RESEARCH ARTICLE

Age-related differences revealed in Australian fur seal *Arctocephalus pusillus doriferus* gut microbiota

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Abstract

The gut microbiota of Australian fur seals (*Arctocephalus pusillus doriferus*) was examined at different age classes using fluorescent *in situ* hybridisation (FISH) and 16S rRNA gene pyrosequencing. The FISH results indicated that in the fur seal groups, the predominant phyla are *Firmicutes* (22.14–67.33%) followed by *Bacteroidetes* (3.11–15.45%) and then *Actinobacteria* (1.4–5.9%) consistent with other mammals. Phylum *Proteobacteria* had an initial abundance of 1.8% in the 2-month-old pups, but < 1% of bacterial numbers for the other fur seal age groups. Significant differences did occur in the abundance of *Clostridia*, *Lactobacilli* and *Bifidobacteria* between 2 months pups and 9 months pups and adult fur seals. Results from the 16S rRNA gene pyrosequencing supported the FISH data and identified significant differences in the composition of *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia* and *Fusobacteria* at all ages. Class *Clostridia* in phylum *Firmicutes* dominates the microbiota of the 2 months and 9 months seal pups, whilst class *Bacilli* dominates the 6 months pups. In addition, a high level of dissimilarity was observed between all age classes. This study provides novel insight into the gut microbiota of Australian fur seals at different age classes.

Introduction

Defining the diverse microbial ecology of the gastrointestinal tract (GIT), collectively referred to as the gut microbiota (Tannock, 2002), is paramount to determining its effects on host metabolism and health (Rinttilä *et al.*, 2004; Blaut & Clavel, 2007). The indigenous gut microbiota assist in the fermentation of indigestible carbohydrates (Hooper *et al.*, 2002), vitamin synthesis, uptake of and biotransformation of dietary nutrients, development of the host immune system (Cebra, 1999; Leser & Molbak, 2009), angiogenesis (Stappenbeck *et al.*, 2002), energy storage (Gibson, 1999; Backhed *et al.*, 2004; Ley, 2010) and embryonic development (Rawls *et al.*, 2004). Disturbances in the gut microbiota drastically result in chronic pathologies such as obesity and inflammatory bowel disease (Guarner & Malagelada, 2003; Swidsinski *et al.*, 2005; Ley *et al.*, 2008; Turnbaugh *et al.*, 2008). Despite the significance of these effects, the biological mechanisms that influence the gut microbiota composition and gut health are largely unknown in vertebrates and invertebrates (Benson *et al.*, 2010; Reid *et al.*, 2011).

The predominant gut microbiota in healthy mammals is dominated by three phyla: *Firmicutes*, *Bacteroidetes* and to a much lesser extent *Actinobacteria* (Lay *et al.*, 2005). *Firmicutes* represents the largest bacterial phylum (around 80% of the gut microbiota) and contains 250 genera, including *Lactobacillus* spp., *Mycoplasma* spp., *Bacillus* spp. and *Clostridium* spp. (Tremaroli & Bäckhed, 2012). The phylum *Bacteroidetes* includes *c.* 20 genera such as the predominantly obligate anaerobes from the genus *Bacteroides*, representing 17–20% of the total gut microbiota (Gibson, 1999). The phylum *Actinobacteria* accounts for about 2–3% of the microbiota and includes *Bifidobacterium* spp. and *Coriobacteriaceae* spp. Despite differences in vertebrate groups and digestive physiology, many members of these phyla are also found as symbionts in the GIT of reptiles and amphibians (Bjorndal, 1997). However, there have been few studies on the natural gut microbiota in marine mammals (Glad *et al.*, 2010).

FEMS MICROBIOLOGY ECOLOGY

Marine pinnipeds (true seals, fur seals and sea lions) evolved from a terrestrial ancestor (Bonner, 1984) and have many physiological and ecological adaptations compared with land-based mammals. The transition from land to water has led to changes in their metabolic activity, lactation patterns and dietary behaviour. As with other carnivores, marine mammals have a high-protein diet and a diet high in polyunsaturated fatty acids (PUFAs; Hume *et al.*, 2004), which may influence the gut microbiota composition (Maslowski & Mackay, 2011). However, the ecology and composition of the gut microbiota of marine mammals have not been fully investigated in Australian fur seals particularly during developmental stages.

The Australian fur seal (*Arctocephalus pusillus doriferus*) breeds in South Eastern Australia on nine islands in the Bass Strait (Kirkwood *et al.*, 2005) and has a diet consisting predominantly of fish and cephalopods (Deagle *et al.*, 2009). They have a lactation period typical to that of otariid seals (fur seals and sea lions) lasting 10–11 months (Arnould & Hindell, 2002). Neonatal pups cannot swim adequately and are restricted to a terrestrial existence, surviving on milk provided by their mother upon return to the colony after foraging (Arnould & Hindell, 1999, 2002; Spence-Bailey *et al.*, 2007). At 9 months of age, pups can dive to the same depths as adults, but they do not have the same physiological capacity as adults and are limited in dive duration (Spence-Bailey *et al.*, 2007; Deacon & Arnould, 2009). Currently, there is no information on how the transition from a milk-based diet to that of fish and cephalopods influences the gut microbiota in marine vertebrates such as fur seal pups and adults.

Diversity and dynamics of the predominant bacterial phylotypes in Australian fur seal colonic microbiota present in faecal samples (scats) at four time points in development stages, that is, 2 months, 6 months, 9 months of age, and adults were analysed using fluorescent *in situ* hybridisation (FISH) and 16S rRNA gene pyrosequencing. In doing so, this study characterises changes in the gut microbiota in Australian Fur seals during development.

Materials and methods

Collection of seal samples

Faecal samples for both fur seal adults and pups were collected from the Kanowna Island breeding colony (39.15′ 47″S 146°31′08″E) located near the southern tip of Wilson's Promontory, Victoria, Australia. Samples were collected from individuals at four different ages: 2 months of age ($n = 4$), 6 months of age ($n = 4$), 9 months of age ($n = 9$), which is around the time of weaning (Arnould & Hindell, 2002), and from female adults ($n = 4$).

All animals ($n = 16$) were captured by hand or by a modified hoop net (Fuhrman Diversified, Seabrook, TX), and all were considered normal healthy animals. Approximately 2–3 g, wet weight, of faecal sample ('scats') was collected by scooping fresh faeces from the cloaca and delivered to the specimen container (Techno Plas). The samples were immediately frozen at −20 °C until they were returned to the laboratory where they were stored at −80 °C (Revco, Legali Refrigeration System) for later FISH and DNA extraction for pyrosequencing. Deakin University animal ethics was approved for the study.

FISH sample preparation

Approximately 0.5 g of the thawed faecal samples from the fur seal pups (2-month-old and 9-month-old pups were selected for this part of the study) and adults were suspended in 4.5 mL of filtered 1X phosphate-buffered saline at 5 °C and then homogenised on a vortex mixer (Raytek Pty Ltd) for 3 min with four glass beads of diameter of 4 mm as described by Smith *et al.* (2006). The sample was then centrifuged at low speed of 1500 ***g*** for 1 min, in a refrigerated centrifuge (Biofuge) to remove the large particles. A 1 mL aliquot of the supernatant, taken at least 1 cm below the surface, was removed and mixed with 3 mL of freshly prepared 4% paraformaldehyde (PFA) solution. The supernatant was fixed overnight at 4 °C. The sample was then mixed thoroughly and divided into 0.6 mL aliquots for storage at −80 °C until use. These represent the PFA stocks and are 40 times diluted.

Microscope slides (25 × 75 mm; Sigma) were coated with Vectabond reagent for tissue section adhesive (Vector laboratories Inc., Burlingame) dried at room temperature then fixed with 96% ethanol for 10 min following the protocol as described by Vector laboratories. The slides were repackaged for storage until further use. Immediately prior to use a series of eight 1 cm × 1 cm grids were drawn on the slides with a hydrophobic pen. These 'wells' allowed the application of multiple samples to the slide without their mixing. Diluted PFA samples were thawed on ice, vortexed thoroughly, and a 10 μL aliquot was spread over the complete surface of a one 1 cm × 1 cm well drawn on the coated slides. The samples were dried at room temperature and then fixed with 96% ethanol for 10 min.

FISH analysis of faecal samples

FISH analysis was performed as previously described (Smith *et al.*, 2006). FITC-labelled 16S rRNA gene probes used in the study are listed in Supporting Information,

Table S1. Relative abundance of the gut bacteria in the different seal samples using the FITC-labelled probes was normalised against the total bacteria probe (Bac338). For hybridization studies, the probes were diluted to a concentration of 10 ng μL^{-1} in hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl: pH 7.5, 0.1% w/v sodium dodecyl sulphate) at 50.0 °C. Aliquots of 20 μL of the diluted probe were then added to each well and allowed to hybridise for 16 h at 50.0 °C. Total counts of cells labelled with DAPI and the Bact338 probe, as well as the Erec482 probe were both performed at dilutions of 1 : 400 or 1 : 800. EC1531 and Bac303 samples varied in the best dilution for hybridisation. The Bif164, Chis150-Clit0135 and Lab158 probes were used at a dilution of 1 : 40. For *Lactobacillus* spp., the Lab158 probe hybridisation involved pretreatment with 10 μL of Labmix (1 mg mL^{-1} lysozyme, 1 mg mL^{-1} lipase) for 50 min at 50 °C to allow cell permeabilization for the probe to bind to the bacterial DNA (Harmsen *et al.*, 1999). The Bac303 probe hybridisation was undertaken for 2 h at 45 °C.

Visual counting

Fluorescently labelled cells for each type of bacteria were determined using a fluorescent microscope (Olympus IX70) with × 100 oil lens; excitation was with a mercury lamp with a UV excitation filter for the DAPI stain and a blue excitation filter for FITC for the probes. Positive cells were identified by the fluorescence present under the microscope based on probe hybridisation to the specific bacteria. Twenty-five fields of view were counted in each 1 × 1 cm well. Photographs were taken of each field of view using the camera on the microscope (Optronics) and computer software (MagnaFire), and the cells were counted using the cell counting software 199 package (Image-Pro express/ImageJ).

DNA extraction and analysis via 16S rRNA gene pyrosequencing

For pyrosequencing, total genomic DNA was extracted from 12 faecal samples, including 2-month-old pups ($n = 4$), 6-month-old pups ($n = 4$) and 9-month-old pups ($n = 4$) using the Qiagen™ QIAamp DNA Stool mini kit (Hilden, Germany) as per the manufacturer's instructions. Quantitation of the genomic DNA was determined by a Nanodrop spectrophotometer. Four samples per age class were chosen for 16S rRNA gene pyrosequencing and prepared at Engencore according to Roche protocol used for pyrosequencing. DNA was also extracted from adult seal faeces, but the DNA was too degraded for sequencing. All samples/age class were pooled together with the attachment of MID tag barcodes (i.e. Barcode 338R_BC0491 'TCAATCTAGCGTCATGCT' was attached to all 2 months). Samples were then amplified using universal primers Roche adapter A (5′-GCC TCC CTC GCG CCA TCA GT-3′) and reverse 338R (5′-CAT GCT GCC TCC CGT AGG AGT-3′). Following amplification, samples were sequenced on the Roche/454 GS FLX Titanium Genome Sequencer by Engencore according to Fierer *et al.* (2008). All sample preparations and sequencings were performed by Engencore according to the Roche 454 and Fierer *et al.* (2008) protocol. Following sequencing, barcodes were removed using Roche sff software (Roche Applied Science, Indianapolis, IN). Rarefaction curves (see Fig. S1) highlighted that 10 000 reads per sample were sufficient for analyses. Output sequence files were converted to fastA format, and data were filtered on qual file, trimmed to remove non-16S rRNA gene sequences and sequence alignment, identification and operational taxonomic unit (OTU) classification was performed by Ribocon GmbH (Germany). The final data set comprised 75 187 sequences (see supplement Data S1). Sequences have been submitted to the EMBL database under accession number ERP001886. Data mining was performed in Calypso version 3 (http://bioinfo.qimr.edu.au/calypso/). Very low abundant OTUs that likely represent sequencing errors or extremely rare bacterial groups were excluded. Only those OTUs were included that represented > 0.5% of reads in at least one sample. The coverage of the original communities by sequence reads was assessed in a rarefaction analysis; 16S rRNA gene sequences were randomly drawn from each sample, and the number of observed OTUs was plotted vs. the number of randomly drawn reads. Subsequently, the number of reads assigned to each OTU were normalised by the total number of reads obtained for each sample and global community composition profiles were compared by principal component analysis (PCA).

Statistical analyses

All statistical analysis was carried out using the spss Statistical Package. A one-way analysis of variance (anova) was used to test each FISH probe for significance between the different groups of seals using a *post hoc* Tukey analysis. Statistical significance was determined as P values < 0.05.

Results

Microbiota composition related to fur seal age

Mean bacterial counts from FISH analysis for the 2 months, 9 months and adult fur seal age groups

Table 1. Composition of the seal microbiota compared in the three different age groups using FISH

	Mean number of cells per gram of faeces (± SEM) $\log_{10}$ CFU g^{-1}		
Stain or Probe	2-month-old pups	9-month-old pups	Adult seals
DAPI	10.24 ± 9.28	10.12 ± 9.46	10.01 ± 8.79
Bact338	10.06 ± 9.23	10.17 ± 8.82	9.68 ± 8.98
Bac303	8.60 ± 7.11	8.27 ± 7.58	9.02 ± 8.33
Erec482	9.43 ± 7.98	9.73 ± 8.29	9.52 ± 9.00
Chis150/Clit 0135	7.07 ± 6.21**	7.09 ± 5.80	6.67 ± 5.28**
EC1531	8.24 ± 7.76	8.03 ± 7.78	7.69 ± 6.81
Bif164	7.42 ± 6.09	7.57 ± 6.75***	6.56 ± 5.97***
Lab158	8.05 ± 7.29	8.31 ± 7.28 ***	7.50 ± 6.16***
ATO 291	8.10 ± 7.49*	8.87 ± 8.04*,***	8.18 ± 7.31***

Results are expressed as mean wet weight $\log_{10}$ CFU g^{-1} ± standard error of the mean (SEM). Tukey *post-hoc* analysis was performed.
*Significance at $P < 0.05$ between 2-month old pups and 9-month old pups.
**Significance at $P < 0.05$ between 2-month old pups and adult seals.
***Significance at $P < 0.05$ between 9-month old pups and adult seals.

analysed are summarised in Table 1. There was little difference in the total DAPI stain count, and the total bacterial population detected using the Bact338 probe from 2-month-old pups to adult fur seals. Consistent with other mammals, the phylum *Firmicutes* (based on the sum of the specific probes, Erec 482 plus Chis 150/Clit 135 plus Lab 158) showed these bacteria to be the predominant bacteria in all age groups (Table 1). Significant differences in the *Clostridium perfringens* and *Clostridium difficile* groups (Chis150/Clit 135 probes) in this phylum were observed between 2 months and adults ($P = 0.050$; Table 1). Significant differences were also observed in the numbers of the genera *Lactobacillus* and *Enterococcus* (Lab 158) between 9 months pups and adult seals ($P = 0.006$; Table 1).

The *Bacteroides–Prevotella* group (Bac 303 probe) was the next predominant phylum followed by phylum *Actinobacteria* and then phylum *Proteobacteria* (Table 1). Significant differences were found in the phylum *Actinobacteria* as judged by both the Ato 291 and Bif 164 probes for between age groups (Table 1). There was a significant change in the *Atopobium* cluster (Ato 291 probe) between 2 months and 9 months ($P = 0.015$) and again between 9 months and adult ($P = 0.016$). The *Bifidobacterium* spp. (Bif164 probe) showed a similar change between the 9 months and adult seals ($P = 0.011$) The EC1531 probe for *Enterobacteriaceae* (phylum *Proteobacteria*) showed a steady and consistent decrease in population numbers from 2 months to adulthood (Table 1).

Comparison of population percentages for each group of faecal bacteria

The relative abundance by FISH analysis of each bacterial phyla, class and genera tested in the seal gut microbiota can be seen in Table 2 where the bacterial numbers detected by the probes were normalised against the Bac338 total bacterial cell count. *Firmicutes* (combined Erec482, Chis150/Clit0135, and Lab 158 probes) as a percentage of total bacterial counts was 22.14%, 38.83% and 67.33%, for the 2 months, 9 months and adult seals, respectively (Table 2). The other probes within *Firmicutes* (Chis150/Clit0135, and Lab 158) showed < 1% of bacterial numbers for all age groups.

Using the Bac303 probe to detect the phylum *Bacteroidetes* (*Bacteroides–Prevotella* group), the percentage of total bacterial counts was 3.11%, 1.54% and 15.45% for the 2 months, 9 months and adult seals, respectively. Phylum *Actinobacteria* was represented by the genera *Atopobium* (ATO 281 probe) in the order *Coriobacteriales* and genera *Bifidobacteria* (Bif164 probe) in the order *Bifidobacteriales*. *Actinobacteria* demonstrated 1.42%, 5.95% and 3.41% for the 2 months, 9 months and adult seals, respectively. The EC 1531 probe, representing phylum *Proteobacteria* (class *Gammaproteobacteria*), demonstrated 1.82% for 2 months pups but < 1% of bacterial numbers for 9 months and adult fur seals and phylum *Fusobacteria* was not studied.

The total microbiota detected using specific probes was 28.49% in the 2 months, 45.58% in 9 months and 87.04% in the adult seals as compared with the total Bact338 cell count. These results suggest that the bacteria enumerated by FISH probes represent a very low percentage of total bacteria initially in the 2 months implying that the probes especially Erec482 (*Firmicutes*) does not facilitate recognition of nor variation in a number of bacterial species from within the phylum *Firmicutes* as well as other bacterial phyla tested in pups compared with the adults. To further examine the bacterial phyla abundances during development, seal pup samples from 2 months,

Table 2. Relative composition of the gut microbiota in seals of different ages using FISH analysis normalised against total bacteria (Bac338) probe in comparison with pyrosequencing

Target microorganism	Comparison of percentage bacteria							
	2-month-old pups		6-month-old pups		9-month-old pups		Adults	
Phylum	FISH probes*	Pyrosequencing	FISH probes*	Pyrosequencing	FISH probes	Pyrosequencing	FISH probes	Pyrosequencing
Actinobacteria	1.42	1.54	nd	2.36	5.95	4.85	3.41	nd
Bacteroidetes	3.11	4.32	nd	6.05	1.54	9.46	15.45	nd
Bacteria candidate division TM7	nd	0.05	nd	0.02	nd	0.00	nd	nd
Cyanobacteria	nd	0.62	nd	1.75	nd	0.08	nd	nd
Firmicutes	22.14	83.3	nd	87.1	38.83	82.5	67.33	nd
Fusobacteria	nd	0.01	nd	0.66	nd	0.01	nd	nd
Planctomycetes	nd	0.00	nd	0.00	nd	0.00	nd	nd
Proteobacteria	1.82	3.86	nd	0.83	0.29	1.58	0.85	nd
Tenericutes	nd	0.07	nd	0.22	nd	0.05	nd	nd
Verrucomicrobia	nd	5.35	nd	0.12	nd	0.02	nd	nd
Unclassified	nd	0.93	nd	0.87	nd	1.41	nd	nd
Sum of bacteria	28.49	100	nd	100	45.58	100	87.04	nd

nd, not determined.

*Sum of specific probes compared with Bact338 for FISH analysis of Phyla; *Actinobacteria*, Bif164 plus ATO291; *Bacteroidetes*, Bac 303; *Firmicutes*, Erec 482 plus Chis 150/Clit 135 plus Lab 158; *Proteobacteria*, EC1351.

6 months and 9 months were examined using 16S-rRNA-gene-based pyrosequencing.

Composition of gut microbiota using 16S rRNA gene pyrosequencing

A total of 12 163 16S rRNA gene sequences for 2 months, 26 234 sequences for 6 months and 37 460 sequences for 9 months were amplified from the faecal samples. Taxonomically, 10 classified phyla consisting of more than 77 OTUs were observed: *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Fusobacteria*, *Planctomycetes*, *Proteobacteria*, *Tenericutes*, *Verrucomicrobia* and the newly classified bacterial candidate division TM7 and unclassified bacteria (Table 2). Consistent with the FISH analyses of the 2 months and 9 months, the most abundant bacterial phyla present in all three pup stages included: *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*, with *Firmicutes* (83.3%), *Bacteroidetes* (4.32%), *Actinobacteria* (1.54%) and *Proteobacteria* (3.86%) dominating the microbiota of the 2 months. *Firmicutes* (87.1%), *Bacteroidetes* (6.05%) and *Actinobacteria* (2.36%) dominate the 6 months, whilst *Firmicutes* (82.5%), *Bacteroidetes* (9.46%) and *Actinobacteria* (4.85%) dominate the 9 months. Phylum *Verrucomicrobia* was present in 2 months (5.35%) but was < 1% in 6 months and 9 months (Table 2). Phylum *Cyanobacteria* (1.75%) and *Fusobacteria* (0.66%) were present in 6 months but were < 1% and 0.02%, respectively in 2 months and 9 months (Table 2).

The Erec482 probe designed to detect the *Ruminococcus–Eubacterium–Clostridium* cluster (cluster XIVa) was unsuitable to identify the major bacteria within the phylum *Firmicutes* in 2 months compared with pyrosequencing (Table 2). 16S rRNA gene sequence data were then further analysed to class, family and genus levels within each of the bacterial phyla but especially to look at major differences in the OTUs of the dominant classes (*Clostridia*, *Bacilli* and *Erysipelotrichi*) of phylum *Firmicutes*. Class *Clostridia* seemed to be dominant in 2 months (71.4%) and 9 months (63.6%) in contrast to class *Bacilli* in 6 months (52.7%; Fig. 1). Analysis of OTUs at the dominant families level between the different age groups demonstrated *Coriobacteriaceae* to be the dominant family within the phylum *Actinobacteria* at 2 months (1.26%), 6 months (2.16%) and 9 months (4.58%), whilst the dominant family in the phylum *Bacteroidetes* was *Bacteroideaceae* (mainly from genus *Bacteroides*) at 2 months (3.84%), 6 months (5.03%) and 9 months (1.09%; Fig. 2). In class *Bacilli*, the dominant families of *Lactobacillaceae* and *Streptococcaceae* varied at 2 months (0.024% and 2.08%, respectively), 6 months (44.47% and 7.94%, respectively) and 9 months (2.99% and 7.45%, respectively). In class *Clostridia*, there were three dominant families: *Lachnospiraceae*, *Peptostreptococcaceae* and *Ruminococcaceae*. Seal pups demonstrated significant variation with the families *Lachnospiraceae* at 2 months (18.5%), at 6 months (7.11%) and at 9 months (23.35%), respectively; in the family *Peptostreptococcaceae* 1.67% at 2 months, 4.54% at 6 months and 0.15% at

9 months; and in the family *Ruminococcaceae* which includes *Faecalibacterium* and *Subdoligranulum* contained 49.6% at 2 months, 17.92% at 6 months and 36.4% at 9 months. In class *Erysipelotrichi*, the family *Erysipelotrichaceae* had 9.33% of bacterial phylotypes at 2 months, 2.42% at 6 months and 8.01% at 9 months (Fig. 2). To explore similarities in OTU data set, a principal coordinates analysis (PCoA) of seal pups at 2 months, 6 months and 9 months showed a high level of dissimilarity between all three seal pup groups based on OTUs (Fig. 3).

Discussion

The dominant gut microbiota in Australian fur seals were a *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* with *Firmicutes* representing 80% of the total microbiota consistent with levels in humans and other mammals but with differing proportions at different ages of the seals. Data obtained in this study by the two methods were comparative for most age groups but whilst FISH analysis can make observations on trends in major bacterial groups, it does not observe specific changes in many genera and species. FISH analysis using the Erec 482 and Chis 150/Clit 0135 probes, specific for the *C. histolyticum* and *C. lituseburense* groups, did not identify many *Clostridiales* in 2 months pups but was more consistent with pyrosequencing results when looking at 9 months pups and adults.

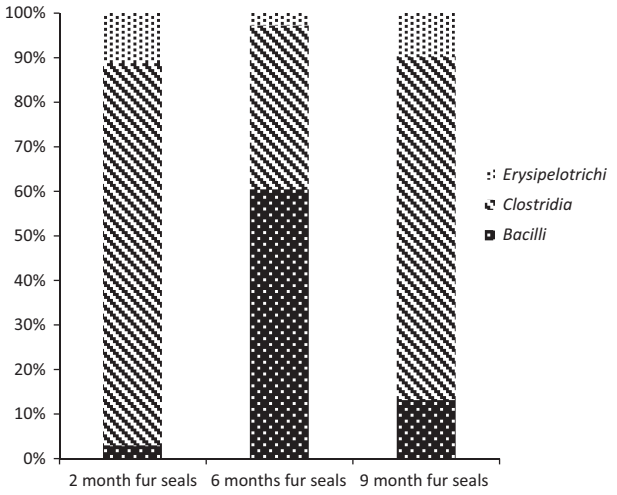

Fig. 1. Variation in abundance of dominant classes within phylum *Firmicutes* in seals at 2 months, 6 months and 9 months of age using pyrosequencing. Dominant classes in phylum *Firmicutes* vary in their composition at the different ages.

Pyrosequencing provided a more detailed and robust analysis of changes in the bacterial phylogenetic diversity during developmental stages of the Australian fur seal. Phylum *Firmicutes* was dominant with class *Clostridia* dominating at 2 months and 9 months and class *Bacilli* at 6 months. Within these classes, there were significant population changes in the seal pups at different ages within the families of *Lactobacillaceae* and *Streptococcaceae* (class *Bacilli*), *Lachnospiracaeae*, *Peptostreptococoaceae* and *Ruminococcaceae* (class *Clostridia*) and *Eryipelotrichaceae* in class *Erysipelotrichi*.

The percentage microbiota were detected by the FISH probes in the 2 months (21.85%) but much higher levels in the 9 months (56.6%, and adult fur seals (77.89%), respectively. The population of *Firmicutes* (*Eubacterium rectale–Clostridium coccoides* group) increased from 9 months to adult fur seals. These bacteria were associated with energy production from carbohydrate utilisation in land-based mammals, which if not used is stored as adipose tissue (Tap *et al.*, 2009).

The microbial composition of bacteria in seal pups compared with adults may be influenced by differences in

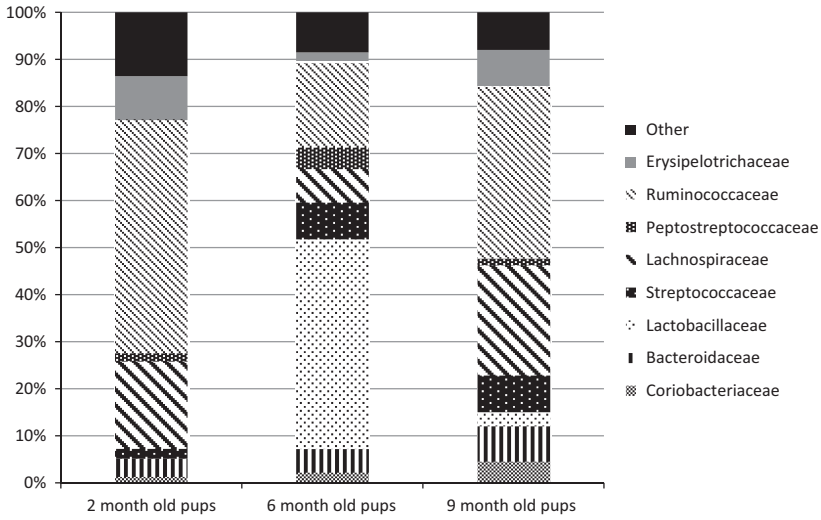

Fig. 2. Comparison of major families of *Erysipelotrichaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, *Streptococcaceae*, *Lactobacillaceae*, *Bacteroidaceae*, *Coriobacteriaceae* and other in bacterial phyla present in Australian fur seal pups at 2 months, 6 months and 9 months of age.

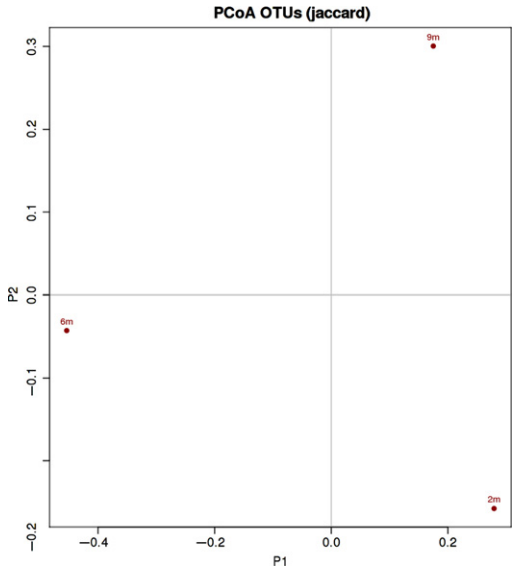

Fig. 3. PCoA of seal pups at 2 months, 6 months and 9 months of age. Analysis shows a high level of dissimilarity between all three seal pup groups based on OTUs. 2m = 2-month-old pups; 6m = 6-month-old pups; 9m = 9-month-old pups.

diet in seals where the initial milk-based diet for seal pups, rich in protein and lipids, is replaced by a solid marine-based diet in weaned adults (Arnould & Hindell, 1999, 2002). Long chain polyunsaturated fatty acids (LC-PUFAs), such as those found in human milk, promote the growth of lactic acid bacteria, such as *Bifidobacteria* and *Lactobacilli* (Das, 2002). Arnould & Hindell (1999) found that fur seal milk has a high lipid content containing PUFAs, and Endo *et al.* (2006) have demonstrated that PUFAs (α-linolenic, eicosapentaenoic and docosahexaenoic acids) strongly inhibit *Lactobacillus* growth, but conjugated FAs with *trans* double bonds promote growth (Endo *et al.*, 2006).

FISH analysis using Lab158 showed there was a significant difference ($P = 0.006$) between the number of *Lactobacillus* at 9 months pups and adults. Pyrosequencing data also support the FISH analyses in that *Lactobacilli* were in low numbers in pups at 2 months of age and again later in adult fur seals. However, there were 11 658 sequences of 26 234 sequences in the scats of pups at 6 months of age corresponding to *Lactobacillus* spp. being 44.47% of all bacteria. Allowing for variation in gut microbiota in different pups, this is significantly different to the abundant sequences representing *Lactobacillus* in 2 months or 9 months pups or adult seals. Also, *Bifidobacterium* spp. do not appear to be abundant in fur seal pups nor in adults in contrast to human infants or adults (Harmsen *et al.*, 2000a, b).

The changes in microbiota may be dependent on the level of dietary PUFAs or changes in the types of PUFAs based during this period of development. Further work needs to be undertaken to examine the influence of these dietary components on the development of seal gut microbiota and assess how diet shapes the gut microbiota and physiology of seals.

Besides the Lactobacilli, compositional differences in the microbiota colonising the seal gut suggest other specific members of the microbiota may also influence host physiological responses (Ivanov *et al.*, 2008; Ivanov & Littman, 2010). Members of the families *Lachnospiraceae* and *Ruminococcaceae* within the class *Clostridia* play a crucial role in host immune function (Ivanov *et al.*, 2008; Thompson *et al.*, 2012), and these were predominant families in all seal pups (Fig. 2). Further work needs to be undertaken to assess whether bacteria in these families influence intestinal immune gene expression in seals during postnatal development such as occurs in germ-free mice after colonisation (Ivanov & Littman, 2010).

The major differences in GIT bacteria occurred in the 9 months pups (around the time of weaning). This indicates a significant change in the gut microbiota in this age group. The 2 months pups are on a strict diet of only milk with no solid food, whereas the adults are on a marine diet based on fish, squid and crustaceans. The 9 months pups diet is partly influenced by both milk and the marine-based foods. Therefore, the difference in GIT microbiota in these animals could be diet related.

The microbial distribution in seal pups and adult Australian fur seals was further compared with data from Glad *et al.* (2010) on Arctic (hooded seal, *Cystophora cristata*) and Sub-arctic seals (harbour seal, *Phoca vitulina*; and grey seal (*Halichoerus grypus*) using phylogenetic analysis of clone libraries and with the metagenome analysis by Lavery *et al.* (2012) on an Australian sea lion (*Neophoca cinerea*). Whilst the methods are significantly different, the FISH analyses (adults) and pyrosequencing (pups) indicate differences in the Australian fur seals to the other seal species. Analysis of four Australian fur seals at each developmental stage provided a suitable comparison and allowed for some control of individual gut microbial variation. Australian fur seals appeared to have similar levels of dominant *Firmicutes* (71.2% for adults and 82.5% for pups using pyrosequencing) to that of the Sub-arctic grey seal (76%) and the Australian sea lion (80%) but much greater levels compared with Sub-arctic harbour seal (50%) and the Arctic hooded seals (22%).

Bacteroidetes were significantly lower in Australian fur seals compared with the other seal types. In the Arctic and Sub-arctic seals, the phylum *Bacteroidetes* varied from 24% to 68%, but from our analyses, the Australian fur seals were between 4% and 9% consistent with the Australian sea lion (2%; Lavery *et al.*, 2012). *Proteobacteria* represented only 1–2% in the fur seals and was similar

with the data from the harbour and grey seal (Glad *et al.*, 2010) but differed to the Australian sea lion and the hooded seals (8–9% of total phylotypes). Pyrosequencing data found no major groups in the phylum *Proteobacteria* except for *Gammaproteobacteria* (410 of 12 163 sequences for 2 months; 195 sequences of 26 234 sequences for 6 months and 333 sequences of 37 460 sequences for 9 months). Our results are also consistent with the low values of *Proteobacteria* in all fur seals using FISH analysis with the 2 months fur seal pups having 1.82%, 9 months fur seal pups having 0.29% and adult fur seals 0.85% using the EC1531 probe. We found no *Aaerobiospirillum* in any age group and < 0.01% sequences for *Sutterella* in pups compared with these diverse genera in the hooded seals (Glad *et al.*, 2010).

We did not test for phylum *Fusobacteria* using FISH but our pyrosequencing demonstrated only 0.66% in 6 months and 0.01% in 2 months and 9 months consistent with the low level of this phylotype (1%) in both the hooded and harbour seals, and no detection in the grey seal (Glad *et al.*, 2010). The phylum *Actinobacteria* group did not appear to be observed in the Arctic and Subarctic seals compared with the Australian fur seals in this study using either FISH probes or pyrosequencing or the Australian Sea lion. Therefore, Australian fur seals appear somewhat different in abundance and phylotypes of gut microbiota compared with other seal species.

Conclusions

The Australian fur seal was a useful model for examining changes in the gut microbiota at different ages. The composition of the gut microbiota in wild Australian fur seals is similar to other mammals where the predominant bacterial phyla is *Firmicutes*, and to lesser extent, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* but diversifies with seal age. FISH analysis applied to determine differences and trends in the data indicated that there were many changes within the different bacterial groups and phyla including some significant changes in phylum *Firmicutes*, especially within the classes *Clostridia* and *Bacilli.* Pyrosequencing provided a more useful profile of abundance of these bacterial phyla in the fur seal pups suggesting changes, especially at 6 months, within the dominant phyla, classes and families during the suckling stage of seal development. However, further information is required to determine what factors, such as diet and genetics, influence the developing gut microbiota of seals.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Rarefaction curves of OTU reads of gut bacteria from 2 month, 6 month and 9 month pups.
Table S1. FISH probe name/target group/reference.
Data S1. Taxonomic breakdown of OTUs.