RESEARCH ARTICLE

Interspecific variations in the faecal microbiota of *Procellariiform* seabirds

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Abstract

Despite the enormous amount of data available on the importance of gut microbiota in vertebrates (especially mammals), there is no information available on the microbiota of seabirds. *Procellariiformes* are long-lived seabirds that consume a diet high in lipids and are characterised by their ability to produce and store large amount of stomach oils through the partial digestion of prey (with the exception of the *Pelecanoididae*). Examining the faecal microbiota of three *Procellariiform* species (short-tailed shearwater, common diving petrel and fairy prion) provided a unique opportunity to not only characterise the gastrointestinal (GI) microbial composition of seabirds but to also examine the influence of stomach oils on the microbial community. The results indicated that *Procellariiform* seabirds host a highly diverse community of faecal microorganisms, dominated by three phyla (*Firmicutes*, *Proteobacteria* and *Bacteroidetes*) and that each species has its own species-specific GI microbiota. In addition, significant differences were observed in the microbial communities of oil-producing and non-oil-producing seabirds. This study is the first whole-community examination and classification of the faecal microbiota of *Procellariiform* seabirds.

Introduction

The order *Procellariiformes* contains some of the world's most threatened seabirds with 47% of the 129 living species listed as threatened by BirdLife International, with 63% of *Procellariiform* species in Australia listed as threatened by the International Union for the Conservation of Nature [International Union for the Conservation of Nature and Natural Resources (IUCN), 1994]. *Procellariiformes* are long-lived seabirds that consume a diet high in dietary lipids and are characterised by their ability to produce stomach oils (with the exception of the *Pelecanoididae*) (Canani *et al.*, 2011). Stomach oils are produced in the proventriculus through partial digestion of prey. The oil fractions of the prey are then concentrated and retained for long periods via delayed gastric emptying (Croxall, 1987). This adaptation reduces the cost of travelling long distances by providing their chick with a highly concentrated food source that is high in energy and fats (Roby *et al.*, 1989). *Procellariiformes* have a highly extensible proventriculus, small gizzard and a unique arrangement of their duodenal loop. The proventriculus acts as a separating funnel, separating the lighter lipid layer from the denser aqueous layer. The dense aqueous layer is the first to pass through the digestive tract, while the lipid layer is retained longer. In diving petrels (*Pelecanoides* spp.), however, the dorsal positioning of the pylorus and gizzard reduces the retention time of the digesta, with the lighter lipid layers emerging first (Speake *et al.*, 1999). The absence of stomach oils in common diving petrels (*Pelecanoides urinatrix*) is explained by differences in their digestive morphology in comparison with other *Procellariiformes*. In diving petrels, the retention time of digesta is thought to preclude the formation of stomach oils (Roby *et al.*, 1989).

The diet of short-tailed shearwaters (*Ardenna tenuirostris*) (STSW), fairy prions (*Pachyptila turtur*) (FP) and common diving petrels (*P. urinatrix*) (CDP) is dominated by krill (*Euphausia* spp. and *Nyctohpanes* spp.) (Prince & Copestake, 1990; Weimerskirch & Cherel, 1998; Klomp & Schultz, 2000; Schumann, 2012). Their krill-based diet is a rich source of n-3 polyunsaturated fatty acids (PUFA),

proteins and minerals (Warham, 1977). Krill is also a rich source of provitamin E, phospholipids, flavonoids, vitamin A, alpha-linolenic acid, astacin and other essential nutrients (Virtue *et al.*, 1995; Nicol *et al.*, 2000; Connan *et al.*, 2005). In avian species, diets high in n-3 PUFA are essential for normal metabolism, growth, heart health and neurological development (Kakuschke *et al.*, 2005; Zhu *et al.*, 2008).

The gut microbiota plays an important role in digestive physiology, including energy extraction, fat metabolism and storage, and host adiposity (Bäckhed *et al.*, 2004; Zoetendal *et al.*, 2004; Zaneveld *et al.*, 2008; Tremaroli *et al.*, 2010). However, the potentially important role of the gastrointestinal (GI) microbiota in digestion by procellariiform seabirds remains unstudied, even though the complex digestive physiology of procellariiform seabirds has been extensively studied (Clarke & Prince, 1976; Imber, 1976; Warham, 1977; Place *et al.*, 1989; Roby *et al.*, 1989, 1997; Connan *et al.*, 2005, 2007; Foster *et al.*, 2010). The objective of this study was to elucidate the molecular diversity and community composition of the procellariiform microbiota using 16S rRNA gene pyrosequencing.

Materials and methods

Sample collection

Faecal samples were collected from short-tailed shearwater (STSW) on Phillip Island (northern Bass Strait, Australia; 38.4833°S, 145.2333°E), common diving petrel (CDP) from Notch Island (northern Bass Strait, Australia; 38.950°S, 146.667°E) and fairy prion (FP) from Lady Julia Percy (western Bass Strait, Australia; 38.420°S, 142.000°E). All individuals were captured when returning to their colony to provision chicks after foraging trips to sea. Individuals were captured by hand in the burrow (STSW), mist nets (CDP) (Prince, 1980; Croxall *et al.*, 1997) and hand net (FP; Garthe & Furness, 2001). Faecal samples were obtained by placing a sterile swab (Copan™, Italy) into the cloaca. Swabs were frozen in liquid nitrogen and then stored at −80 °C as per Dewar *et al.* (2013).

Sample analysis

Genomic DNA was extracted using the Qiagen™ QIAamp DNA Stool Mini Kit (Hilden, Germany) following the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed to examine the abundance of four phyla (*Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*). The primer sequences and annealing temperatures for the chosen bacterial groups can be found in Dewar *et al.* (2013). The qPCR was performed on the Stratagene MX3000P. Each PCR mixture comprised of 5 μL of Brilliant II SYBR green (Stratagene™), 20 pmol μL^{-1} of forward and reverse primer, 2 ng of template DNA and made up to a final volume of 20 μL with nuclease-free water. The cycling conditions were 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s, followed by annealing and extension temperature for 30 s with all samples were run in triplicate as per Dewar *et al.* (2013). Bacterial concentration was determined by comparing the threshold value (c_t values) with a standard curve. The standard curve was created using a serial 10-fold dilution from DNA extracted from a pure culture of *Escherichia coli* ranging from 10^4 to 10^{10} colony-forming units (CFU) per gram as per Dewar *et al.* (2013).

For 16S rRNA gene pyrosequencing, four samples per species were pooled (with attachment of MID multiplexing barcode) and 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′) to amplify the V2–V3 region as previously described in Dewar *et al.* (2013). Following amplification, samples were sequenced on a Roche/454 GS FLX Titanium Genome Sequencer by Engencore according to Fierer *et al.* (2008).

Data processing and analysis

Quality control, removal of chimeras (Chimera Slayer), clustering of sequences into operational taxonomic units (OTUs; uclust_ref approach, sequences were aligned to Greengenes database using UCLUST with 97% sequence identity cut-off) and taxonomic classification assignment (RDP-Classifier) were performed using QIIME (Caporaso *et al.*, 2010) (for rarefraction curve, and quality score and quality score distribution see Figures S3–S5). The 16S rRNA gene sequences reported in this study have been submitted to EMBL under accession number ERA ERP002293. Low-abundant OTUs were excluded from subsequent analysis, that is only those OTUs were included that had > 0.005 relative abundance (assigned reads/total number of reads, Table S1) in at least one sample. Data-mining and statistical analysis was carried out in CALYPSO version 3 (https://bioinfo.qimr.edu.au/calypso/). Differentially abundant OTUs between stomach oil-producing (SOP) and nonstomach oil-producing (NSOP) seabirds were identified by ANOVA (Fig. 5). The statistical power, however, was quite low due to pooling of samples. Shannon diversity, and richness of SOP and NSOP seabirds were compared in CALYPSO by ANOVA on OTU level.

As the qPCR data did not follow a normal distribution, they were log-transformed in SPSS. To determine whether there were significant differences among procellariiform seabirds for the major phyla for qPCR, a one-way ANOVA

was performed in SPSS, and a significance level of $P < 0.05$ was chosen.

Results

The abundance of *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* was detected from DNA extracted from faecal samples of CDP ($n = 12$), FP ($n = 5$) and STSW ($n = 20$) using primers specific for each of the four phyla. Overall, there were significant differences among all *Procellariiform* seabirds for *Firmicutes* and *Bacteroidetes*, but no significant difference in *Proteobacteria* and *Actinobacteria* (Fig. 1). STSW had a significantly higher abundance of *Firmicutes* in comparison with FP ($P = 0.0001$) and a significantly high abundance of *Bacteroidetes* in comparison with FP ($P = 0.021$) and CDP ($P = 0.023$). In addition, FP had a significantly higher abundance of *Firmicutes* in comparison with CDP ($P = 0.016$), but no significant difference between *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* (Fig. 1).

Comparison of the major phyla in *Procellariiform* using multidimensional scaling analysis (MDS) indicates similarity of the microbiota between different species of procellariiform seabirds (Fig. 2). However, despite a generally similar microbiota of all three species, the MDS indicates that procellariiform seabirds have a species-specific gut microbiota as the samples of each species tend to be grouped into distinct, species-specific clusters (Fig. 2). The MDS analysis also shows a tight clustering of all FP samples indicating little variation amongst individual FP. For STSW and CDP, there is loose clustering of all individuals indicating that there is more individual variation within these two species (Fig. 2). The MDS also indicates that there is little intraspecies variation in the intestinal microbial composition of all three bird species.

Characterisation of the *Procellariiform* microbiota by 16S rRNA gene high-throughput sequencing

The faecal microbiota of *Procellariiform* seabirds was further characterised by 16S rRNA gene pyrosequencing of whole-community 16S rRNA gene which provides an in-depth overview of the microbial community structure. A total of 2216 OTUs from 21 phyla were identified in

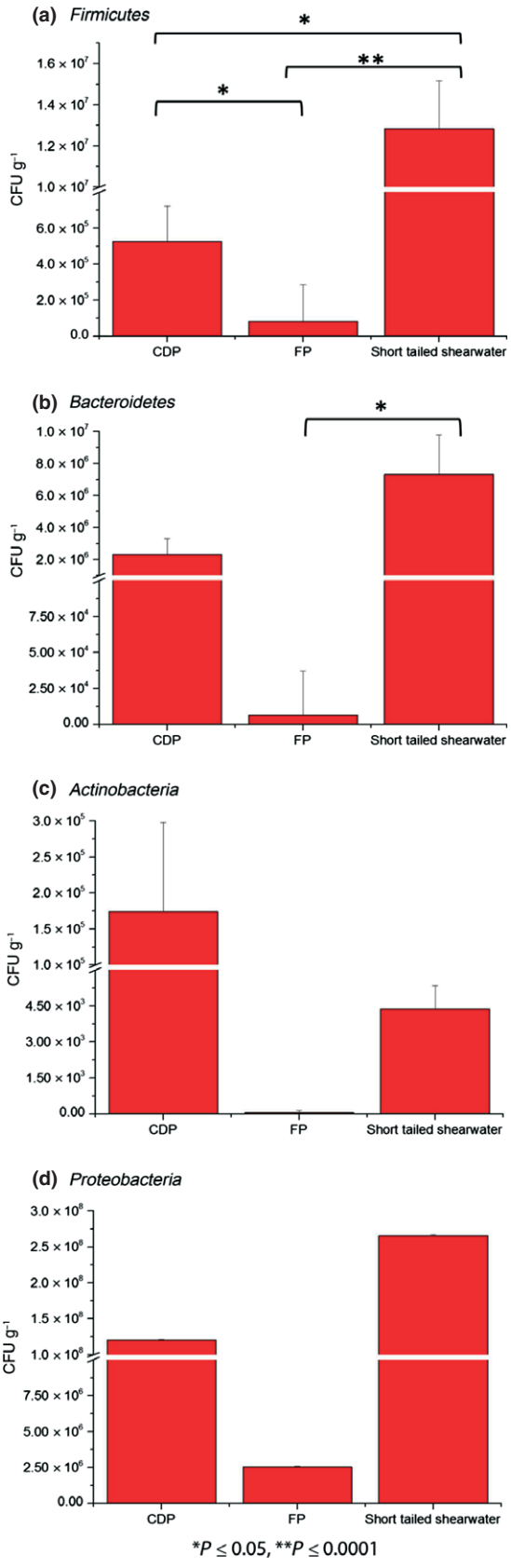

Fig. 1. Variation in the abundance of the major phyla; *Firmicutes* (a), *Bacteroidetes* (b), *Actinobacteria* (c) and *Proteobacteria* (d) in CDP, FP and STSW. Phyla abundance was assayed by quantitative real-time PCR using phyla-specific primers. Abundances of *Firmicutes* and *Bacteroidetes* were significantly different (*$P \leq 0.05$, **$P \leq 0.0001$) among the three seabird species, but *Actinobacteria* and *Proteobacteria* were not (*$P < 0.05$).

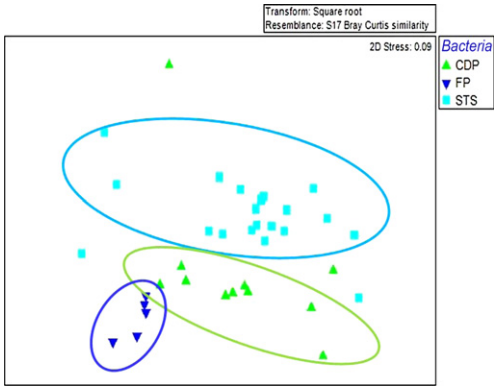

Fig. 2. Similarity of the major bacterial phyla in *Procellariiform* seabirds using qPCR. Three different seabird species were included: STSW (*Ardenna tenuirostris*), FP (*Pachyptila turtur*) and CDP (*Pelecanoides urinatrix*). MDS analysis shows tight clustering of individual FP indicting little individual variation of the main bacterial phyla. Three different seabird species were included: STSW (*Ardenna tenuirostris*), FP (*Pachyptila turtur*) and CDP (*Pelecanoides urinatrix*). While most bacterial communities from STSW and CDP clustered together, at least one-third do not cluster together, indicating extensive variation among communities from different host individuals.

the microbiota of the *Procellariiform* seabirds ($n = 4$ species). The majority of OTUs were rare, and only five OTUs had a relative abundance > 2% in at least one sample. The majority of 16S rRNA gene sequences were classified as *Firmicutes*, *Proteobacteria* and *Bacteroidetes*, with *Firmicutes* and *Proteobacteria* dominating the microbial communities of all three *Procellariiform* seabirds (Fig. 3). At family level, *Ruminococcaceae*, *Lachnospiraceae* and *Porphyromonadaceae* dominate the microbial composition of CDP with *Faecalibacterium*, uncultured *Lachnospiraceae*, *Petrimonas* and *Barnesiella* being the dominant known genera. In FP and STSW, *Leuconostocaceae* and *Streptococcaceae* are the dominant families, with *Leuconostoc*, *Weissella* and *Lactococcus* the dominant genera in FP and *Leuconostoc* and *Lactococcus* the dominant genera in STSW (Fig. 3). Microbial communities from the three different host species had a different composition on a global, whole-community level. A microorganism–seabird association network, providing an overview of the associations identified from the three species of *Procellariiform* seabirds, visualised in CALYPSO is shown in (Fig. 4). The network association identified co-occurrence relationships between gut microbial communities of all three seabird species.

Influence of stomach oil

The faecal microbiota of SOP seabirds (FP and STSW) and NSOP seabirds (CDP) was compared using the 16S rRNA gene sequence data. A microorganism interaction network analysis demonstrated that SOP and NSOP seabirds have a distinct gut microbiota composition and that a large number of genera are specific for either stomach oil or nonstomach oil producers (Fig. 4). Also the Bray–Curtis distance indicates that the intestinal microbiota of the two SOP seabirds is more similar on the global, whole-community level than the intestinal microbiota between SOP and NSOP seabirds (Bray–Curtis distance FP-STSW: 0.43; CDP-FP: 0.95; CDP-STSW: 0.92). The network shows co-occurring (yellow edges) and mutual exclusive (blue edges) bacterial genera in the gut microbiota of the three seabird species. The bacterial genera found in the three seabirds form three distinct clusters, and each cluster is associated with one of the three seabird species. Positive correlations (yellow edges) between the clusters associated with STSW and FP indicate that some bacterial genera are found in both of these SOP seabirds. The cluster associated with CDP on the other hand is clearly distinct from the clusters associated with STSW and FP. These results indicate that each seabird has its own, species-specific gut microbiota. While the SOP STSW and FP show some overlap between their gut microbiota, the NSOP CDP have a clearly distinct intestinal microbiota. Nine phylotypes (OTUs) were shown to be significantly differentially abundant in SOP and NSOP seabirds (Fig. 5). SOP seabirds also show a significant higher overall diversity (Shannon index, $P = 0.01$, ANOVA) and richness (OTU richness, $P = 0.2$, ANOVA; Figs S1 and S2).

Discussion

This paper describes the first whole-community examination and classification of the faecal microbiota of *Procellariiform* seabirds, and provides evidence that the species examined differ significantly in the composition of their gut microbiota. In accordance within a range of other vertebrates including polar bears, pinnipeds, terrestrial mammals and avian species (including penguins, chickens and gulls) (Lan *et al.*, 2002; Eckburg *et al.*, 2005; Ley *et al.*, 2008a, b; Costello *et al.*, 2010; Glad *et al.*, 2010a, b; Kohl, 2012; Lavery *et al.*, 2012; Nelson, 2012; Dewar *et al.*, 2013; Nelson *et al.*, 2013), the *Procellariiform* microbiota appears to harbour a highly diverse community of microorganisms, dominated by three phyla: *Firmicutes*, *Proteobacteria* and *Bacteroidetes*. Microorganisms in the *Procellariiform* faeces are abundant, with estimates of the total abundance of the major phyla examined with qPCR estimated at over 1.2×10^6 CFU mL^{-1} for FP, 2.6×10^8 CFU mL^{-1} for CDP and 2.8×10^8 CFU mL^{-1} for STSW.

In accordance with previous studies on other marine vertebrates such as polar bears (*Ursus maritimus*) and Australian sea lions (*Neophoca cinerea*), the faecal microbiota of STSW and FP is highly dominated by the

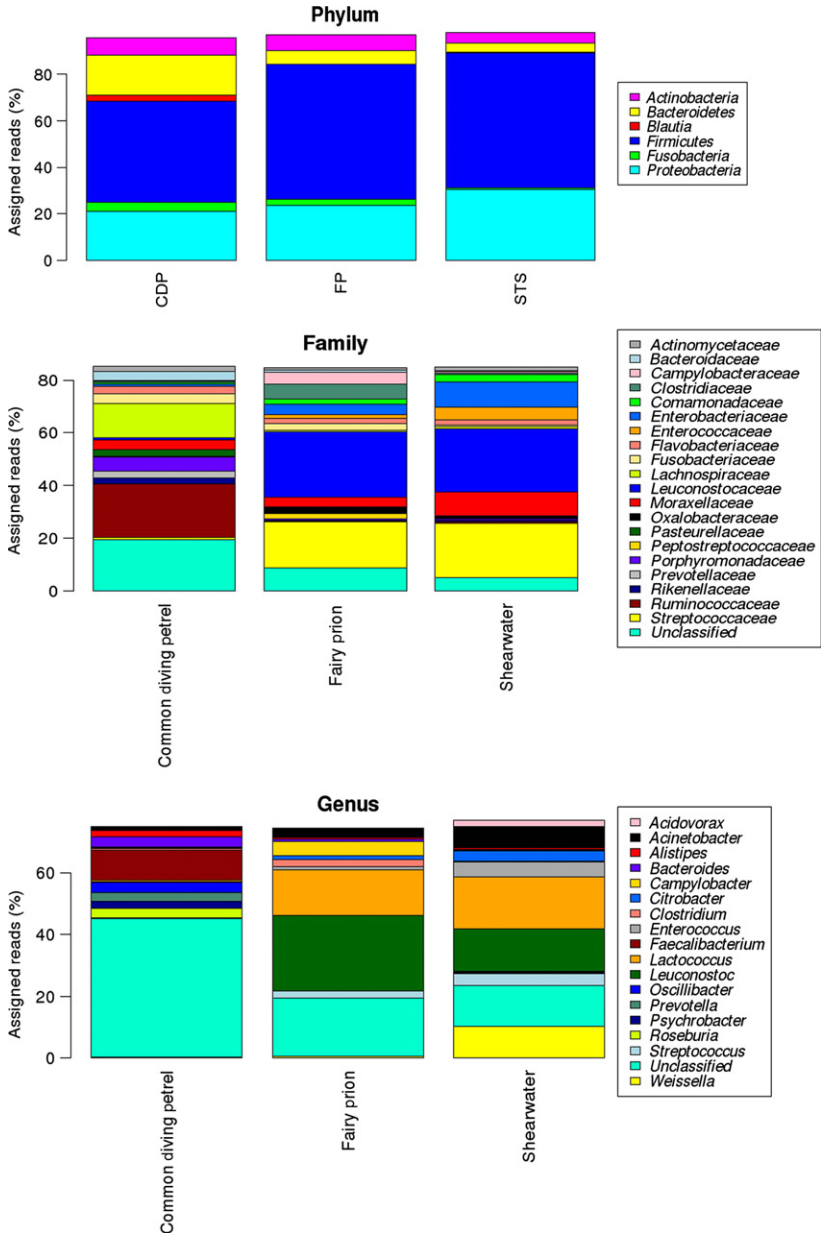

Fig. 3. Relative abundance of major taxa in *Procellariiform* faecal microbiota assayed by 16S high-throughput sequencing. Taxa with relative abundances < 2% were not included.

phylum *Firmicutes* (Glad *et al.*, 2010a, b; Lavery *et al.*, 2012), whereas the domination of *Firmicutes* and *Bacteroidetes* in CDP is similar to that of other vertebrates including penguins (Lan *et al.*, 2002; Ley *et al.*, 2008a, b; Banks *et al.*, 2009; Costello *et al.*, 2010; Glad *et al.*, 2010a, b; Kohl, 2012; Nelson, 2012; Dewar *et al.*, 2013). Members of the phylum *Firmicutes* are associated with the breakdown of complex carbohydrates, polysaccharides, sugars and fatty acids, which are then utilised by the host as an energy source (Flint *et al.*, 2008; Tap *et al.*, 2009). Members of the phylum *Bacteroidetes* in humans have been associated with vitamin synthesis, polysaccharide metabolism and membrane transport (Gross, 2007). Although the CDP microbiota in this study is similar to that of other vertebrate species, they do differ from other vertebrates in regard to the relatively high abundance of *Proteobacteria* (5–30%).

Most of the bacteria detected in STSW and FP microbiota are lactic acid bacteria (*Leuconostoc* and *Lactococcus*), known to have probiotic properties (*Weissella*), and are commonly associated with the vertebrate GI tract and skin microbiota (Walter *et al.*, 2001; Rawls *et al.*, 2004; Björkroth & Holzapfel, 2006; Casalta & Montel, 2008; Ogier *et al.*, 2008). In CDP, the microbiota is associated with butyrate-producing microorganisms (*Fusobacteria* and *Ruminococcus*; Potrykus *et al.*, 2008; Atarashi *et al.*, 2011) and

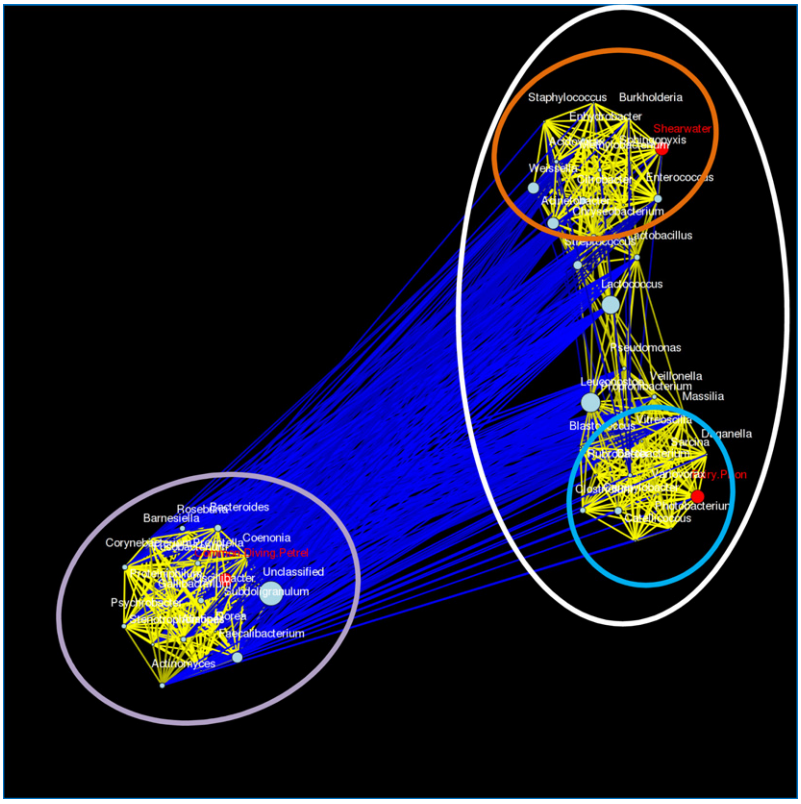

Fig. 4. Pearson's correlation network for seabird microbiota. A network analysis was carried out in CALYPSO. Genera are represented as nodes, and lines connecting nodes (edges) represent significant positive (yellow, $r > 0.6$) or negative (blue, $r < -0.6$) associations as defined by the Pearson's correlation coefficient. An overview of the associations identified from three species of *Procellariiform* seabirds is provided (visualised in CALYPSO). The network association identifies the co-occurrence relationships between gut microbial communities of STSW (orange circle), FP (blue circle) and CDP (purple circle). Gut microbial communities of SOP (white circle) and NSOP (purple circle) *Procellariiform* seabirds form distinct clusters.

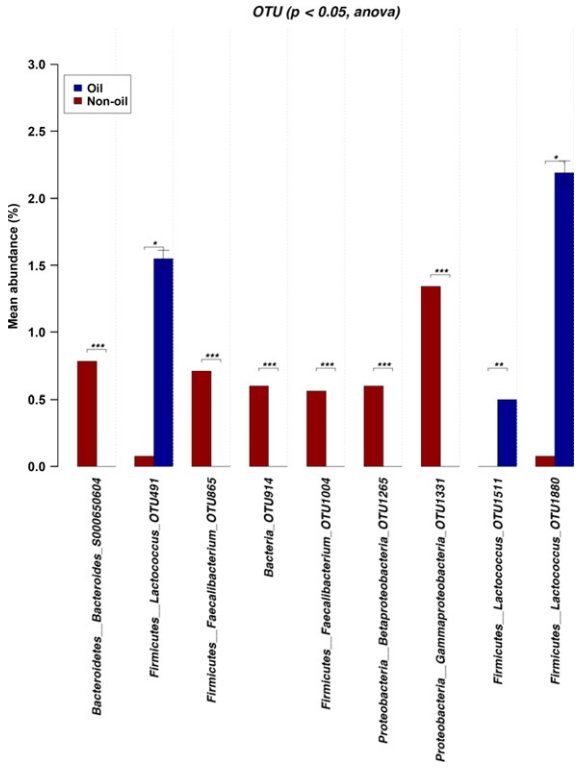

Fig. 5. Significant differences between SOP and NSOP *Procellariiformes* at OTU level. (*$P = 0.01$, **$P = 0.05$ and ***$P = 0.001$).

microorganisms associated with anti-inflammatory properties (*Faecalibacterium*) (Louis *et al.*, 2007). Butyrate is an essential short-chain fatty acid produced in the colon by bacteria. The main effects butyrate has on the intestinal tract in humans include influence of ion absorption, cell proliferation and differentiation, immune regulation, and are an important anti-inflammatory agent (Canani *et al.*, 2011). In chickens, butyrate supplementation leads to a significant increase in host defence peptide gene expression, enhanced antibacterial properties of monocytes against pathogenic bacteria, boosting host immunity and increasing host adiposity (Panda *et al.*, 2009).

Stomach oil vs. nonstomach oil

Significant differences were found in the abundance of several OTUs and global community composition of the microbiome between the oil- and non-oil-producing *Procellariiform* seabirds. These results, along with the negative correlation observed by the Pearson's network correlation, highlight the potential influence of stomach oils on the composition of the microbiota of *Procellariiform* seabirds (Fig. 4). These negative correlations indicate that differences in digestive physiology and retention time could influence the microbial composition. Ley *et al.* (2008a, b) identified that host phylogeny and gut physiol-

ogy are significant predictors of host microbial composition. In addition, retention time of digesta has also been shown to influence the microbial composition of the GI tract (Stevens & Hume, 1998). It is well known that the digestive physiology in diving petrels differs to that of other *Procellariiformes*, but retention time of digesta is also significantly shorter than other *Procellariiformes* (Roby *et al.*, 1989). For example, in the Georgian diving petrel *Pelecanoides georgicus,* the retention time for lipids was 2.3 h, whereas in shy albatross (*Macronectes giganteus*) and other large *Procellariiformes,* the retention time was 12.5 h, and in the smaller oil-producing *Procellariiform* Antarctic prion (*Pachyptila desolata*), the average retention time was 15 h (Roby *et al.*, 1989).

Conclusion

This study has identified that there is large variation within the microbiota of *Procellariiformes*, which correlates with the presence or absence of stomach oils. *Firmicutes* and *Proteobacteria* dominate the GI microbiota of oil-producing *Procellariiformes*, whilst *Firmicutes*, *Proteobacteria* and *Bacteroidetes* dominate the GI microbiota of non-oil-producing species. Although the cause of these differences is yet to be determined, host phylogeny, digestive physiology and retention time could potentially play major roles in determining the final microbial composition of individual seabird species (Stevens & Hume, 1998; Ley *et al.*, 2008a, b; Banks *et al.*, 2009).

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Supporting Information

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

Fig. S1. Community diversity OTU (ANOVA).
Fig. S2. Community diversity OTU.
Fig. S3. Rarefraction curve.
Fig. S4. Quality scores across bases.
Fig. S5. Quality score distribution over all sequences.
Table S1. Taxonomic assignment of bacterial sequences.