same animal during the trapping period generally grouped together although with no significant similarity in band intensity. The profile of the bacteria in the faecal sample collected during August 2007 did not group with any of the profiles of bacteria in the faecal samples collected in April 2007.

The number of bacterial species in the faeces of *I. macrourus* collected in April and August 2007 were uniformly distributed, as shown by the high evenness values (≥0.91) (Table 2). The Shannon and Simpson indices indicated high bacterial species evenness and richness in the faeces of *I. macrourus*.

Faecal samples collected from *I. macrourus* during August 2007 showed several common bacterial bands across all profiles (Fig. 7). Cluster analysis for band intensities showed no significant trends for similarities. Bacterial profiles of faecal samples from recaptured bandicoots in August did not group together.

The numbers of bacterial species present in faeces from *I. macrourus* were uniformly distributed across all samples, as shown by the high evenness values (≥0.92) (Table 3). The Shannon and Simpson indices indicated high bacterial species evenness and diversity.

Phylogenetic analysis of DNA from cloned bacterial sequences

A faecal sample from one *I. macrourus* (Fig. 7, lane 9) was selected as a representative sample to be used for cloning.

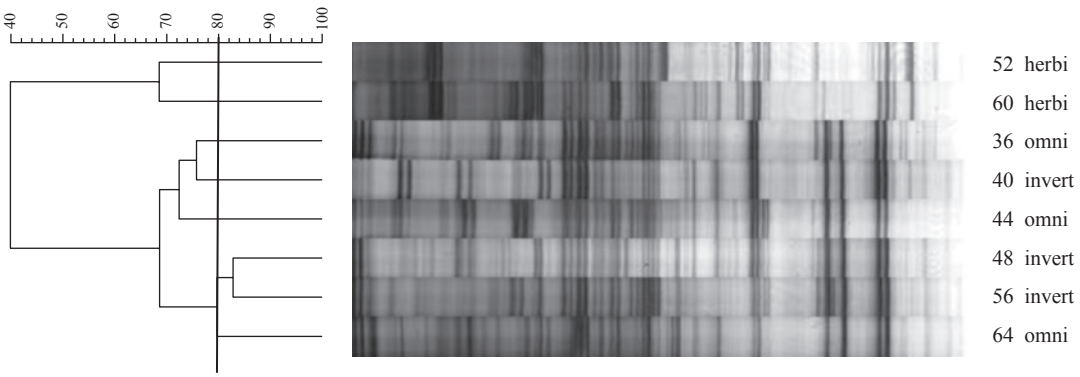

Fig. 4. Cluster analysis (Pearson correlation) of DGGE profiles of bacteria present in the proximal colon of *I. macrourus* fed three experimental diets (two-digit numbers = sample ID, pc = proximal colon, herbi = herbivorous diet, invert = insectivorous diet, omni = omnivorous diet).

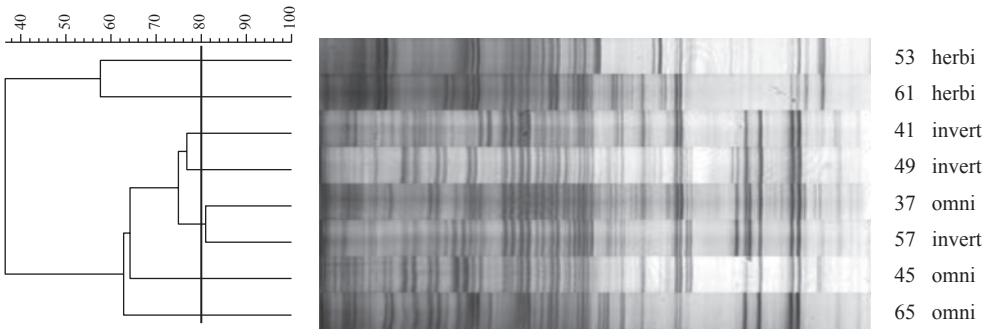

Fig. 5. Cluster analysis (Pearson correlation) of DGGE profiles of bacteria present in the distal colon of *I. macrourus* fed three experimental diets (two-digit numbers = sample ID, herbi = herbivorous diet, invert = insectivorous diet, omni = omnivorous diet).

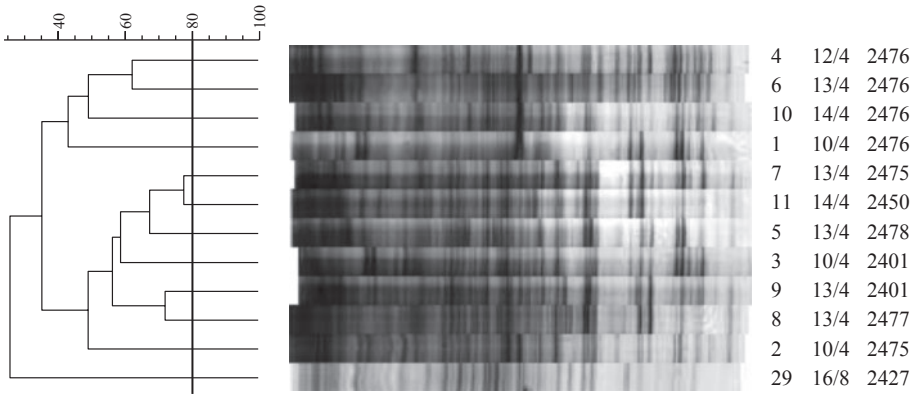

Fig. 6. Cluster analysis (Pearson correlation) of DGGE profiles of bacteria present in the faeces collected from free-living *I. macrourus* captured in April and August 2007 (one- or two-digit numbers = sample ID, four-digit number = bandicoot ID).

Table 2. Species richness (total no. of bands), Shannon and Simpson diversity indices and species evenness for samples obtained from faeces of free-living *I. macrourus* captured in April 2007

Sample ID	Bandicoot ID	Capture date	No. of bands	Shannon Index	Evenness	Simpson Index
9	2401	13/4	44	3.59	0.950	30.9
3	2401	10/4	47	3.68	0.955	34.4
10	2476	14/4	48	3.66	0.945	32.9
7	2475	13/4	41	3.55	0.957	30.4
5	2478	13/4	43	3.53	0.938	28.1
11	2450	14/4	45	3.65	0.960	33.6
1	2476	10/4	40	3.38	0.917	23.0
8	2477	13/4	47	3.62	0.941	30.2
4	2476	12/4	46	3.64	0.952	33.3
2	2475	10/4	48	3.70	0.955	35.2
6	2476	13/4	56	3.86	0.959	41.6
29	2427	16/4	40	3.44	0.932	26.6
	mean ± s.d.		45.4 ± 4.44	3.61 ± 0.12	0.95 ± 0.01	31.7 ± 4.70

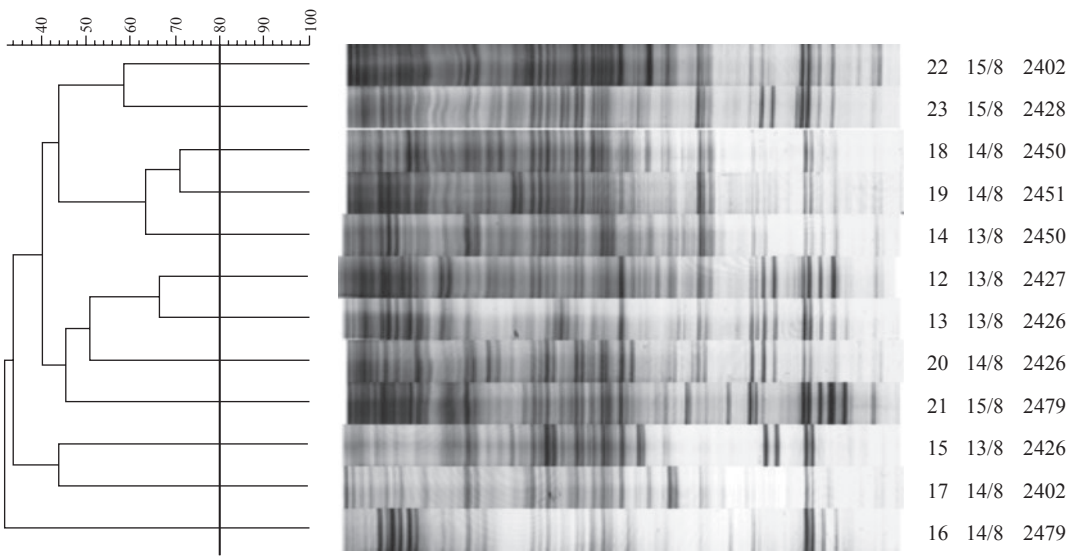

Fig. 7. Cluster analysis (Pearson correlation) of DGGE profiles of bacteria present in the faeces of free-living *I. macrourus* captured in August 2007 (two-digit numbers = sample ID, four-digit number = bandicoot ID).

Sample 21 clustered significantly with more than half of the faecal samples obtained in August 2007 and its profile represented most bacterial species present.

Sequence data were obtained from 29 clones. Bacterial species identified from cloned sequences belonged to two phyla, Bacteriodetes (5 clones) and Firmicutes (24 clones) (Figs 8 and 9 respectively).

Only one bandicoot faecal clone in the Bacteriodetes achieved a similarity of more than 98% to a cultured species (Clone 10) (Fig. 8). Clone 10 was 98.9% similar to *Parabacteroides*

Table 3. Species richness (total no. of bands), Shannon and Simpson diversity indices and species evenness for samples obtained from faeces of free-living *I. macrourus* captured in August 2007

Sample ID	Bandicoot ID	No. of bands	Shannon Index	Evenness	Simpson Index
9	2427	53	3.78	0.953	37.2
3	2428	44	3.58	0.946	31.2
10	2450	49	3.78	0.972	39.6
7	2426	41	3.44	0.925	25.6
5	2479	35	3.23	0.907	19.3
11	2402	41	3.49	0.939	27.8
1	2450	52	3.77	0.953	38.3
8	2451	53	3.78	0.951	36.6
4	2426	46	3.65	0.953	33.9
2	2479	52	3.74	0.946	36.0
6	2402	51	3.74	0.952	35.5
29	2428	49	3.68	0.945	33.5
	mean ± s.d.	47.2 ± 5.81	3.64 ± 0.18	0.95 ± 0.01	32.9 ± 5.96

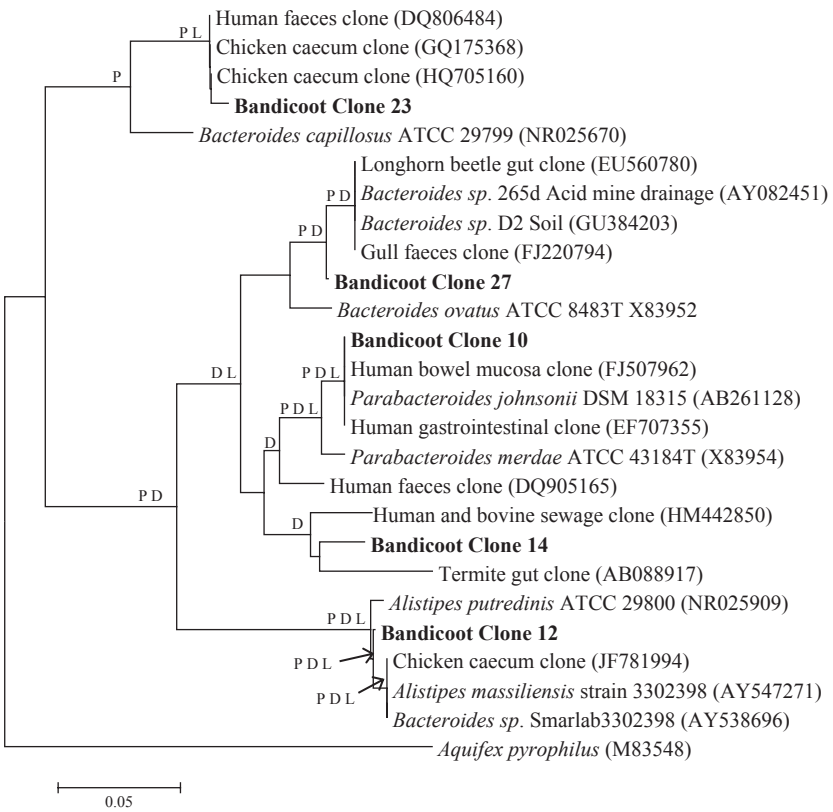

Fig. 8. Phylogenetic dendrogram illustrating the relationship of the 16S rRNA V3-region sequences derived from DGGE band clones (in bold font) and selected reference sequences belonging to the phylum Bacteriodetes. Bootstrap values for parsimony (P, 1000 replicates), distance (D, 1000 replicates) and likelihood (L, 100 replicates) are indicated with a corresponding letter if they exceeded 60% support. *Aquifex pyrophilus* (M83548) was used as the out-group. The bar represents a sequence divergence of 5%. GenBank accession numbers are given after each reference sequence designation.

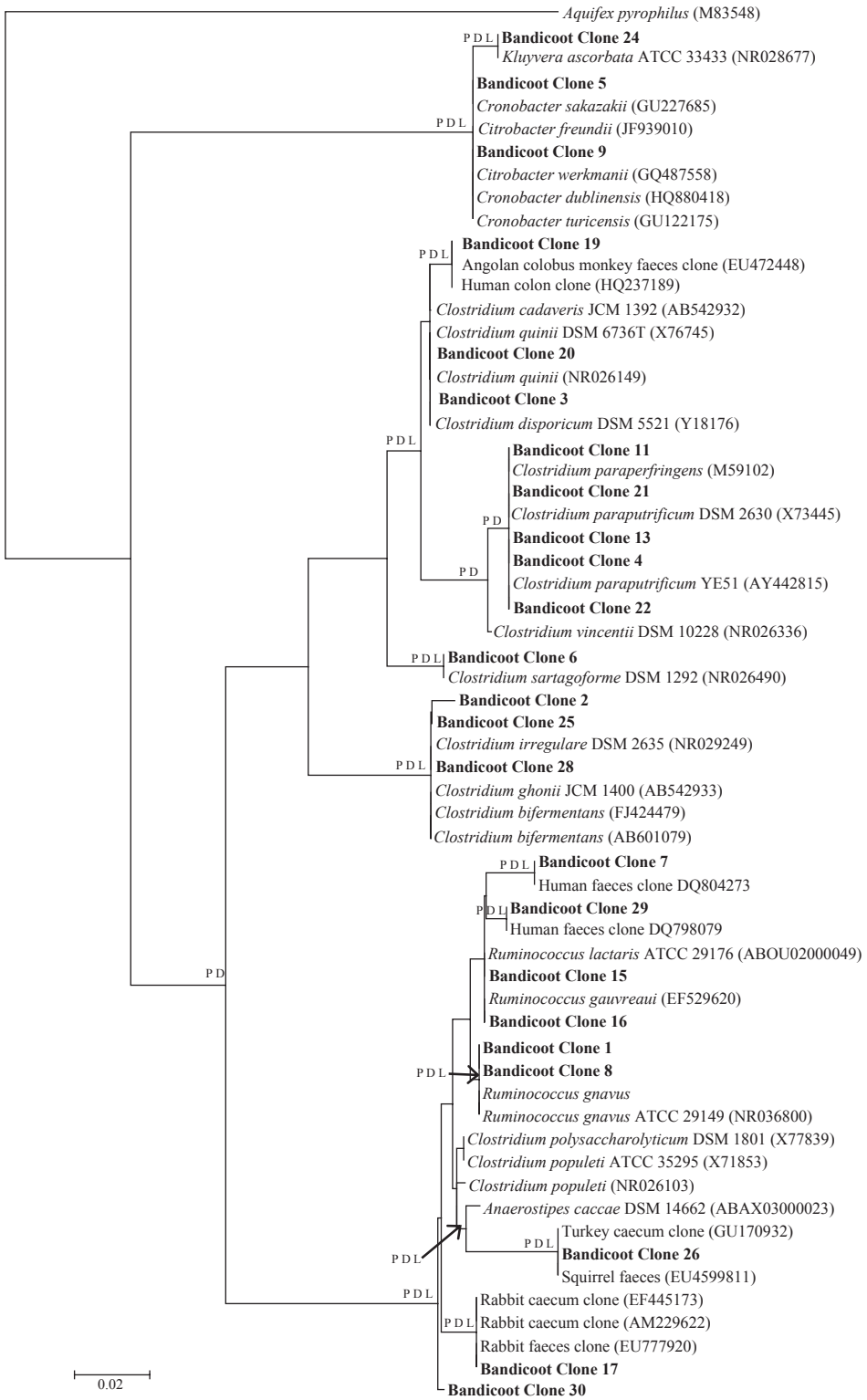

Fig. 9. Phylogenetic dendogram illustrating the relationship of the 16S rRNA V3-region sequences derived from DGGE band clones (in bold font) and selected reference sequences belonging to the phylum Firmicutes. Bootstrap values for parsimony (P, 1000 replica replicates), distance (D, 1000 replicates) and likelihood (L, 100 replicates) are indicated with a corresponding letter if they exceeded 60% support. *Aquifex pyrophilus* (M83548) was used as the out-group. The bar represents a sequence divergence of 2%. GenBank accession numbers are given after each reference sequence designation.

johnsonii isolated from human faeces (Sakamoto *et al*. 2007). Clone 27 was 97.3% similar to *Bacteroides ovatus*, which was isolated from the human gastrointestinal tract (unpublished, GenBank NZ_AAXF02000050.1). The remaining three Bacteriodetes bandicoot clones (12, 14 and 23) had less than 97% similarity with cultured bacterial species. Clone 12 was 96.8% similar to *Alistipes putredinis*, which has been isolated from the human appendix and faeces (Rautio *et al*. 2003). Clone 14 is most closely related to *Parabacteroides merdae* but with a similarity of only 92.5% it is unlikely to be within the same genus (unpublished, GenBank NZ_AAXE02 000110.1). In the phylogenetic tree, Clone 14 grouped closely with a gastrointestinal tract clone, *Candidatus Symbiothrix dinenymphae*, isolated from a termite. *Ca. S. dinenymphae* attach to the unicellular microorganisms, or protists, within the gastrointestinal tract of termites (Hongoh *et al*. 2007). However, *Ca. S. dinenymphae* did not appear in the database results as an identity match so it is unlikely that Clone 14 and this species belong to the same genus. Clone 23 was most closely related to *B. capillosus* (95.5%), which has been isolated from an unknown source (unpublished, GenBank NR_025670.1).

Of the 24 bandicoot faecal clones for the Firmicutes, 16 matched sequences of culturable bacteria with 100% similarity (Fig. 9). On the basis of sequence comparisons of 167 to 192 bp, some bandicoot clones produced multiple matches of 100%.

Of the 24 clones in the Firmicutes, 12 clustered with *Clostridium* species and 6 clustered with *Ruminococcus* species (Fig. 9). *C. paraputrificum* showed 100% similarity to bandicoot Clones 4, 11, 13, 21 and 22. Clones 1 and 8 were 100% similar to *Ruminococcus gnavus*. Clone 17 grouped with unidentified caecum and faecal clones from rabbits. GenBank searches showed that Clone 24 was similar (100%) to several *Citrobacter* species; however, the phylogenetic dendrogram clustered Clone 24 with *Kluyvera ascorbata* (Mollet *et al*. 1997) although it showed 99.5% similarity in database searches. An unidentified faecal clone from an Angolan colobus monkey (*Colobus angolensis*) (Ley *et al*. 2008) provided the closest phylogenetic match for bandicoot Clone 19. Clone 30 was not closely related to any bacteria, cultured or cloned.

From comparisons of the bandicoot clones and the DGGE bacterial profile from a free-living *I. macrourus*, it was possible to identify 17 individual bands to at least genus level (Fig. 10). The *Clostridium* species (Clones 2, 25 and 28) were present in all faecal samples collected in this study. Half of the total bands that could be identified were *Clostridium* species. It was not possible to accurately identify bands in the top half of the bacterial profile due to the density of bands.

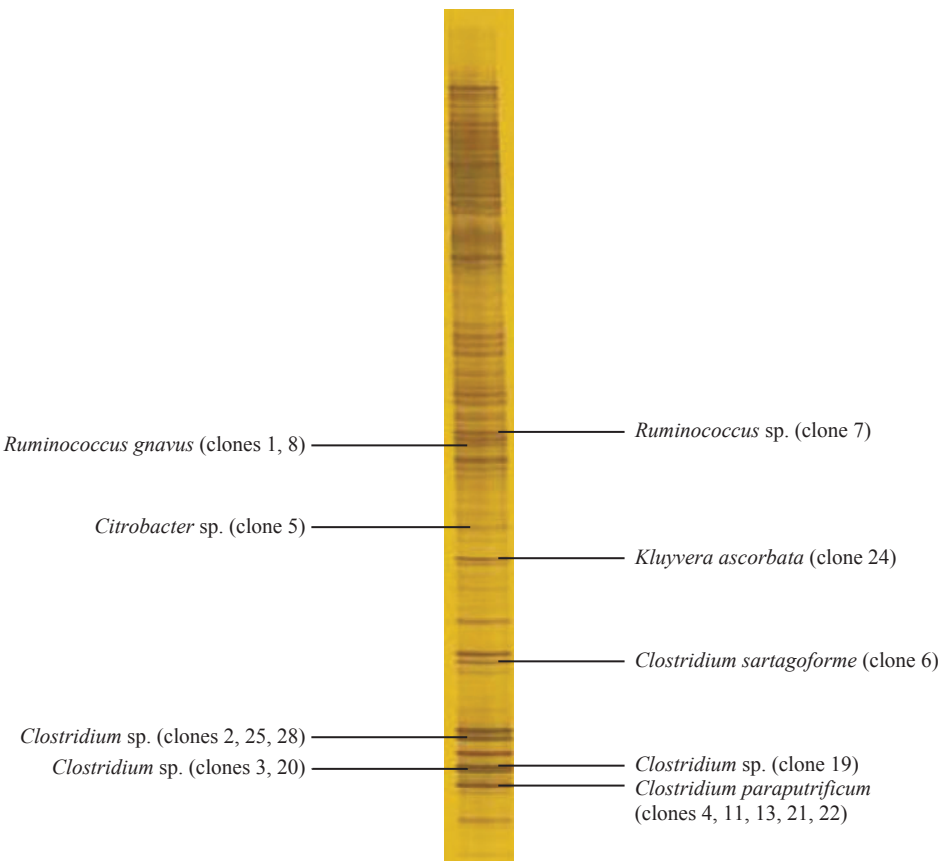

Fig. 10. Identification of 17 individual bands in the DGGE bacterial profile of a representative faecal sample collected from a free-living *I. macrourus*.

Discussion

The bacterial profile of the small intestine of captive *I. macrourus* was consistently different from the bacterial profiles of samples from the caecum, proximal colon and distal colon irrespective of diet. Profiles of bacteria in the small intestine showed less diversity than the bacterial profiles of the caecum, proximal colon and distal colon, indicating the hindgut as the major site of microbial fermentation. Diet showed some effect on the overall diversity of bacteria along the gastrointestinal tract. Profiles of bacteria for the proximal colon and distal colon generally clustered together by diet. The bacterial profiles of bandicoots fed the herbivorous diet generally clustered together although with less consistency than the profiles of bacteria of *I. macrourus* fed the insectivorous and omnivorous diets. This may be due to individual feed consumption and selectivity by bandicoots, particularly for animals fed the omnivorous diet. While the herbivorous dietary components were blended to minimise selectivity, the mealworms were not. For bandicoots fed the omnivorous components, 50% of their diet was blended herbivorous dietary components and 50% live mealworms. The *I. macrourus* fed the omnivorous diet generally consumed all the mealworms and consumed varying quantities of the herbivorous ration and this may account for the variability in bacterial profiles from bandicoots fed the insectivorous and omnivorous diets.

Most peramelid species exhibit seasonal dietary preferences associated with feed availability. Changes in the major dietary components in a free-living population of *I. macrourus* would likely alter their gastrointestinal microorganisms. It was expected that gastrointestinal bacteria would alter during the provision of each of the experimental diets to captive *I. macrourus*. Even though the bacterial profiles from captive bandicoots fed the herbivorous diet generally clustered together and the omnivorous and insectivorous diets together, the results from this study did not show distinct bacterial profiles for each experimental diet. Plant material is consumed year round and increases in proportion during winter for free-living *I. macrourus* (Gott 1996) so it could be assumed that purely herbivorous- and purely insectivorous-based captive diets do not accurately mimic diets consumed by free-living bandicoots. It can be assumed that the bacterial profiles presented in this study, from the captive *I. macrourus* fed omnivorous diets, provide a likely representation of the gastrointestinal bacteria present in free-living bandicoots. In this current study on *I. macrourus* most clones have been shown to belong to the phylum Firmicutes and significantly less belong to the phylum Bacteriodetes.

Although somewhat limited in scope, it was expected that bacterial profiles for faecal samples from the same *I. macrourus* collected during the same trapping period would be similar and cluster together. This was not the case. Bacterial profiles from faecal samples from the first capture of an individual free-living *I. macrourus* clustered separately from bacterial profiles from faecal samples from subsequent captures of the same bandicoot in the same trapping period. It was expected that bacterial profiles of faecal samples collected from bandicoots between trapping periods (i.e. April and August) would be different and not cluster together. There is some evidence that this may have been the trend.

A positive correlation could be expected between gastrointestinal bacteria and those present in faecal samples. The analysis of faecal samples from free-living *I. macrourus* provides a relatively non-invasive method to monitor changes in bacteria from the gastrointestinal tract in free-living and captive bandicoots. This study showed the consistent clustering, hence similarity, between bacterial profiles from the proximal colon and distal colon samples. The profiles for the proximal colon and distal colon were, in turn, similar to the bacterial profile of the caecum in captive *I. macrourus.* The distinction between the proximal colon and distal colon was taken to be the point at which the ingesta were formed as faeces (McClelland *et al*. 1999; Gibson and Hume 2000). It is likely that the bacterial profiles from faecal samples of free-living *I. macrourus* represent the bacteria present in the lower gastrointestinal tract.

Clostridium species are common bacteria and have a wide range of functions within the gastrointestinal tract (Stewart *et al*. 1997). *C. paraputrificum* (Clones 4, 11, 13, 21 and 22) is a gas-producing, anaerobic, gram-positive bacterium that has been isolated from the forestomach of *Macropus giganteus* (eastern grey kangaroo) (Ouwerkerk *et al*. 2005). In humans, *C. paraputrificum* has been linked to negative effects on gastrointestinal health but has also been reported to utilise chitin (Šimůnek *et al*. 2002). A large proportion of the exoskeleton of arthropods, which includes insects, is made up of chitin (Hume 1999). As the diet of *I. macrourus* predominantly comprises invertebrates, the role of *C. paraputrificum* is assumed to have a similar chitinolytic role as previously described in humans.

Ruminococcus are non-motile, gram-positive, anaerobic bacteria that are generally responsible for the fermentation of cellulose in the rumen (Stewart *et al*. 1997). *R. gnavus* has been isolated from the rumen of suckling lambs (Rieu-Lesme *et al*. 1996) and has regularly been found in the gastrointestinal tract of humans (Hata and Smith 2004). *R. gnavus* (Clones 1 and 8) and other unidentified *Ruminococcus* clones (7, 15, 16 and 29) in the gastrointestinal tract of *I. macrourus* are likely to have roles in degrading dietary plant material.

The incidence of identity matches of bacteria isolated from the faeces of free-living *I. macrourus* with culturable bacteria was high in this study. Many decades of research, largely on domestic animals, have provided a substantial database of culturable bacteria. It is not common to have such a high occurrence of matches for culturable bacteria from cloned material. For example, recent research on the association with rumen microbial protein and the microbial community structure within the rumen did not identify a single match with any culturable bacteria at a high similarity (>97%) (Harper 2011). However, four sequences showed a 100% homology with previously reported but uncultured gastrointestinal tract bacteria from grazing animals (Harper 2011). Greater sequencing effort in future research is likely to increase the accurate identification of more bacterial species in *I. macrourus*.

The faecal samples collected from free-living *I. macrourus* in both April and August exhibit common bands in their bacterial profiles. Some of these bands are present in the bacterial profiles from segments of the gastrointestinal tract of captive *I. macrourus*. This suggests that these bands may form

part of the core gastrointestinal microbiome of *I. macrourus*. However, this will require further research to corroborate.

This study has provided the first investigation into the bacteria present in the gastrointestinal tract and faeces of a peramelid marsupial. Identifying the microbial community is an important first step in studying the gastrointestinal ecosystem (Zoetendal *et al*. 2004). The understanding of the microbial community structure of an animal greatly increases our knowledge of nutritional physiology. The effects of changing diets or dietary components and the subsequent changes in the microbial community structure and the roles of individual bacteria may help explain nutrient absorption and the overall effect these factors have on the host animal. Decades of research on gastrointestinal tract microbes in domestic animals has led to changes in the management of these animals, particularly in regard to diet. Research on marsupials can benefit from the discoveries of previous research on domestic animals and can identify the 'normal' microbial community structure and improve our knowledge of the nutritional physiology of these species. The bacterial profiles of various gastrointestinal segments and faecal samples from *I. macrourus* are diverse. *Clostridium* and *Ruminococcus* species dominated the identifiable bacteria in this research. Several individual bacterial species have been identified but further research, particularly utilising next-generation sequencing, is required to expand this knowledge and to catalogue the microbiome of *I. macrourus*.

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