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SHORT COMMUNICATION

Analysis of the microbial diversity in faecal material of the endangered blue whale, *Balaenoptera musculus*

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Abstract Using bacterial and fungal tag-encoded FLX-Titanium amplicon pyrosequencing, the microbiota of the faecal material of two blue whales living in the wild off the coast of California was investigated. In both samples the most predominant bacterial phylum was the Firmicutes with *Clostridium* spp. being the most dominant bacteria. The most predominant fungi were members of the phylum Ascomycota with *Metschnikowia* spp. being the most dominant. In this study, we also preliminarily characterised the culturable anaerobic bacteria from the faecal material, using traditional culture and 16S rRNA gene sequencing approaches. In total, three bacterial species belonging to the phylum Firmicutes were identified.

Keywords *Balaenoptera musculus* · Blue whale · Faecal material · Microbiota

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Introduction

The blue whale (*Balaenoptera musculus*) is an endangered species (Reilly et al. 2008). These marine mammals feed mostly on low trophic level prey (Pauly et al. 1998) in spite of having high energetic requirements (Barlow et al. 2008). There are three widely recognised *B. musculus* subspecies found in the Southern Hemisphere (*B.m. intermedia*), Indian Ocean (*B.m. brevicauda*) and in the North Pacific and North Atlantic (*B.m. musculus*) (Rice 1998).

The gut microbiomes of mammals are very important to the health, and perhaps the fitness, of the animal (Ley et al. 2008; McFall-Ngai et al. 2013). For example, it has been shown that alterations in the gut microbiota of humans are associated with obesity (Le Chatelier et al. 2013; Ley et al. 2006), type 2 diabetes (Qin et al. 2012), inflammatory bowel disease (IBD) (Manichanh et al. 2012), rheumatoid arthritis (Vaahtovuo et al. 2008) and allergies (Russell et al. 2012). In this study, we set out to better understand the intestinal microbial diversity of the blue whale. Although several studies have suggested that the intestinal microbiota differs along various regions of the gastrointestinal tract (Suchodolski et al. 2005; Ritchie et al. 2008), faecal samples are much easier and more practical to collect. We characterised the community composition of the faecal material of two blue whales using bacterial and fungal tag-encoded FLX-Titanium amplicon pyrosequencing. We compared the bacterial diversity to that of other baleen whales described in

previous studies. To our knowledge this is the first study examining the fungal diversity found in whale faecal material.

Materials and methods

Sample collection

The faecal material from two adult blue whales, blue whale#1 and blue whale#2, living in the Pacific Ocean off Southern California were collected on June 25, 2015 and June 20, 2015, respectively. Visual surveys from a small rigid-hull inflatable boat were being conducted at the time of collection. When faeces were spotted on the surface in the vicinity of surfacing whales, a sample was collected with a dip net and transferred into a vial. Immediately upon return to land, the samples were stored at −80 °C.

Microbial diversity

The two blue whale faecal samples used in this study were analysed using both bacterial tag-encoded flexible-Titanium (FLX) amplicon pyrosequencing (bTEFAP®) (MR DNA Shallowater, TX) and fungal tag-encoded FLX amplicon pyrosequencing. Total genomic DNA was extracted from the faecal samples using a mini kit and the manufacturer's recommended methods (QIAamp stool DNA mini kit; Qiagen, Valencia, CA). The DNA sample was then quantified (Nanodrop Spectrophotometer; Nyxor Biotech, Paris, France).

All DNA samples were diluted to 20 ng/μL. A 1 μL aliquot of each sample DNA was used for a 25 μL PCR reaction. The 16S universal Eubacterial primers 27F (forward28F: GAG TTT GAT CNT GGC TCA G; reverse519R: GTN TTA CNG CGG CKG CTG) and fungal ITS1-F CTTGGTCATTTAGAGGAAGTAA.

ITS2 GCTGCGTTCTTCATCGATGC were used for bacterial and fungal DNA amplification, respectively. A single-step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) were used under the following conditions: 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s; 53 °C for 40 s and 72 °C for 1 min; after which a final elongation step at 72 °C for 5 min was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilising Roche 454 FLX titanium instruments and reagents and following the manufacturer's guidelines.

Sequences were depleted of barcodes and primers, quality filtered and chimera checked using the open-source computational package Qiime 1.8.0. Operational taxonomic units were defined by clustering at 3 % divergence (97 % similarity). Finally, OTUs were taxonomically classified using BLASTn against a curated database (MR DNA, Shallowater, TX) derived from GreenGenes, RDPII and NCBI (www.ncbi.nlm.nih.gov; DeSantis et al. 2006; http://rdp.cme.msu.edu).

Isolation of cultivable bacteria

In total 100 mg of faecal material was added to 9.9 ml of tris-buffered saline. 100 μl aliquots of suspension was then plated on sheep blood agar plates (Remel, Santa Fe CA, USA). The plates were incubated anaerobically at 37 °C for 2 days. Bacterial colonies were sub-cultured two separate times to ensure a pure culture was obtained.

Sequencing of the 16S rRNA gene and data analysis

To determine the sequence of the 16S rRNA gene, bacterial colonies were sent to Genewiz Inc. (South Plainfield NJ, USA). Sequences were assembled using the software DNA Baser version 3 (HeracleSoftware). For sequence identification, the assembled sequences of approximately 1300 bp were pairwise compared using the BLAST algorithm to the 16S rRNA genes of known organisms deposited in the NCBI Genbank database.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences have been deposited in the GenBank nucleotide database under the accession numbers KU531485 to KU531493. The NGS data have been deposited in the Sequence Read Archive under accession number SRP071906.

Results and discussion

Pyrosequencing for bacterial and fungal organisms

bTEFAP® was originally described by Dowd et al. (2008) and has been used to determine the bacteria present in the faecal material of a variety of animals, including cattle (Dowd et al. 2008; Callaway et al. 2010; Hristov et al. 2012), cats and dogs (Handl et al. 2011). fTEFAP has been used to determine the fungi present in animals, including poultry (Nonnenmann et al. 2010). In the present study, for bacteria a total of 25451 sequences were analysed for both animal samples with 22716 quality sequences. There were a total of 123 OTUs assigned for blue whale#1 and 140 OTUs for blue whale#2. The Shannon index, a measure of diversity, was 2.68 and 2.82 for blue whales#1 and #2, respectively. For fungi a total of 6730 sequences were analysed for both animals with 5467 quality sequences. There were a total of 102 OTUs assigned for blue whale#1 and 107 OTUs for blue whale#2. The Shannon index was 3.27 and 3.23 for blue whales#1 and #2, respectively.

The predominant bacterial phylum was Firmicutes (≥98.0 % of sequences) and the predominant fungal phylum was Ascomycota (≥99.9 % of sequences) in both blue whales (Table 1). For bacteria level, minor levels of Proteobacteria, Bacteroidetes and Actinobacteria were also detected (Table 1). At the genus level *Clostridium* spp. were the most dominant bacteria and *Metschnikowia* spp. were the dominant fungal species in both blue whales (Table 1).

Determining cultivable bacteria

To identify cultivable bacteria, an approximate 1300 bp portion of the 16S rRNA gene from five colonies obtained from the faecal material of blue whale#1 and 4 colonies from the faecal material of blue whale#2 were sequenced. From blue whale#1 all five sequences (BW1#1 to BW1#5; GenBank accession numbers KU531485 to KU531489) were determined to be from colonies of *Enterococcus faecalis*. Two sequences from blue whale#2 (BW2#1 and BW2#4; GenBank accession numbers KU531490 and KU531493) were also determined to be from *E. faecalis*. One sequence (BW2#2; GenBank accession number KU531491) was determined to be from *Clostridium perfringens* and another (BW2#3; GenBank accession number KU531492) was determined to be from *Clostridium sordellii*.

Very few studies have looked at the bacterial diversity in whales (Herwig et al. 1984; Olsen et al. 1994, 2000; Sanders et al. 2015). To our knowledge, the bacterial diversity in blue whales has not been examined. In addition, the fungal diversity present in the faecal material of any whale species has not apparently been examined. In our study, approximately ≥98 % of the sequences mapped to the Firmicutes whilst a relatively small percentage of the sequences mapped to Bacteroidetes. The gut bacterial communities in both terrestrial and marine mammals contain varying abundances of bacteria from the phyla Firmicutes and Bacteroidetes (Nelson et al. 2013). In a recent study (Sanders et al. 2015; J.G. Sanders, personal communication), using 454 pyrosequencing, this was shown to be the case in baleen whales. For example in a sei whale (*Balaenoptera borealis*) Bacteroidetes was the most dominant phylum, followed by Firmicutes. In three of four right whales the most dominant phylum was Firmicutes, followed by Bacteroidetes. In one sample the opposite was true.

Using the clone library method it was also shown in different mammals that Bacteroidetes was at a much higher percentage of the sequences compared to this study. For example, Bacteroidetes made up 24–68 % of the sequences in three seal species (Glad et al. 2010a) and have been shown to be one of the dominant groups in many faecal and intestinal studies from a variety of animals, such as the yak (*Bos grunniens*) (An et al. 2005), the Jinnan cow (*Bos taurus*) (An et al. 2005), the horse (Daly et al. 2001), the reindeer (*Rangifer tarandus platyrhynchus*) (Sundset et al. 2004) and the wild wolf (*Canis lupus*) (Zhang and Chen 2010).

The results in this study revealed that *Clostridium* spp. were the dominant bacteria in the faecal material of both blue whales (Table 1). In a study using the faecal material of polar bears all sequences were affiliated with the phylum Firmicutes, with 70 % affiliated to the genus *Clostridium* (Glad et al. 2010b). Here, a total of 123 OTUs were identified for blue whale#1 and 140 OTUs for blue whale#2. The number of OTUs can be compared to other aquatic mammals. In four Yangtze finless porpoises the number of OTUs ranged from 22 to 36 (Wan et al. 2016). The faecal material of 36 Florida manatees was examined in the early winter, mid-winter and late winter. The number

Table 1 The bacterial and fungal community composition in two blue whales at the genus level

Phylum (bacteria)	Genera (bacteria)	Blue whale#1[a]	Blue whale #2[a]	Phylum (fungi)	Genera (fungi)	Blue whale#1[a]	Blue whale #2[a]
Firmicutes	*Clostridium*	88.08	87.19	Ascomycota	*Metschnikowia*	98.17	94.55
Firmicutes	*Sarcina*	6.89	7.86	Ascomycota	*Arthroderma*	1.83	0.00
Proteobacteria	*Phocoenobacter*	1.42	0.02	Ascomycota	*Fusarium*	0.00	4.60
Firmicutes	*Enterococcus*	0.97	0.11	Ascomycota	*Trichophyton*	0.00	0.39
Firmicutes	*Oscillospira*	0.49	1.15	Ascomycota	*Pseudevernia*	0.00	0.13
Proteobacteria	*Photobacterium*	0.40	0.00	Ascomycota	*Clavispora*	0.00	0.20
Firmicutes	*Ruminococcus*	0.34	0.36	Basidiomycota	*Leucoagaricus*	0.00	0.13
Firmicutes	*Eubacterium*	0.28	1.06				
Firmicutes	*Faecalibacterium*	0.24	0.63				
Firmicutes	*Anaerovorax*	0.10	0.12				
Firmicutes	*Solobacterium*	0.10	0.14				
Actinobacteria	*Atopobium*	0.09	0.08				
Firmicutes	*Butyrivibrio*	0.09	0.05				
Firmicutes	*Granulicatella*	0.07	0.04				
Firmicutes	*Dorea*	0.07	0.01				
Firmicutes	*Erysipelothrix*	0.07	0.07				
Firmicutes	*Sporobacter*	0.06	0.00				
Bacteroidetes	*Bacteroides*	0.06	0.27				
Bacteroidetes	*Prevotella*	0.04	0.11				
Firmicutes	*Turicibacter*	0.03	0.07				
Firmicutes	*Peptococcus*	0.03	0.01				
Firmicutes	*Anaerofilum*	0.02	0.06				
Firmicutes	*Peptostreptococcus*	0.02	0.05				
Actinobacteria	*Gordonibacter*	0.01	0.06				
Firmicutes	*Acetivibrio*	0.01	0.06				
Bacteroidetes	*Barnesiella*	0.01	0.02				
Firmicutes	*Bulleidia*	0.01	0.05				
Firmicutes	*Pseudoflavonifractor*	0.00	0.04				
Firmicutes	*Blautia*	0.00	0.04				
Firmicutes	*Subdoligranulum*	0.00	0.06				
Bacteroidetes	*Parabacteroides*	0.00	0.08				
Firmicutes	*Gracilibacter*	0.00	0.06				
Bacteroidetes	*Alloprevotella*	0.00	0.04				

[a] Relative (proportion) percentage of sequences

of OTUs ranged from 155 to 638 (Merson et al. 2014). In the faecal material of two wild dugongs there were 102 and 106 OTUs (Eigeland et al. 2012).

In our study, the fungal microbiome was also characterised. Sequences associated with the phylum Ascomycota represented ≥99.9 % of the sequences in both whales. Ascomycota are found in many diverse environments, such as soils around the world (Qiu et al. 2012; Abed et al. 2013; Al-Sadi et al. 2015), food products (Liu et al. 2015), vascular plants in the High Arctic zone (Zhang and Yao 2015) and decomposing logs (Ottosson et al. 2015). In animals, this was the main fungal phylum in the vagina of Nellore cattle (Laguardia-Nascimento et al. 2015), the faecal material of dogs (Foster et al. 2013; Handl et al. 2011) and the faecal material of cats (Handl et al. 2011). In the

faecal material of four giant pandas ranging in age from 7 to 34 years, the predominant phyla were Ascomycota, with the percentage of total sequences varying from approximately 50–91 %, and Basidiomycota, with the percentage of total sequences varying from approximately 9–50 % (Tun et al. 2014). Finally, we attempted to cultivate bacteria, under anaerobic conditions, from the faecal material of the two whales. In total, five of the nine sequences were from *E. faecalis*. *E. faecalis* is widely distributed in nature, being found in soil, water, plants and foods (Byappanahalli et al. 2012; Franz et al. 2011). It is also found in the gastrointestinal tract of humans and animals (Lebreton et al. 2014). This bacterium has been isolated from the faecal material of a wild magellanic penguin (*Spheniscus magellanicus*) (Prichula et al. 2016) and swine manure (Mangalappalli-Illathu et al. 2010). We also recovered *C. perfringens* and *C. sordellii*. *C. perfringens* is found in the gastrointestinal tract of humans and many animals and can cause gas gangrene, food poisoning, myositis, enterotoxemic, and diarrheagenic diseases (Hirsh and Biberstein 2005). *C. sordellii* has also been shown to cause several different diseases in animals, such as enteritis-like disease in commercial broiler chicken flocks (Rimoldi et al. 2015), septicemia in a brown bear (*Ursus arctos*) (Balseiro et al. 2013), perinatal mortality in foals (Ortega et al. 2007) and malignant oedema in sheep (Costa et al. 2007).

The bovine rumen contains a diverse population of microorganisms that converts plant biomass to protein, short chain volatile fatty acids, and gases by ways of fermentation. End-products of rumen microbial fermentation provide the host with essential nutrients for metabolism (Flint 1997; Flint et al. 2008; Mackie and White 1990). Similar to artiodactylates, whales also possess multi-chambered stomachs (Gatesy et al. 2013; Langer 2001). A recent study by Sanders et al. (2015) showed that baleen whale microbiomes are similar in comparison to terrestrial herbivores in the abundance profiles of genes associated with fermentation. In future studies, more blue whale faecal material samples will be collected and a metagenomic analysis of the faecal microbiota will be done to answer the question which genes are associated with fermentation and other metabolic pathways. How this data compares to other whale species and to other animals needs to be determined.

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