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Citation: Yew, Wen Chyin, Pearce, David, Dunn, Michael J., Samah, Azizan Abu and Convey, Peter (2017) Bacterial community composition in Adélie (Pygoscelis adeliae) and Chinstrap (Pygoscelis antarctica) Penguin stomach contents from Signy Island, South Orkney Islands. Polar Biology, 40 (12). pp. 2517-2530. ISSN 0722-4060

Published by: Springer

URL: https://doi.org/10.1007/s00300-017-2162-8 <https://doi.org/10.1007/s00300-017-2162-8>

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Bacterial community composition in Adélie (*Pygoscelis adeliae*) and Chinstrap (*Pygoscelis antarctica*) Penguin stomach contents from Signy Island, South Orkney Islands

W. C. Yew [1, 2] • D. A. Pearce [1, 3, 4] • M. J. Dunn [3] • A. A. Samah [1] • P. Convey [1, 3, 4]

1. National Antarctic Research Centre, Institute of Graduate Studies, University of Malaya, 50603 Kuala Lumpur, Malaysia
2. Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
3. British Antarctic Survey, NERC, High Cross, Madingley Road, Cambridge CB3 0ET, United Kingdom
4. Department of Applied Sciences, Faculty of Health and Life Sciences, University of Northumbria at Newcastle, Ellison Building, Northumberland Road, Newcastle upon Tyne, Tyne and Wear NE1 8ST, United Kingdom

Corresponding Author
Wen Chyin Yew
Email address: wen.c.yew@gmail.com
Telephone: +603-79674634
Fax: +603-79605935

Abstract

Penguin stomach microbiota and its variability are important as these microbes may contribute to the fitness of the host birds and their chicks, and influence the microbial ecosystem of the surrounding soils. However, there is relatively little knowledge in this area, with the majority of studies focused on their deposited faeces. Here we investigated whether similar foraging strategies in adjacent colonies of different penguin species lead to similar temporarily conserved stomach microbiota. To do this, we studied the inter- and intra-specific variations in bacterial community composition in the stomach contents of sympatrically breeding Adélie (*Pygoscelis adeliae*) and Chinstrap (*P. antarctica*) Penguins, which consumed a diet of 100 % Antarctic krill (*Euphausia superba*) under a similar foraging regime on Signy Island (maritime Antarctic), using a high-throughput DNA sequencing approach. Our data show that Adélie and Chinstrap Penguins shared 23 - 63 % similarity in the stomach bacterial community composition, with no significant differences observed in the α-diversity or the assemblages of frequently-encountered groups of operational taxonomic units (OTUs). The most frequently encountered OTUs that were shared between the species represented members of the phyla Fusobacteria, Firmicutes, Tenericutes and Proteobacteria. OTUs which were unique to individual birds and to single species formed approximately half of the communities identified, suggesting that stomach microbiota variability can occur in penguins that forage and breed under similar environmental conditions.

Keywords Antarctic • High-throughput sequencing • Internal gut • Inter-individual • Inter-specific • Microbiota

Introduction

Based on a range of studies that have focused on poultry and captive birds, avian gut microbiota are known to benefit their host bird's health, growth and ultimately reproductive success, mainly by degrading and converting consumed food to nutrients thereby providing energy to the host (Robrish et al. 1991; Chen et al. 2002; Bjerrum et al. 2006; Stanley et al. 2012; Roggenbuck et al. 2014), and by excreting antibiotics against pathogens (Portrait et al. 2000; Van Der Wielen et al. 2000; Chen et al. 2013). Although phylogenetic factors may also play a role (Grond et al. 2014; Waite and Taylor 2014), the environment has been claimed to exert a strong influence on avian gut microbiota, with factors such as bird diet and habitat being important (Lucas and Heeb 2005; Maul et al. 2005; Hammons et al. 2010; Hird et al. 2014; Roggenbuck et al. 2014).

In Antarctic penguins, several gut microbiota studies have sought to increase our knowledge base, mainly relying on cloacal swabs (Soucek and Mushin 1970; Potti et al. 2002; Banks et al. 2009; Dewar et al. 2014; Barbosa et al. 2016) and faecal samples collected on the ground (Zdanowski et al. 2004; Dewar et al. 2013), as these methods allow data collection without harming the study birds. These studies have identified pathogenic microbes that are present in the penguin guts using a culture-dependent method (Soucek and Mushin 1970), and the association of penguin gut microbiota and/or its variability with fasting and moulting behaviours (Dewar et al. 2014), growth (Potti et al. 2002), age (Barbosa et al. 2016) and phylogeny (Banks et al. 2009; Dewar et al. 2013) of the host bird using either culture-dependent or molecular approaches. However, avian gut microbiota were found to differ between different parts of a gastrointestinal tract, and hence cloacal or faecal samples may not provide a suitable proxy for the study of internal gut microbiota (Gong et al. 2002, 2007; Wilkinson et al. 2016). To the best of our knowledge, a single study available in the literature of stomach microbial communities was reported in King Penguins (*Aptenodytes patagonicus*) (Thouzeau et al. 2003a), in which these microbes were found to be restricted in growth during food preservation (Thouzeau et al. 2003a, b).

Like other seabirds, penguins are one of the top marine consumers in Antarctica (Brooke 2004), and their populations are vulnerable to changes in the marine environment (Forcada and Trathan 2009; Boersma and Rebstock 2014). Prey-associated and some marine bacteria may enter the penguin stomachs during foraging and feeding. As penguins are able to store and temporarily conserve large amounts of food in their stomach for chick feeding, the growth of bacteria associated with the temporarily conserved-food (e.g. prey-associated and marine bacteria) in the stomachs might have an immediate impact on the chicks relying on regurgitate for food. Furthermore, as penguins feed in the sea and breed on the land, besides their deposited materials being the key contributors of nutrients to the typically nutrient-poor Antarctic soils and subsequently for the microbial succession in the regional

terrestrial ecosystem (Ugolini 1972; Heine and Speir 1989; Sun et al. 2000, 2004; Ma et al. 2013; Zhu et al. 2015), their stomach microbes could possibly also be input to the surrounding soil microbial ecosystem through regurgitation or defecation. In order to examine how the stomach microbiota influences both penguins, chicks and the surrounding terrestrial ecosystem, it is important first to understand which microbes are present in penguin stomachs, and the factors that shape these communities.

Signy Island, part of the South Orkney Island archipelago, hosts sympatrically breeding populations of Adélie (*Pygoscelis adeliae*) and Chinstrap (*P. antarctica*) Penguins with total island populations of 18,333 and 19,530 pairs, respectively (Dunn et al. 2016). Although Adélie Penguins begin their annual breeding cycle approximately one month earlier than Chinstrap Penguins on the island, the chick-rearing period of both penguin species overlap (Lynnes et al. 2002; Black 2016). The two penguin species also forage at sea over similar temporal and spatial scales (Lynnes et al. 2002; Takahashi et al. 2003), and feed almost entirely on Antarctic krill (*Euphausia superba*) (Lynnes et al. 2002, 2004; British Antarctic Survey unpublished data). Previous studies reported that both Adélie and Chinstrap Penguins capture prey using pursuit dive strategies (Watanuki et al. 1997; Takahashi et al. 2003) and, on Signy Island, Lynnes et al. (2002) found such pursuit diving taking place during penguin foraging trips with distances from their breeding colonies at Gourlay Peninsula of between 3 – 177 km for Adélie Penguins, and 19 – 112 km for Chinstrap Penguins. This study also showed that although the summer foraging ranges of each penguin species did overlap, in years of lower prey availability there was inter-species variation in the entire foraging range utilised.

In this study, we aimed to examine the inter- and intra-specific variations in the stomach bacterial community composition of two *Pygoscelis* penguins that breed in a similar environment. To achieve this, we employed a high-throughput sequencing approach (Illumina MiSeq) to investigate the bacterial community composition of stomach contents (obtained as regurgitated ingesta samples) of Adélie and Chinstrap Penguins from Signy Island that consumed 100 % Antarctic krill. The use of this recent but well-established sequencing method in generating 16S rDNA short regions (Caporaso et al. 2011) should provide a higher resolution taxonomic comparison of the bacterial community composition between samples than is possible with a "shotgun" method (Suenaga 2012). As Adélie and Chinstrap Penguins shared the same diet composition under a very similar foraging and breeding environment (Lynnes et al. 2002, 2004; British Antarctic Survey unpublished data), we predicted similar bacterial community compositions both between these two different species of penguins, and between individuals of the same species.

Materials and methods

Study area, sample collection and DNA extraction

Fieldwork was carried out during the 2013/14 chick-rearing period of Adélie (December - January) and Chinstrap (January - February) Penguins (Lynnes et al. 2004;

British Antarctic Survey unpublished data) at Gourlay Peninsula (60°43.586' S, 45°35.063' W) on Signy Island, South Orkney Islands (Fig. 1). Gourlay Peninsula is located at the south-east of Signy Island, and hosts the largest population of Adélie and Chinstrap Penguins on the island, with breeding colonies ranging in size from 15 to more than 2,000 pairs (Dunn et al. 2016). Although these two penguin species differ in their nest topography preference and form distinct species-specific rookeries adjacent to one another (White and Conroy 1975; Waluda et al. 2014), they breed sympatrically at Gourlay Peninsula with overlapping chick-rearing periods (Lynnes et al. 2002; Black 2016) and foraging area (Lynnes et al. 2002; Takahashi et al. 2003), and feed almost exclusively on Antarctic krill (Lynnes et al. 2002, 2004; British Antarctic Survey unpublished data).

As part of the standard sampling protocol of the long-term monitoring programme of the Convention for the Conservation of Antarctic Marine Living Resources (CCAMLR) Ecosystem Monitoring Programme (CEMP) on Signy Island, five or six independent healthy adult individuals of each penguin species that returned from the sea were captured every five days (depending on weather and logistic constraints) at the shore close to the colonies (Lynnes et al. 2004). On the spot, stomach ingesta samples of these captured birds were collected using the water flushing method (Wilson 1984) following CEMP Standard Methodology (CCAMLR 2003). As Antarctic penguin's body temperature is approximately 38 °C (Thouzeau et al. 2003a), in order to minimise harm to the captured penguins, temperature of the flushing-water was adjusted by mixing boiled and un-boiled seawater collected at the sampling shore (where the birds came ashore after foraging in the sea), prior to flushing the stomach of the penguins. To avoid cross contamination in samples between captured birds, a fresh bucket of flushing-water was prepared, and all tools that were used for the penguin stomach flushing were cleaned with 70 % ethanol, before the stomach ingesta samples of each and every individual bird were sampled. The samples were immediately sub-sampled into 50-mL sterile Falcon tubes, and rapidly returned to the laboratory at the British Antarctic Survey's Signy Island research station (1 - 3 h), where total DNA was extracted from individual samples using the DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. In an initial trial study, comparing the effectiveness of the hexadecyltrimethylammonium bromide (CTAB) method that was previously used to extract DNA from squid stomach contents (Deagle et al. 2005), and the QIAGEN kit used for DNA extraction in Antarctic krill samples (Passmore et al. 2006) and human stomach contents (Bik et al. 2006), the latter achieved better yields and concentration of DNA extract (data not shown).

16S V4 gene fragment amplification, Illumina MiSeq and filtering of MiSeq datasets

The DNA samples of a total of twelve individual birds captured (Adélie = 6 and Chinstrap = 6) that consumed 100 % Antarctic krill as their dietary component (British Antarctic Survey unpublished data) were further studied. The variable region 4 (V4) of the 16S rRNA gene, targeting bacteria and archaea, was amplified using the adapted PCR primers (F515 and R806) and the polymerase chain reaction (PCR) as described by Caporaso et al. (2011). DNA quality was checked using a NanoDrop 2000c (Thermo Scientific, Waltham, Massachusetts, USA) and quantified using a Qubit® 2.0 Fluorometer (Invitrogen,

Carlsbad, California, USA). DNA libraries were prepared and performed in the MiSeq system for paired-end runs following the manufacturer's instructions (Illumina, San Diego, California, USA). The generated raw datasets were demultiplexed and were trimmed for the presence of Illumina adapter sequences using MiSeq Reporter Software version 2.5 (Illumina, San Diego, California, USA), and were further trimmed at a Phred Score of Q30 using Trimmomatic (Bolger et al. 2014). Trimmed data were then deposited into the open source software Quantitative Insights into Microbial Ecology (QIIME) version 1.9.1 (Caporaso et al. 2010, 2011) for sequence assembly, chimera removal, operational taxonomic unit (OTU) picking, taxonomic classification and analyses.

Sample coverage, bacterial community composition and statistical analyses

OTU data with taxonomic classification were generated using the Greengenes database implemented in QIIME, with a minimum sequence identity cut-off was set at 97 % (Caporaso et al. 2011; McDonald et al. 2012). In order to limit the impact of sequencing errors, OTUs represented by only one read (singletons) were removed as possible artifacts (Goodrich et al. 2014), and were not considered further. To ensure the OTU data provide complete and thorough coverage for subsequent analyses, a rarefaction analysis was generated using the observed species metrics in QIIME to estimate the sampling effort for individual samples (Caporaso et al. 2011). In addition, the percentage sample coverage for all samples was calculated using Good's formula (Good 1953).

As Illumina MiSeq is not a quantitative but a semi-quantitative method (Hirsch et al. 2010), our analyses focused on α-diversity (OTU richness and evenness) of samples, bacterial taxonomic composition (presence/absence data of annotated OTUs), and the assemblage pattern of frequently-encountered groups of OTUs (OTUs with relative abundance ≥ 1 %), rather than the absolute abundance of annotated OTUs. The α-diversity of individual samples was calculated as the Shannon diversity index as this is more sensitive to the richness rather than the abundance of OTUs (Hughes and Bohannan 2004), while both the bacterial taxonomic composition and the assemblages of frequently-encountered groups of OTUs were analysed at three different classification levels (phylum, family and genus).

To examine both the inter- and/or intra-specific variations in stomach bacterial community composition, sample α-diversity data were checked for normality before an independent sample T-test (IBM SPSS Windows version 19.0, Armonk, New York, USA) was used. In addition, the Jaccard index was used on the bacterial presence/absence data between individual Adélie and Chinstrap Penguins to calculate the percentage of taxonomic composition similarity, while Spearman rank multiple correlation analysis was conducted to examine similarity in the assemblage patterns of frequently-encountered groups of OTUs between individual Adélie and Chinstrap Penguins.

To compare inter- versus intra-specific variation in stomach bacterial community composition, a principal coordinate analysis (PCoA) with Bray-Curtis distance metric was performed using QIIME to visualise the similarity/dissimilarity matrix across all stomach ingesta samples based on normalised OTU data (Caporaso et al. 2011). Further, to test

whether there was a significant difference in the mean values of taxonomic composition similarity and the assemblages of frequently-encountered groups of OTUs at inter- and intra-specific levels, one-way analysis of variance (ANOVA) with a *post-hoc* comparison using Tukey's honestly significant difference (HSD) test (IBM SPSS Windows version 19.0, Armonk, New York, USA) was applied to the Jaccard indices and Spearman rank multiple correlation coefficients obtained.

Nucleotide sequence accession numbers

All sequences were deposited in an open source metagenomics RAST server (Meyer et al. 2008) with accession numbers listed in Table 1.

Results

Sample coverage

Rarefaction analyses showed similar accumulation curves for all samples (Fig. 2), suggesting suitable diversity coverage to undertake the intra and inter-specific comparisons. This was further supported by a preliminary calculation using Good's coverage (Table 1), showing that the sampling completeness averaged 99.5 % (ranging from 99.3 to 99.7 %). A total of 128 OTUs were identified at the genus classification level, with individual samples ranging between 18 and 53 OTUs (Table 1). All OTUs identified shared > 97 % similarity in the Greengenes database available in QIIME, and belonged to a total of 14 phyla and 60 families. No archaea were identified in any samples. The complete list of assigned OTUs, along with abundance of each OTU in individual bird samples, is provided in the electronic supplementary material (Online Resource 1).

Bacterial community comparison between Adélie and Chinstrap Penguins

The α-diversity values obtained showed no significant difference (independent sample T-test, $t10 = 1.36$, $p = 0.205$) between Adélie ($X \pm SE = 2.23 \pm 0.17$, $n = 6$) and Chinstrap ($X \pm SE = 2.62 \pm 0.23$, $n = 6$) Penguins, although variable α-diversity values were obtained across individual bird samples (ranging from 1.51 to 3.02) (Table 1).

Jaccard indices showed that taxonomic composition similarity between these two penguin species was higher at phylum ($X \pm SE = 68.64 \pm 2.02$ %, $n = 36$), and lower at family ($X \pm SE = 35.22 \pm 1.39$ %, $n = 36$) and genus ($X \pm SE = 34.66 \pm 1.15$ %, $n = 36$) classification levels (Online Resource 2). Approximately 33 % of the individuals compared at phylum level, 50 % at family level, and 61 % at the genus level showed a significant positive correlation (Spearman rank correlation, $rs = 0.683$ - 1.000, $n = 36$, $p < 0.05$) in the assemblages of frequently-encountered groups of OTUs between these two penguin species (Online Resource 2).

Excluding unclassified bacteria, 39 % of the bacterial community members were found in both penguin species, and 37 % were unique to Adélie Penguins and 24 % to

Chinstrap Penguins. Amongst the overlapping members, only 50 % of phyla, 14 % of families and 21 % of genera were encountered frequently (relative abundance > 1 %) in both Adélie and Chinstrap Penguins. The unique members each accounted for < 1 % of relative abundance, and are thus considered as the 'rare' group in the samples studied. The overlapping and unique OTUs at the different classification levels, with the frequently encountered overlapping OTUs listed in bold, are shown in Table 2.

Bacterial community composition within Adélie Penguins

Excluding unclassified bacteria, a total of 13 phyla, 54 families and 47 genera were identified from Adélie Penguins. However, only 38 % of annotated phyla, 15 % of families and 13 % of genera were present in all individual birds sampled. These bacteria included members of *Cetobacterium*, *Psychrobacter*, *Chelonobacter*, *Clostridium* (family: Clostridiaceae), *Mycoplasma* and *Ornithobacterium*. However, none of these bacteria were unique to Adélie Penguins. Frequently encountered OTUs (relative abundance ≥ 1 %) with their relative abundance in individual bird samples at different classification levels, are shown in Fig. 3.

Jaccard indices showed that taxonomic composition similarity across individual Adélie Penguins was greatest at the phylum (X ± SE = 64.11 ± 3.22 %, n = 15), followed by the family (X ± SE = 33.35 ± 1.63 %, n = 15) and genus (X ± SE = 33.83 ± 1.44 %, n = 15) classification levels (Online Resource 3). About 27 % of the individuals compared at phylum level, 53 % at family level, and 60 % at the genus level showed a significant positive correlation (Spearman rank correlation, rs = 0.606 - 1.000, n = 36, p < 0.05) in the assemblages of frequently-encountered groups of OTUs between individuals of Adélie Penguins (Online Resource 3).

Bacterial community composition within Chinstrap Penguins

Not including unclassified bacteria, a total of 9 phyla, 35 families and 39 genera were identified from Chinstrap Penguins. Approximately 44 % of annotated phyla, 17 % of families and 18 % of genera were present in all individual birds sampled. These included closest matches to *Cetobacterium*, *Chelonobacter*, *Clostridium* (family: Clostridiaceae), *Fusobacterium*, *Mycoplasma*, *Psychrobacter* and *Sutterella*, and again none of these were unique to Chinstrap Penguins. Frequently encountered OTUs (relative abundance ≥ 1 %), with their relative abundance in individual Chinstrap Penguins at different classification levels, are shown in Fig. 3.

Jaccard indices showed that taxonomic composition similarity between individual birds was greatest at the phylum (X ± SE = 70.69 ± 2.78 %, n = 15), followed by family (X ± SE = 41.73 ± 1.77 %, n = 15) and genus (X ± SE = 41.27 ± 1.16 %, n = 15) levels (Online Resource 4). Approximately 40 % of the individuals compared at phylum level, 53 % at family level, and 60 % at the genus level showed a significant positive correlation (Spearman rank correlation, rs = 0.699 - 1.000, n = 15, p < 0.05) in the assemblages of frequently-encountered groups of OTUs between individuals of Chinstrap Penguins (Online Resource 4).

Inter- versus intra-specific variation

Excluding unclassified bacteria, penguin species-specific and individual-specific bacteria were identified at phylum (43 % and 36 %, respectively), family (52 % and 38 %) and genus classification levels (61 % and 45 %). PCoA (Fig. 4) showed no apparent differences between bacterial communities in either inter- and/or intra-specific comparisons in Adélie and Chinstrap Penguins. When Jaccard similarities at different bacterial classification levels were analysed for data from both penguin species separately and for the entire dataset from both species, no significant difference (one-way ANOVA, $F(2,63) = 1.229$, $p = 0.299$) was observed between inter- and intra-specific level in the bacterial phylum taxonomic composition. However, significant differences in the composition of the bacterial families (one-way ANOVA, $F(2,63) = 5.299$, $p = 0.007$) and genera (one-way ANOVA, $F(2,63) = 5.650$, $p = 0.006$) were found in inter- and intra-specific comparisons in the two penguins. At both family and genus classification level, *post hoc* comparisons with Tukey's HSD indicated that the mean Jaccard similarities between individuals of Chinstrap Penguins were significantly higher than those of Adélie Penguins (family level $X \pm SE = 8.39 \pm 2.78$, $p = 0.010$; genus level $X \pm SE = 7.44 \pm 2.55$, $p = 0.014$) or those between the two penguin species (family level $X \pm SE = 6.52 \pm 2.34$, $p = 0.019$; genus level $X \pm SE = 6.62 \pm 2.15$, $p = 0.009$). In the analysis of Spearman coefficients, inter- and intra-species comparisons showed no significant difference in the assemblages of frequently-encountered bacterial phyla (one-way ANOVA, $F(2,63) = 2.028$, $p = 0.140$), families (one-way ANOVA, $F(2,63) = 0.697$, $p = 0.502$) or genera (one-way ANOVA, $F(2,63) = 0.121$, $p = 0.886$).

Discussion

At a 97 % confidence threshold bacterial genus level, Adélie and Chinstrap Penguins harboured different bacterial community composition in their stomach contents both between the two penguin species and between individuals of the same species, although no significant differences were found in the α-diversity values (i.e. OTU richness and evenness) or the assemblages of frequently-encountered groups of OTUs (relative abundance $\geq$ 1 %). In addition, approximately half of the communities identified overall were either species-specific or individual-specific. In this study, sympatrically breeding Adélie and Chinstrap Penguins are known to have the same diet composition (100 % Antarctic krill), and the food source is from a similar foraging environment at Signy Island in the maritime Antarctic (Lynnes et al. 2002, 2004; Takahashi et al. 2003), yet individual still have different stomach bacterial community compositions both between and within each penguin species. Dietary component alone, therefore, is unlikely to be the key determinant of the bacterial community present in the birds' stomachs. When considering the foraging environment, both Adélie and Chinstrap Penguins forage using pursuit diving in the same general geographic area; however in years of lower prey availability, Adélie Penguins tend to forage farther from the island compared to Chinstrap Penguins (Lynnes et al. 2002). Furthermore, although the chick-rearing periods of both penguin species overlap, Adelie Penguins begin their breeding cycle with chicks hatching approximately one-month earlier than Chinstrap Penguins (Lynnes et al.

2002; Black 2016). Such spatial and temporal variations in the foraging area and timing between the two penguin species (and potentially between individuals of the same species) could possibly contribute to the differences observed between their stomach bacterial community compositions. In addition, one alternative hypothesis may be Adélie and Chinstrap Penguins have different gut structures and digestive tract environments, which might have the selection for specific microorganisms.

Inter- or intra-specific variation in the faecal microbiota has previously been reported in other bird species (Grond et al. 2014; Waite and Taylor 2014), including Antarctic penguins (Banks et al. 2009; Dewar et al. 2013). Grond et al. (2014) found two different species of migratory shorebirds differed in their faecal bacterial communities although they shared similar environmental conditions, and suggested that the gut microbiota might be species-specific. Waite and Taylor (2014) re-analysed previously-studied cloacal and/or faecal bacterial sequence datasets from a variety of bird species, and suggested that host bird species played a more significant role in the establishment of gut microbiota in birds, while the sampling site, diet and captivity status also contributed. In studies of Antarctic penguins, Dewar et al. (2013) addressed inter-specific variation in the faecal bacterial communities between King (*A. patagonicus*), Gentoo (*Pygoscelis papua*), Macaroni (*Eudyptes chrysolophus*), and Little (*E. minor*) Penguins, although the causes contributing to variation remained unclear in their study because the species studied were from different breeding islands. However, Banks et al. (2009) identified host phylogeny as a greater influence than geographical location in the intra-specific variation in cloacal bacterial communities of Adélie Penguins, and suggested that bacterial communities can be inherited. In this study, when comparing inter- versus intra-specific variations observed, variation between individuals of Chinstrap Penguins (but not Adélie) was significantly higher than those between the two penguin species. This suggests that each individual penguin has its own unique community of gut microbiota, and further supports the finding of Banks et al. (2009). The establishment of avian gut microbiota begins during egg incubation (Barnes et al. 1980), and only reaches a stable stage in adulthood (Mills et al. 1999; Lu et al. 2003). Besides the potential spatial and temporal variations in the foraging area between individuals mentioned earlier, the vertical transmission of bacteria through regurgitation during chick feeding (Kyle and Kyle 1993) is also likely to contribute to the unique gut microbiota of individual penguins.

The frequently encountered OTUs present in the stomachs of both penguin species belonged to the phyla Firmicutes, Fusobacteria, Proteobacteria and Tenericutes, while Actinobacteria, Bacteroidetes, Verrucomicrobia and the bacterial candidate GN02 were less frequently encountered. Most of these phyla (in particular the predominant communities) have also previously been identified in the guts of a variety of bird species (Kohl 2012; Waite and Taylor 2014) and Antarctic penguins (Zdanowski et al. 2004; Banks et al. 2009; Dewar et al. 2013, 2014; Barbosa et al. 2016). This further supports the review of Kohl (2012), in which the bacterial communities at a higher taxonomic level (i.e. phylum) are very similar between species of birds and mammals. However, bacterial communities analysed at the genus level showed different results. In comparisons with previously studied penguins that

forage and breed elsewhere in Antarctica, approximately 46 % of the bacterial communities reported from King Penguin stomachs from Possession Island (Thouzeau et al. 2003a), 37 % from Adélie Penguin cloacae from the Ross Sea region (Banks et al. 2009), and 63% from King (Bird Island, South Georgia) and Little (Phillip Island, Australia) Penguins (Dewar et al. 2014) were also present in the samples studied here. These bacteria included *Acinetobacter*, *Actinomyces*, *Bacillus*, *Campylobacter*, *Cetobacterium*, *Chryseobacterium*, *Clostridium* (family: Clostridiaceae), *Corynebacterium*, *Erysipelothrix*, *Flavobacterium*, *Helicobacter*, *Moraxella*, *Mycoplasma*, *Peptostreptococcus*, *Porphyromonas*, *Psychrobacter* and *Streptococcus*, which most probably represent the common inhabitants in Antarctic penguin guts. When comparing the data of Thouzeau et al. (2003a), differences in the community composition observed could possibly caused by the differences in penguin species and location studied, and the analytical approach used. When comparing the data reported by Banks et al. (2009) and Dewar et al. (2014), besides the former causes mentioned, the differences in the community composition observed might be due to environmental differences in the different body parts. This further supports the contention that cloacal or faecal microbiota are not representative of internal gut microbiota (Gong et al. 2002, 2007; Wilkinson et al. 2016). In addition, although the data comparison was not between samples obtained from the same bird, the composition similarity shown between the compared cloacae/faeces and stomachs suggests that there could possibly be a microbial link between the stomachs, cloacae and faeces. Previously, Ma et al. (2013) and Zhu et al. (2015) reported that penguin deposited materials may change the geochemical component in Antarctic soils for microbial succession. The information obtained here is therefore useful for further study to understand the transfer and establishment of microbes from penguin internal guts to deposited materials and subsequently input to the surrounding soil microbial ecosystem. On the other hand, about 73 % of the bacterial genera found in this study have not been reported previously in Antarctic penguin guts (Online Resource 1), indicating the presence of many uncharacterised bacterial groups that might play an important role in the guts of Antarctic penguins, which also require further studies.

As classical culture studies are well known to isolate only a proportion of bacteria from natural communities, their role in the inference of function is limited. High-throughput sequencing studies may therefore provide a greater insight into potential functions in specific communities. For instance in this study, among the 39 % of the overall diversity that was shared between Adélie and Chinstrap Penguins, and amongst the bacterial genera that were present in all individual birds studied, *Cetobacterium*, *Chelonobacter*, *Clostridium* (family: Clostridiaceae), *Fusobacterium* and *Mycoplasma* occurred more frequently, and are thus more likely to be dominant bacteria in the functioning community in the penguin stomachs. Excepting *Chelonobacter*, these bacteria have been reported as common inhabitants in the guts across a variety of bird species (Bjerrum et al. 2006; Strong et al. 2013; Grond et al. 2014; Roggenbuck et al. 2014; Kreisinger et al. 2015), including Antarctic penguins (Thouzeau et al. 2003a; Banks et al. 2009; Dewar et al. 2014), however, the majority of their role in the guts remain unclear. *Chelonobacter*, a new bacterial genus belonging to the family Pasteurellaceae, was first discovered from diseased tortoises (Gregersen et al. 2009), and has been found in human stomachs (Delgado et al. 2013) but so far has not been reported in

406 penguin or other avian gut samples. As for *Clostridium* (family: Clostridiaceae), some species
407 strains have been identified to have ability to degrade chitin (Chen et al. 2002), which is a
408 main component of crustaceans including Antarctic krill (Clarke 1980; Nicol and Hosie
409 1993). A variety of species or strains of the genus *Fusobacterium* have been reported to be
410 involved in prey tissue decomposition (Roggenbuck et al. 2014), carbohydrate metabolism
411 (Robrish et al. 1991; Bjerrum et al. 2006) and bacteriocin production (Portrait et al. 2000) in
412 the guts of birds.

413 As expected, prey-associated and marine bacteria were also detected in the samples
414 studied. These bacteria were closely related to members of genera previously identified from
415 Antarctic krill, including *Acinetobacter*, *Bacillus*, *Corynebacterium*, *Moraxella* and
416 *Pseudomonas* (Kelly et al. 1978), and from Antarctic sea ice and marine samples, including
417 *Brachybacterium*, *Gelidibacter*, *Loktanella*, *Oleispira*, *Polaribacter*, *Polaromonas*,
418 *Pseudoalteromonas*, *Psychrobacter* and *Sphinogomonas* (Zdanowski and Donachie 1993;
419 Irgens et al. 1996; Bowman et al. 1997a, b; Junge et al. 1998; Yakimov et al. 2003; Dickinson
420 et al. 2016; Luria et al. 2016). As penguins forage in the marine environment, they are likely
421 to take in these bacteria together with their consumed prey and associated sea water.
422 Nonetheless, the frequency of encountering these OTUs in our samples was low, with prey-
423 associated bacteria and marine bacteria accounting for 8 % and 16 % respectively, of the
424 overall diversity, and they may be transient in penguin stomachs. Penguin stomachs are warm
425 (38 °C), acidic (pH < 4), and contain antimicrobial peptides known as spheniscins, which
426 function to restrict the growth of microbes in the stomach and thereby aid food preservation
427 (Thouzeau et al. 2003a, b).

428 In this study, data were analysed at the bacterial phylum, family and genus
429 classification levels. When comparing the three classification levels, the data showed that
430 both inter- and intra-specific variations in the penguin stomach bacterial community
431 composition became more significant with progression from the phylum to the family or
432 genus level. This finding is in line with the study of Yarza et al. (2014), who reported that for
433 bacterial community studies inferred using the 16S rDNA, the taxa recovery is better at a
434 lower classification level (e.g. family or genus) than a higher classification level (e.g.
435 phylum). However, most comparative studies have used a higher classification level, which
436 therefore might not able to report a sufficient resolution of microbiota to serve as baseline
437 information for future studies.

438 In summary, through the application of a high-throughput DNA sequencing approach,
439 this study revealed comparable depth and quality to those previously obtained in either
440 stomach, cloacal or faecal studies, providing a more extensive dataset of penguin gut
441 microbiota than previously available. In addition, this study demonstrated diversity in
442 penguins' gut microorganisms, which might explain differential susceptibilities of these
443 animals to gut pathogens.

444

445 **Acknowledgments**

This study was funded by the Sultan Mizan Antarctic Research Foundation (YPASM) and the National Antarctic Research Centre, University of Malaya Research Grant (UMRG: RP007-2012A). Laboratory resources were provided by British Antarctic Survey (BAS) and Northumbria University. We thank Stacey Adlard for her assistance in the field sampling. We also thank the editor and the three anonymous reviewers for their constructive comments. Wen Chyin Yew is a recipient of MyBrain scholarship (MyPhD) funded by the Ministry of Higher Education Malaysia. Peter Convey and Michael J Dunn are supported by NERC core funding to the BAS "Biodiversity, Evolution and Adaptation" and "Ecosystems" teams, respectively. This paper also contributes to the Scientific Committee on Antarctic Research "State of the Antarctic Ecosystem" research programme (AntEco).

Compliance with ethical standards

All procedures involving animals followed internationally recognised CCAMLR CEMP standard methods and were in accordance with the ethical standards of the British Antarctic Survey.

Competing interests

The authors declare no competing interests.

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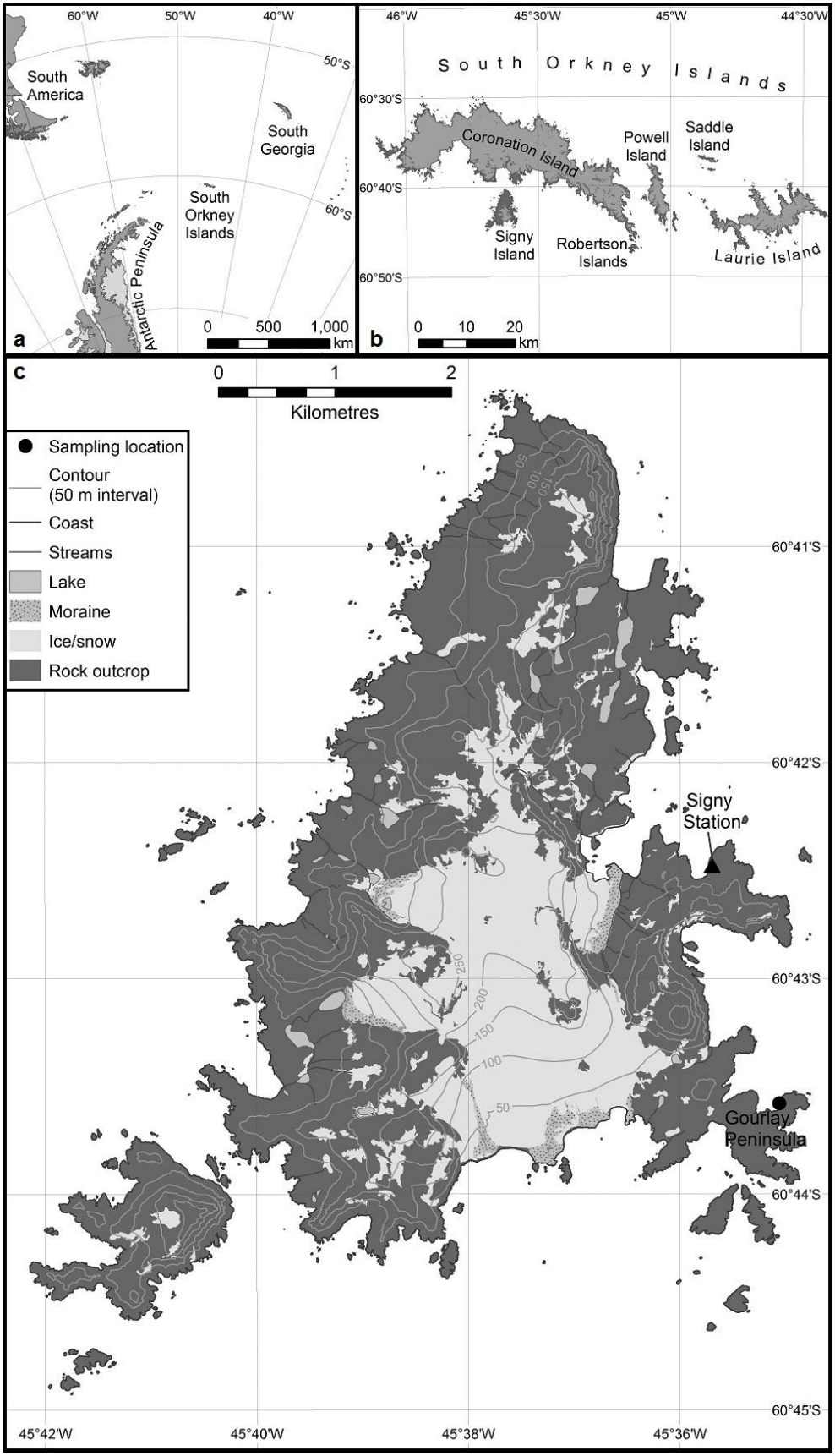

Fig. 1 The locations of **a** South Orkney Islands in the maritime Antarctic, **b** Signy Island within the South Orkney Island archipelago, and **c** Gourlay Peninsula on Signy Island. Map provided by Laura Gerrish, Mapping and Geographic Information Centre, British Antarctic Survey.

Table 1 Information analysed from MiSeq dataset of individual Adélie (A1 - A6) and Chinstrap (C1 - C6) Penguin stomach ingesta samples

Sample	Accession number	Krill (%)	Good's coverage (%)	Number of OTU	Shannon index
A1	4705524.3	100	99.7	28	2.060
A2	4709469.3	100	99.4	45	1.744
A3	4705597.3	100	99.6	33	1.805
A4	4715573.3	100	99.4	53	2.782
A5	4715572.3	100	99.5	51	2.531
A6	4705483.3	100	99.6	20	2.460
C1	4705526.3	100	99.7	24	1.511
C2	4705618.3	100	99.3	50	2.856
C3	4705575.3	100	99.6	25	2.551
C4	4705632.3	100	99.6	28	2.997
C5	4705639.3	100	99.5	23	3.022
C6	4705449.3	100	99.6	18	2.805

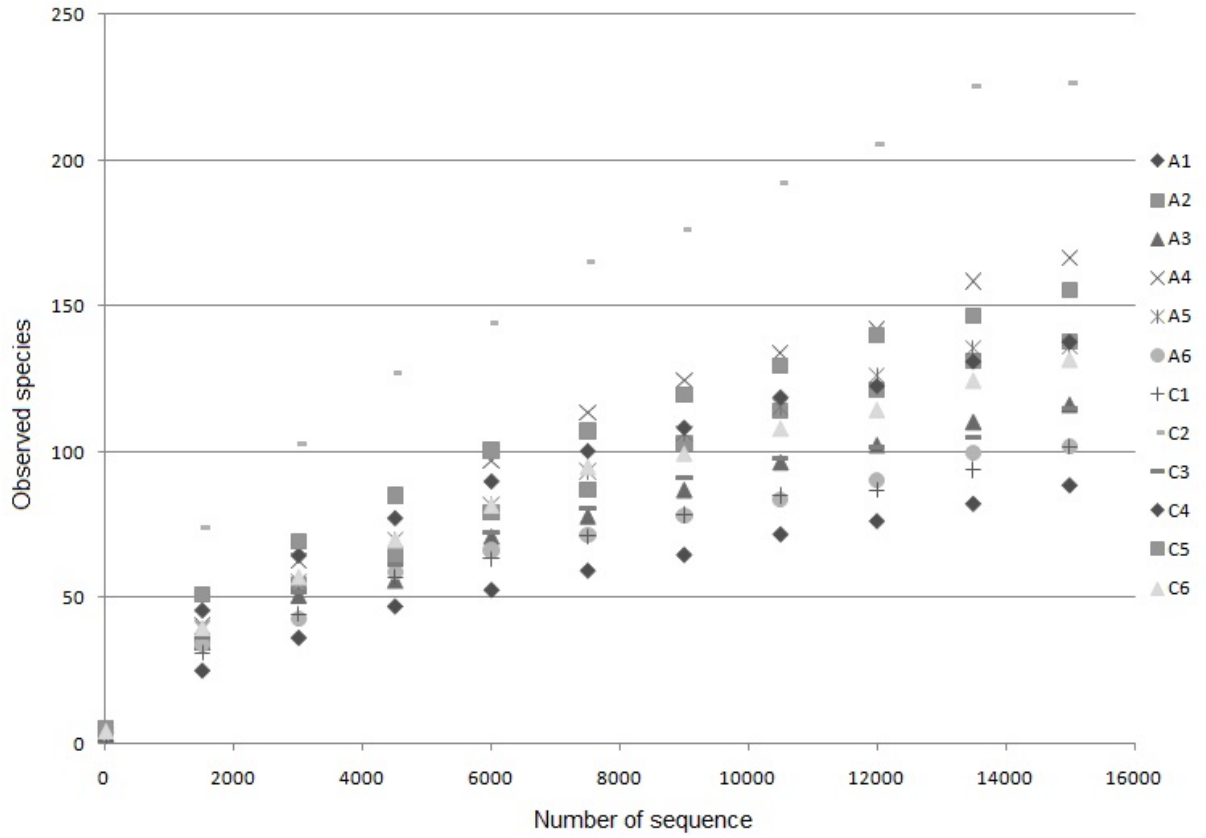

Fig. 2 Rarefaction curve of individual Adélie (A1 – A6) and Chinstrap (C1 – C6) Penguin stomach ingesta samples

Table 2 Composition of the overlapping and the unique stomach bacterial communities of Adélie (A) and Chinstrap (C) Penguins that were assigned at phylum, family and genus classification levels. Frequently encountered groups of OTUs (with an average relative abundance > 1 %) that present in both penguin species were listed in bold

Phylum			Family			Genus		
In A only	In A and C	In C only	In A only	In A and C	In C only	In A only	In A and C	In C only
Acidobacteria	Actinobacteria	Gemmatimonadetes	Acidobacteriaceae	Actinomycetaceae	Carnobacteriaceae	*Alicyclobacillus*	*Acinetobacter*	*Actinobacillus*
Cyanobacteria	Bacteroidetes		Aeromonadaceae	Alcaligenaceae	Gemmatimonadaceae	*Bacillus*	*Actinomyces*	*Aliivibrio*
FBP	**Firmicutes**		Alicyclobacillaceae	Bacteroidaceae	Moritellaceae	*Brachybacterium*	*Aequorivita*	*Caloramator*
Planctomycetes	**Fusobacteria**		Aurantimonadaceae	Campylobacteraceae	Piscirickettsiaceae	*Bradyrhizobium*	*Aggregatibacter*	*Carnobacterium*
SR1	GN02		Bacillaceae	Cardiobacteriaceae	Propionibacteriaceae	*Brumimicrobium*	*Arcobacter*	*Coprococcus*
	Proteobacteria		Bradyrhizobiaceae	Chitinophagaceae	Vibrionaceae	*Campylobacter*	*Bacteroides*	*Erysipelothrix*
						**Clostridium*		
	Tenericutes		Burkholderiaceae	**Clostridiaceae**		(Lachnospiraceae)	*Capnocytophaga*	*Gemmatimonas*
	Verrucomicrobia		Cellulomonadaceae	Colwelliaceae		*Corynebacterium*	***Cetobacterium***	*Loktanella*
			Corynebacteriaceae	Comamonadaceae		*Finegoldia*	***Chelonobacter***	*Lysobacter*
			Cryomorphaceae	Erysipelotrichaceae		*Flavobacterium*	*Chryseobacterium*	*Mannheimia*
							[a] ***Clostridium***	
			Cytophagaceae	Flavobacteriaceae		*Haemophilus*	(Clostridiaceae)	*Moritella*
			Dermabacteraceae	**Fusobacteriaceae**		*Hymenobacter*	*Dokdonella*	*Peptostreptococcus*
			Enterobacteriaceae	Helicobacteraceae		*Legionella*	***Fusobacterium***	*Perlucidibaca*
			Isosphaeraceae	Lachnospiraceae		*Luteolibacter*	*Gelidibacter*	*Psychromonas*
			Legionellaceae	Leptotrichiaceae		*Moraxella*	*Helicobacter*	*Tenacibaculum*
			Micrococcaceae	Moraxellaceae		*Oleispira*	***Mycoplasma***	
			Mogibacteriaceae	**Mycoplasmataceae**		*Paludibacter*	*Ornithobacterium*	
			Nocardiaceae	Oceanospirillaceae		*Pedobacter*	*Polaribacter*	
			Oxalobacteraceae	**Pasteurellaceae**		*Rhodococcus*	*Polaromonas*	
			Pirellulaceae	Peptostreptococcaceae		*Sediminibacterium*	*Porphyromonas*	
			Sphingobacteriaceae	Porphyromonadaceae		*Sphingomonas*	*Pseudoalteromonas*	
			Sphingomonadaceae	Pseudoalteromonadaceae		*Streptococcus*	*Pseudomonas*	
			Streptococcaceae	Pseudomonadaceae		*Suttonella*	*Psychrobacter*	
			Streptomycetaceae	Psychromonadaceae			*Sutterella*	
			Verrucomicrobiaceae	Rhodobacteraceae				
				Ruminococcaceae				
				Tissierellaceae				
				Weeksellaceae				
				Xanthomonadaceae				

[a] *Clostridium* assigned in this study belongs to either the family Clostridiaceae or Lachnospiraceae

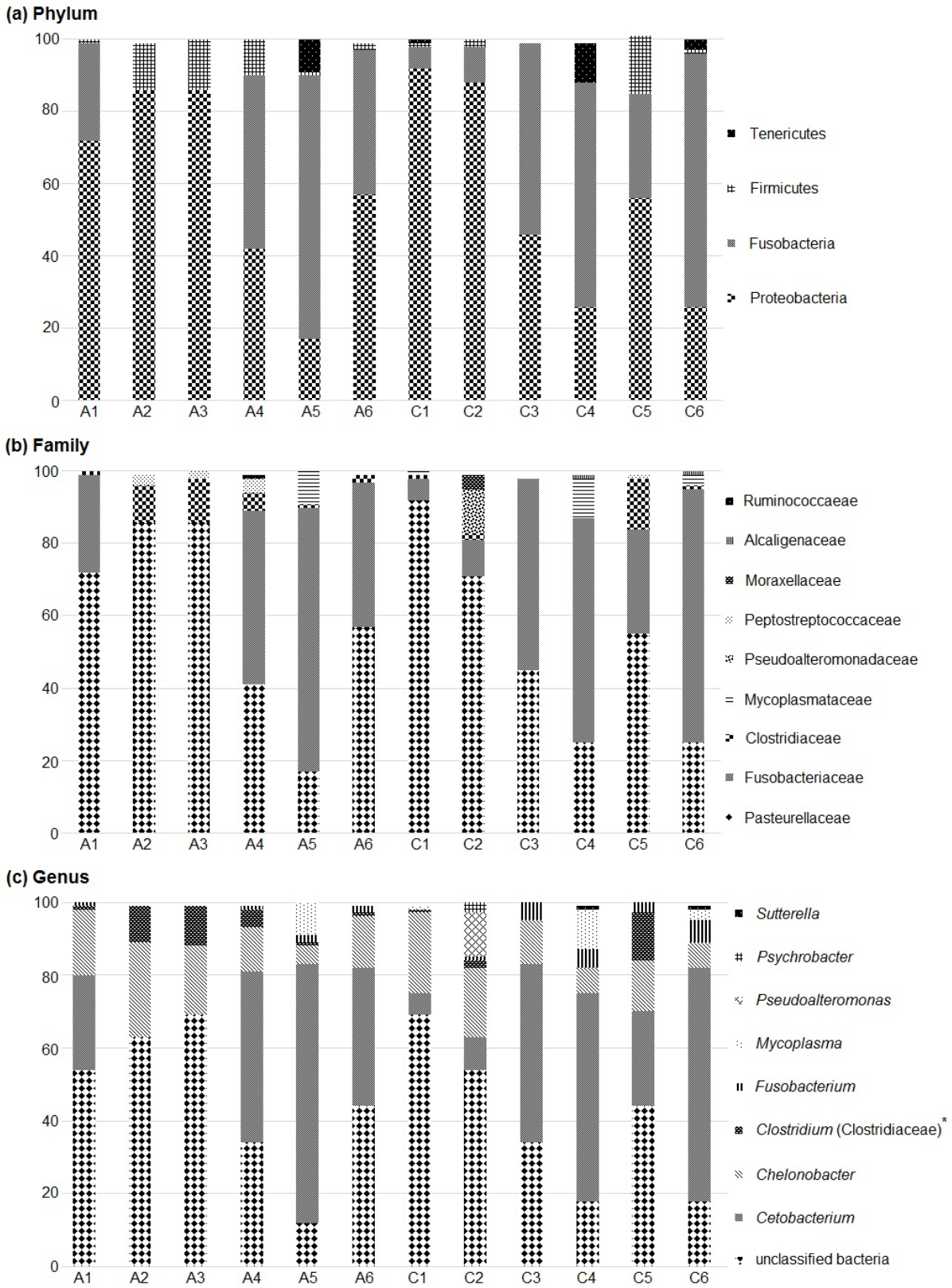

Fig. 3 Assemblages of frequently encountered stomach bacterial communities (relative abundance > 1 %) of individual Adélie (A) and Chinstrap (C) Penguins that were assigned at (a) phylum, (b) family and (c) genus classification levels. **Clostridium* assigned in this study belongs to either the family Clostridiaceae or Lachnospiraceae

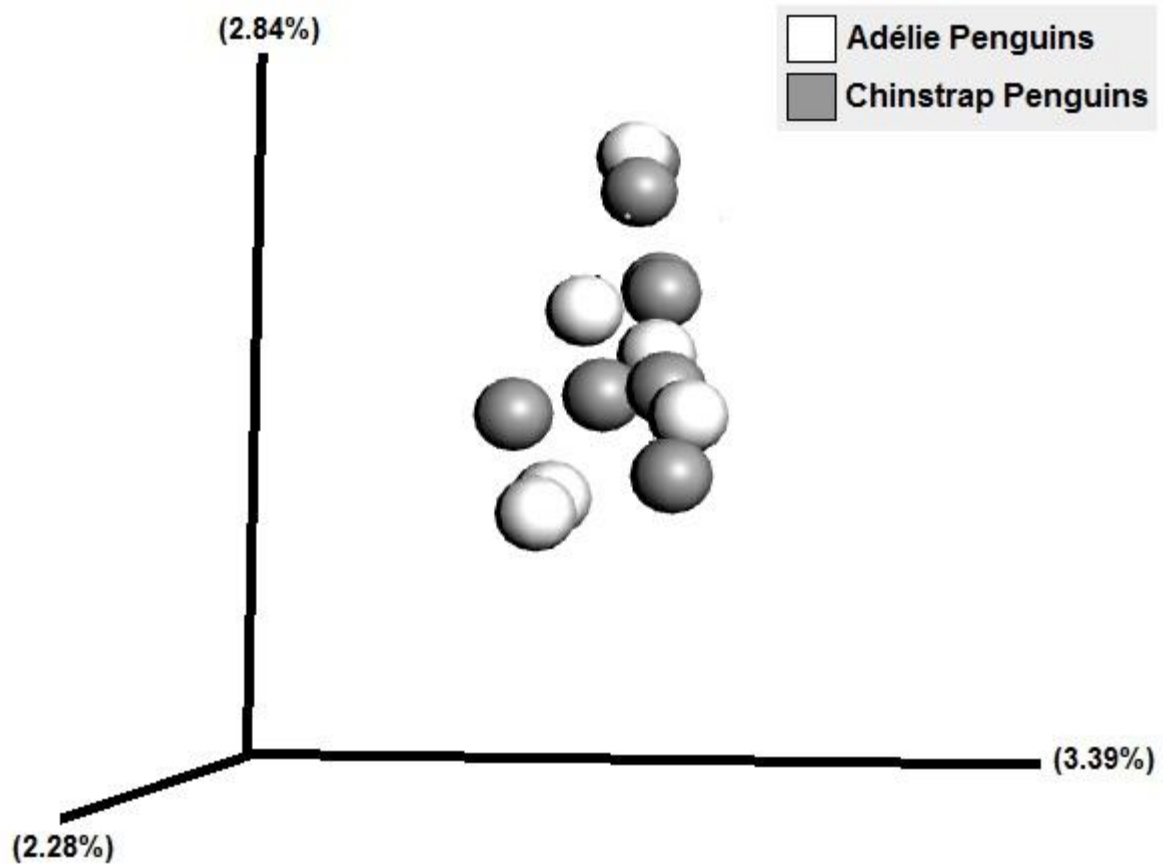

Fig. 4 Principal coordinate analysis (PCoA) of penguin stomach bacterial communities calculated using Bray-Curtis distance matrix on normalised OTU assignment data