Title:

Patterns in the Juan Fernandez fur seal faecal microbiome

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Abstract:

As apex predators, pinnipeds are considered to be useful bioindicators of marine and coastal environments. Endemic to a small archipelago in the South Pacific, the Juan Fernandez fur seal (JFFS) is one of the less-studied members of the pinniped family *Otariidae*. This study aimed to characterise the faecal microbiome of the JFFS for the first time, to establish a baseline for future studies of host-microbial-environment interactions and monitoring programs. During two consecutive reproductive seasons, 57 faecal samples were collected from 7 different JFFS colonies within the Juan Fernandez Archipelago, Chile. Bacterial composition and abundance were characterised by sequencing the V4 region of the 16S rRNA gene. The overall microbiome composition was dominated by five phyla: *Firmicutes* (40 % ± 24), *Fusobacteria* (30 % ± 17), *Bacteroidetes* (22 % ± 10), *Proteobacteria* (6 % ± 4) and *Actinobacteria* (2 % ± 3). Alpha diversity was higher in Tierras Blancas. However, location was not found to be a dominant driver of microbial composition. Interestingly, the strongest signal in the data was a negative association between the genera *Peptoclostridium* and *Fusobacterium*, which explained 29.7 % of the total microbial composition variability between samples. The genus *Peptoclostridium* has not been reported in other pinniped studies and its role here is unclear, with interpretation challenging due to a lack of information regarding microbiome functionality in marine mammals. As a first insight into the JFFS faecal microbiome, these results contribute towards our understanding of the natural microbial diversity and composition in free-ranging pinnipeds.

Keywrods: *Arctophoca philippii*, scatology, microbiome, pinnipeds, marine mammals

Introduction:

Marine environments are complex and interconnected systems subject to various environmental impacts. Pollution, climate change, disruption of the food network and pathogen dissemination are a few examples of problems currently affecting ocean integrity and function (Halpern *et al.*, 2019). Integrated approaches at the macro- and micro-ecological levels are needed to properly understand and manage environmental threats in these kinds of complex systems. Identification and investigation of potential environmental sentinel species such as marine mammals can provide a better understanding of the deterioration or improvement of ocean health (Bossart, 2011; Hazen *et al.*, 2019). However, to effectively use wild populations as sentinels, it is first necessary to establish a baseline.

In the last couple of decades, the study of the microbiome in wild populations has increased, due to the profound impact of host-microbial interactions on host physiology and the growing affordability of sequencing technology (Redford *et al.*, 2012; Trevelline *et al.*, 2019). The gastrointestinal tract, especially the colon, is recognised as one of the largest microbial reservoirs (O'Hara and Shanahan, 2006). This microbial community fulfils essential functions in digestion, metabolic activity and immunity, and differences in species composition and abundance can therefore provide much information about the host organism. For example, following its initial acquisition during birth and lactation, the microbiome is constantly modified by factors such as age, sex, and diet (Ley, *et al.*, 2008a; Ley, *et al.*, 2008b; Nicholson *et al.*, 2012). Similar factors shaping the gut microbiome in terrestrial mammals influence that of marine mammals (Nelson *et al.*, 2013; Smith *et al.*,

2013; Pacheco-Sandoval *et al.*, 2019; Stoffel *et al.*, 2020). However, studies have also shown substantial differences between marine and terrestrial mammal gut microbiomes, even when these two groups share a similar diet (e.g., herbivore, carnivore) (Nelson, Rogers and Brown, 2013; Bik *et al.*, 2016). Thus, even though research into the microbiome of terrestrial mammals is at a relatively advanced stage, this information cannot be easily extrapolated to marine mammals whose microbiomes remain poorly understood particularly, those in non-captive, natural populations. Consistent characterisation of the core microbiome of these populations is therefore required as a fundamental baseline before we can attempt to understand it's functions, roles, interactions, and possible uses (Shade and Handelsman, 2012).

The faecal microbiome has been characterised for eight pinniped species inhabiting the southern hemisphere, including three out of the eleven members forming the subfamily *Arctocephalinae* (fur seals): *Arctocephalus pusillus doriferus* (Smith *et al*., 2013), *Arctocephoca australis australis* and *Arctophoca tropicalis* (Medeiros *et al*., 2016). Also part of the *Arctocephalinae* subfamily is the Juan Fernandez fur seal (*Arctophoca philippii philippii*) (JFFS) which is endemic to the Juan Fernandez Archipelago, a group of islands located in the middle of the Pacific Ocean 600 km away from the Chilean continental coast (Fig 1). The archipelago is a hotspot for biodiversity with a high number of endemic marine species, including the JFFS (Aguayo, Maturana and Torres, 1971; Pompa, Ehrlich and Ceballos, 2011; Friedlander *et al.*, 2016). These fur seals are the only native mammals in the archipelago and like other pinnipeds occupy upper trophic levels in the marine food web (Ochoa Acuña and Francis, 1995; Trites, 2019). Their feeding behaviour, lifespan, fat storage, and their amphibian lifestyle, which links marine and coastal environments, are

some of the characteristics that make this species a great candidate to act as a marine bioindicator. However, despite showing a significant population recovery since the late 1960s and becoming an icon for local tourism, little is known about this species.

This study aimed to characterise the JFFS faecal microbiome for the first time, as a baseline for understanding the host-microbial interactions in this species. To investigate, we performed sequencing of the 16S rRNA gene, a highly conserved region of the bacterial genome, which provides a reliable overview of bacterial community composition.

Methods:

Ethics statement

All faecal samples were collected from the environment in a non-invasive manner. Disturbance of the colonies was kept to a minimum and no animal was handled or harmed in the process. Permits for the collection of samples were given by CONAF (Certificate 009217) and SERNAPESCA (R.E.X.N 43). Permission for importation of samples into the United Kingdom was also obtained (ITIMP16.1158).

Sample collection

Faecal samples were collected from seven reproductive colonies of Juan Fernandez fur seals situated throughout the Juan Fernandez archipelago, Chile (coordinates: 33°38′29″S 78°50′28″W) (Fig. 2). Six of the seven colonies included in this study were located on Robinson Crusoe island: El Arenal (EA) (n =9), Bahia El Padre (BP) (n=23), Piedra Carvajal (PC) (n=1), Punta Trueno (PT) (n=1), Tierras Blancas (TB) (n=12) and Vaquería (V) (n=1). One colony was located on Santa Clara island (SC). Samples were collected

during two consecutive reproductive seasons (2017 and 2018), which took place between mid-January to the end of February. Collection of samples took place before noon to limit sun exposure. The samples were collected based on consistency and colour to reduce the variability caused by the delay between the defecation and collection. A disposable wooden spatula was used to expose the centre of the faeces to avoid collecting material in direct contact with the surrounding elements. Using a sterile Copan FLOQSwab®, a sample from the core of the faeces was placed into RNAlater® (Sigma-Aldrich) (Vlčková *et al.*, 2012; Blekhman *et al.*, 2016). No animal was observed defecating. Thus, it was not possible to distinguish sex or age at the time of sample collection. We used visual cues and GPS location to decrease the risk of collection from the same individual. Samples were stored at -20 ° C within 32 hours post collection for 1-2 months until arrival in the laboratory, where they were transferred to -80 ° C until further analysis.

DNA extraction and sequencing

Samples were processed as soon as possible after collection (2017 and 2018 respectively). Due to the possible batch effect introduced by processing samples in different years, comparisons between years of collection will not be explored in this study.

Samples were thawed on ice and centrifuged at 10,000 x ***g*** for 15 min to pellet the sample out of RNAlater®. Genomic DNA was extracted from each pelleted sample (approx. 180 micrograms) using the MO BIO PowerSoil DNA Isolation kit (QIAGEN) according to the manufacturer's instructions. Isolated DNA was quantified on a Qubit fluorometer (Invitrogen).

The bacterial 16S rRNA gene was PCR (Polymerase Chain Reaction) amplified targeting

a 250 bp region covering the V4 variable region. PCR amplification, barcode tagging, and library preparation was performed according to Kozich *et al*. (Kozich *et al.*, 2013). Libraries were constructed using the TrueSeq DNA kit and sequenced on a MiSeq™ platform (Illumina®). The read length target changed between the two sampling years. Sequencing was performed using the v2 chemistry producing 2x 250 bp paired-end reads in the 2017 samples while the 2018 sequences were 2x 150 bp paired-end reads.

Sequence data analysis and taxonomic classification

Raw sequence quality was manually assessed with FastQC v. 0.11.5 (Simon Andrews, 2010). All 57 samples contained reads of consistent length (respective to the sequencing year) and the average read quality score was above 30. A drop in base quality was observed at the ends of reads (4 - 5 and 8 - 10 respectively). Demultiplexed raw sequences were imported into QIIME2-2019.10 (Bolyen *et al.*, 2019) where quality control, de-replication, read truncation and paired read merging was performed using the DADA2 (Divisive Amplicon Denoising Algorithm) qiime2 plugin (Callahan *et al.*, 2016). Instead of generating operational taxonomic units (OTUs) by clustering sequences based on similarity, the final output of DADA2 is a table with exact sequence variants also known as amplicon sequence variants (ASVs), which are generated by modelling and correcting Illumina sequencing errors. This step was carried out separately according to the year of collection. However, to normalise between datasets, the 250 bp reads produced from 2017 samples were truncated so that the paired reads matched the length of the paired reads from 2018 samples. To confirm consistency in paired read lengths between the two years, representative sequences generated from both years were aligned in Geneious Prime® 2020.0.5 (https://www.geneious.com) by Multiple Alignment using the Fast Fourier

Transform (MAFFT) plug-in with default settings and then assessed by eye (Katoh and Standley, 2013).

Next, a mid-point rooted, approximately-maximum-likelihood phylogenetic tree for diversity analysis was generated using the qiime2 phylogeny plug-in which uses MAFFT and the FastTree program (Price, Dehal and Arkin, 2010). Finally, taxonomies were assigned to the ASVs using a 16S-V4-specific classifier trained against the Silva132 database clustered at 99 % sequence similarity (Quast *et al.*, 2013).

Data processing and statistical analysis

Statistical analysis was performed in duplicate, once using all available data and again with data corresponding to the core microbiome only. The core microbiome was defined here as all the ASVs present in at least 50 percent of the samples.

Data processing and statistical analysis were carried out in R version 3.6.0 (R Core Team, 2019). To prepare the data by identifying unassigned ASVs and removing contaminants and samples with insufficient depth of sampling prior to analysis, multiple filtering steps were applied to the data using the phyloseq package (McMurdie and Holmes, 2013). 1) Unassigned ASVs at the Kingdom level, were manually inspected with the Basic Local Alignment Search Tool (BLAST) before filtering based on both BLAST results (those with non-bacterial matches) and prevalence (ambiguous taxonomy at the phylum level with a prevalence of 1 and total abundance less than 5 reads) (Altschul *et al.*, 1990). 2) Based on the rarefaction curve (Supplementary fig. 1), 3 samples were identified as having insufficient depth of sampling and were therefore removed from the statistical analysis. A threshold of 13,980 reads was used as a cut-off. Removed samples were identified as

17JFFS16 (BP, 4463 counts), 17JFFS23 (TB, 2602 counts) and 17JFFS23 (EA, 2042 counts). 3) Possible contamination signals were also removed by running a correlation analysis and comparing clusters with a list of previously identified reagent contaminants (Salter *et al.*, 2014). 4) Finally, the data was rarefied using the same threshold used for filtering samples (Supplementary Table 1) (McKnight *et al.*, 2019).

The overall microbiota composition was characterised by summing the non-normalized read counts and obtaining the relative abundance at different taxonomic levels.

Alpha diversity

Estimates of within-sample diversity (alpha diversity) were calculated using the phyloseq package. Three indices were included: a richness estimator, which estimates the total number of species in each sample (Chao1) and two different diversity estimators (Shannon-Weiner and Simpson index). The latter two approaches consider richness as well as abundance. However, the effect of richness and rare species strongly impact the Shannon-Weiner index, whereas the Simpson index is mainly influenced by evenness and common species.

Non-rarefied data was used to explore the alpha diversity. To compare locations, a one-way analysis of variance test (ANOVA) or a non-parametric Kruskall Wallis test were performed for each estimate. ANOVA assumptions were tested by visualisation of the data and statistical testing. A Shapiro-Wilk test was used to confirm normality and a Levene's test for heteroscedasticity. When exploring Shannon-Weiner and Simpson indices sample 18JFFS23 (SC) was identified as an outlier (standard residual >3) and was removed for these indices only. Finally, data visualisation suggested samples collected from TB differed

from the other locations thus, a post-hoc analysis was performed with Dunnett's or the non-parametric Dunn's test to compare each location to TB. Samples from PC, PT and V were not included in the location comparison due to their limited sample size (n = 1).

Beta diversity

To investigate variation between samples (beta diversity) two different distances were calculated using the rarefied full as well as the core datasets. Bray-Curtis dissimilarity distance was used to look at the differences between samples based on the ASVs abundances. Weighted UniFraq distance was used to explore the phylogenetic divergence between ASVs by also taking into account the abundance of these (with an emphasis on dominant ASVs). Respective distance matrices were visualised using principal coordinate analysis plots (PCoA).

To further explore the clustering of samples (Cluster 1 versus Cluster 2) observed in the Bray-Curtis PCoA, a permutational multivariate ANOVA (PERMANOVA) was computed with 999 permutations to test for statistically significant differences between the clusters. Finally, a Similarity Percentages breakdown analysis (SIMPER) was performed between the clusters to identify the genera that most contributed to the difference between clusters. Genera that highly contributed to dissimilarities between groups were further explored with the non-parametric Mann-Whitney U test.

Spearman rank correlation coefficient (ρ) was used to explore any possible associations between the different taxa and also between the first two components of the Bray-Curtis ordination analysis. Correlations were visualised in a correlation matrix plot and only those significantly and strongly correlated (Rho (ρ) $\geq |0.6|$) were explored further. For this

method, only the core microbiome dataset was used at the genus level.

Results:

Following removal of low-quality sequences and merging the 2017 and 2018 datasets, a total of 2,074,038 paired reads, grouped into 595 ASVs were imported into R studio for statistical analysis. A total of 54 samples, with 2,062,763 sequences clustered into 558 ASVs remained after the filtering steps (Supplementary Table 1). Three samples were removed from the analysis due to rarefaction analysis indicating insufficient depth of sequencing. The rarefied dataset ended up with 518 ASVs and a total of 754,974 reads.

Composition of the Juan Fernandez fur seal faecal microbiome

A total of 10 bacterial phyla were detected in the faeces of the JFFSs. From the total ASV counts *Firmicutes* (41.9 %), *Fusobacteria* (28.2 %), *Bacteroidetes* (22.1 %), *Proteobacteria* (5.5 %) and *Actinobacteria* (1.5 %) dominated the bacterial composition. The total ASV counts from individual samples were very similar to the average relative abundance: *Firmicutes* (40 % ± 24), *Fusobacteria* (30 % ± 17), *Bacteroidetes* (22 % ± 10), *Proteobacteria* (6 % ± 4) and *Actinobacteria* (2 % ± 3) (Supplementary Table 2). Eighty-two bacterial families could be assigned, of which 14 had a relative abundance ≥ 1 % of the total ASV count. Five bacterial families accounted for 78.5 % of all read counts: *Fusobacteriaceae* (28.2 %) belonging to the phylum *Fusobacteria*, *Bacteroidacea*e (15.5 %) from the phylum *Bacteroidetes*, and *Ruminococcaceae* (15.0 %), *Lachnospiraceae* (10.4 %) and *Peptostreptococcaceae* (9.4 %) from the phylum *Firmicutes* (Fig. 3A and 3B) (Supplementary Table 3). Forty-six ASVs were present in at least 50 % of the samples

(Supplementary Table 4). While fourteen ASVs were present in > 90 % of samples, only three ASVs were present in all the samples, all of which were assigned to the genus *Fusobacterium* (14.9 %, 6.5 % and 3.7 % of the total reads respectively) (Table 1).

Alpha diversity

Three alpha diversity indices (Chao1, Shannon-Weiner and Simpson) were used to compare within-sample diversity between locations (Supplementary Table 5). Despite Tierras Blancas showing a trend towards higher diversity in all analyses, the one-way ANOVA results showed no significant differences between locations according to Chao 1 index ($F(3/47) = 2.45$, $p = 0.07$, ges = 0.08) and Shannon-Weiner index ($F(3/46) = 2.65$, $p = 0.06$, ges = 0.09). The Simpson index (chi-squared = 8.26, $p < 0.05$, ges = not provided) on the other hand, showed a significant difference between locations. Post-hoc Dunnet's and Dunn's tests consistently showed that samples from TB had higher mean and mean rank values (respectively) than the other locations, especially when compared to Tierras Blancas (Fig. 4, Supplementary fig. 2).

Beta diversity

Based on weighted Unifrac dissimilarity distance, 51.0 % (full dataset) and 53.8 % (core dataset) of the total variation between samples could be explained by the first principal component (PC1). No clustering of individual samples by location or year of collection was observed. Similarly, Bray-Curtis dissimilarity, which quantifies the differences in ASV abundance, found that the first principal components in both the full and core datasets explained 23.9 % and 29.8 % of the total variation respectively. In both data sets, a group of samples (cluster 2) were clearly separated from the main cluster (cluster 1) along PC1

(Fig. 5, Supplementary fig. 3).

Based on the relative average abundance of the dominant phyla, evident differences in the overall microbial composition were visualised between the two clusters (Fig. 6). PERMANOVA evidenced a significant difference in the microbial composition between the two clusters. This was consistent in both full (F= 10.1, Pr (>F) = 0.001, R^2 = 16.3 %) and core datasets (F= 13.6, Pr (>F) = 0.001, R2 = 20.88 %). SIMPER analysis identified five genera that together contributed 71 % to the observed compositional difference between the clusters. As expected, both *Fusobacterium* and *Peptoclostridium* were the largest contributors (24 and 25 % respectively). Furthermore, the abundance of *Fusobacterium* and *Peptoclostridium* were significantly different between clusters. Full results of the SIMPER and Mann-Whitney U-tests are summarised in Table 2.

Correlation analysis

Spearman correlation analysis revealed that the genera *Bacteroides*, *Fusobacterium* and *Peptoclostridium* were strong drivers of PC1 in both Bray-Curtis and Weighted Unifrac PCoA analyses. In addition, the genera *Ruminoclostridium 9* and *Ruminococcaceae NK4A214* were also found to be influential for PC1 in Bray-Curtis analysis (Fig. 7, Supplementary Table 6). PCoA analyses showed strong negative correlations between PC1 and *Bacteroides* (Bray-Curtis, $\rho = -0.67$, $p \leq 0.001$); and between PC1 and *Fusobacterium* (Bray-Curtis, $\rho = -0.92$, $p \leq 0.001$ and weighted Unifrac, $\rho = -0.94$, $p \leq 0.001$). *Peptoclostridium*, on the other hand, was positively correlated with PC1 (Bray-Curtis, $\rho = 0.81$, $p \leq 0.001$, and weighted Unifrac, $\rho = -0.75$, $p \leq 0.001$).

Discussion:

Marine mammal microbiome studies of free-ranging wild populations are rare, with many of these studies being limited to a small number of individuals. Instead, most studies of marine mammals have relied on data from dead or captive animals. To our knowledge, this is one of the most extensive studies of the faecal microbiome in free-ranging pinnipeds and the first of JFFS. Our approach focused on characterising the core members of the JFFS faecal microbiome, identified at the genus level, providing a baseline for understanding host-microbial interactions in this species. However, interpreting unexpected phenomena in a dataset such as ours is made difficult by a lack of literature with results generated using similar methodologies, as well as the various uncontrollable factors influencing wild populations.

Consistent with previous reports in other pinniped species, five phyla dominated the JFFS faecal microbiome: *Firmicutes*, *Fusobacteria*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* (Nelson *et al*., 2013; Bik *et al*., 2016; Numberger *et al*., 2016; Pacheco-Sandoval *et al*., 2019; Kim, Cho and Lee, 2020; Stoffel *et al*., 2020). When comparing our results to other southern pinnipeds, different microbial patterns were found in faeces from other fur seal species (Medeiros *et al*., 2016, Smith *et al*., 2013). The faecal microbiome described for both the South American (*Arctophoca australis australis*) and the sub-Antarctic fur seals (*Arctophoca tropicalis*) is almost entirely dominated by *Firmicutes* (88.56 % and 85.02 %). *Fusobacteria*, on the other hand, represents less than 1 % of the bacterial community for both species (Medeiros *et al*., 2016). The study involving these two species collected samples from dead juvenile individuals. Thus, it is expected to find altered microbiomes. Smith *et al*. (2013) characterised the faecal microbiome of Australian

fur seal (*Arctocephalus pusillus doriferus*) pups and adult females. The adult samples showed similar proportions of *Firmicutes, Bacteriodetes* and *Actinobacteria* as those observed here for JFFS. *Fusobacteria* was not detected in any of the adults. However, the authors only relied on fluorescent *in situ* hybridisation (FISH) to detect these bacteria for this age group.

Overall, pinniped gut microbiomes are very variable between and within species, possibly due to differences in their geographic range (e.g., polar versus subtropical), diet (benthic vs pelagic hunters, generalist versus specialist), or mating systems. One or more of *Fusobacteria, Firmicutes* and *Bacteroides* (all three in the case of JFFS and harbour seals), have been found to consistently dominate the overall microbial composition of pinnipeds, followed by *Proteobacteria* and *Actinobacteria* (Nelson *et al*., 2013; Pacheco-Sandoval *et al.*, 2019). The latter two are usually at lower abundance and *Actinobacteria*, in particular, has not been described in every pinniped species studied. Another interesting observation, common to all the studies reviewed, including ours, is that when *Firmicutes* dominates, the abundance of *Fusobacteria* and *Bacteroidetes* decreases, suggesting some degree of competition. The *Firmicutes : Bacteroidetes* ratio has been well documented in human and mice. In these land mammals, the ratio increases in response to diets high in lipids and decreases in response to large amounts of protein (Turnbaugh *et al.*, 2006; Hildebrandt *et al.*, 2009; Pu *et al.*, 2016). We also observed changes in the relative abundance of *Fusobacteria* were similar to those observed in *Bacteroidetes.* This suggests some functionally redundant roles.

The phylum *Firmicutes* is common in mammalian gut microbiomes (Ley, *et al.*, 2008a; Ley, *et al.*, 2008b). Members of this taxonomic group are well known for their role in

obesity in humans and mice, which is associated with an increase in *Firmicutes* and a decrease in *Bacteroidetes* (Turnbaugh *et al.*, 2006; Hildebrandt *et al*., 2009; Pu *et al*., 2016). The energy harvesting role of *Firmicutes* has also been identified in the zebrafish gut microbiome, where these bacteria are associated with an increase in lipid droplet numbers in epithelial cells (Semova *et al.*, 2012). Fat is fundamental for marine mammal survival, as it is needed for energy storage and thermoregulation and may explain why *Firmicutes* is consistently among the most dominant phyla across all pinniped species (Guerrero and Rogers, 2019).

The phylum *Fusobacteria* consists of facultative or strict anaerobes that produce various organic acids from amino acids or carbohydrates fermentation (Olsen, 2014). This phylum is usually found at high relative abundance in the gut microbiomes of strict carnivores adapted to diets rich in proteins, purines and polyunsaturated fatty acids (Zhu *et al.*, 2018; Guo *et al.*, 2020). Similar to other marine carnivores, *Fusobacteria* was one of the most abundant phyla in JFFS (Pacheco-Sandoval *et al.*, 2019). Most of the knowledge generated around the specific role *Fusobacteria* may play in mammalian intestinal tracts is based on human-centred research. Even though some genus members seem to play a beneficial role in the human gut microbiome, the presence of relatively high levels of the genus *Fusobacterium* is more often associated with health issues (Potrykus, White and Bearne, 2008; Garrett and Onderdonk, 2014; Huh and Roh, 2020). Conversely, the high relative abundance of this bacterial genus in the gut of carnivores suggests a rather symbiotic relationship where *Fusobacterium* is likely to play a role in protein metabolism (Potrykus, White and Bearne, 2008).

Similar to *Fusobacteria*, the phylum *Bacteroidetes*, especially members of the genus

Bacteroides are associated with diets high in animal proteins (Zhu *et al.*, 2018; Guo *et al.*, 2020). This genus, known for its capacity to degrade animal-derived glycans (Eilam *et al.*, 2014), was the most abundant Bacteroidetes. Similar to previous reports, JFFS samples high in *Firmicutes* contained lower relative abundances of *Bacteriodetes* and *Fusobacteria*. This phenomenon suggests differences in nutritional needs and will be discussed later in the text.

Within sample diversity

Initially, we hypothesised that the alpha diversity of samples collected from BP, a key access point to Robinson Crusoe island, was going to be different from other colonies. BP is the most transited area in this study; it connects the airfield with the town and is a popular leisure location for the local community (Fig. 1). We found instead that BP did not differ from other less-visited locations such as EA and SC. Therefore, this finding is different to a previous report showing an association between exposure to anthropogenic stressors and reduced alpha diversity in harbour seals (Pacheco-Sandoval *et al.*, 2019). The colony at TB was the only location with higher alpha diversity, indicating that samples collected from TB had a richer and more evenly distributed microbial composition than other samples. Bacterial richness has been previously associated with population density due to the increase in microbial sharing (Li *et al.*, 2016). Alternative studies have suggested that overcrowding might also negatively affect microbial diversity due to higher levels of stress (Bharwani *et al.*, 2016; Partrick *et al.*, 2018). Lower diversity of the skin microbiome in denser populations was also observed in *Arctocephalus gazella*, a closely related species (Grosser *et al.*, 2019). Population density of JFFS and its effects on the microbiome has not been studied. However, superficial observations from the field did not suggest

differences in population density between the colonies. It may therefore be that other stressors were limiting alpha diversity in the other locations. For instance, the colony on TB was relatively sheltered compared to the other colonies, as it was situated on an open platform a few meters above sea level; in contrast, the other colonies were on narrow strips of land with greater exposure to sea storms, rockfalls and landslides. Additionally, the colony on TB is rarely visited by humans due to the complicated access. However, the effects of location on alpha diversity were marginal. Nevertheless, the stress hypothesis could be tested in future studies by measuring markers of stress (e.g., cortisol) in the faeces (Wasser *et al.*, 2000).

Despite the trend showing how TB differed from the other locations, only one of the three alpha diversity estimates (Simpson) showed TB to be statistically significantly different to the other locations. The other two diversity estimates (Chao 1 richness and Shannon-Weiner) did not reach our significance cut-off. Both these estimates are affected by the detection of rare taxa and larger library and sample sizes are more likely to input rare taxa to the data set. ANOVA was also used to compare locations with these diversity estimates. ANOVA is sensitive to differences in sample size and so small group sizes may have affected statistical power.

Variation between samples

The Bray-Curtis dissimilarity PCoA revealed two distinct clusters. Seventy-five per cent of the samples clustered together in what we named cluster 1. The remaining samples were grouped as cluster 2. This variation between clusters was mostly explained by the

differences in the relative abundance of the genera *Fusobacterium* and *Peptoclostridium*. Samples in cluster 1 had a high relative abundance of *Fusobacterium* and very low *Peptoclostridium* relative abundance, whilst samples in cluster 2 showed the opposite pattern: increased *Peptoclostridium* and a significant drop in *Fusobacterium* relative abundance. To our knowledge, this is the first time the genus *Peptoclostridium* (phylum *Firmicutes*, class *Clostridia*) has been reported in a pinniped gut microbiome. The family *Peptostreptococcaceae,* to which *Peptoclostridium* belongs, has been reported in previous studies, but representing no more than 8 % of the total composition, and more often less than 4 % (Nelson *et al.*, 2013; Delport *et al.*, 2016; Pacheco-Sandoval *et al.*, 2019). On average, *Peptoclostridium* represented 29 % of the microbial composition observed in Cluster 2 versus the average 3 % observed in Cluster 1.

The genus *Peptoclostridium* was initially proposed in 2013 and validated in 2016 (Galperin *et al.*, 2016). This poorly characterised taxonomic group is believed to metabolize amino acids and oligopeptides and has been isolated from both wastewater-mud and marine sediments (Galperin *et al.*, 2016). The SILVA 132 taxonomy reference database used in this study included 144 members in the *Peptoclostridium* clade from which only 11 were classified within the four known species of this genus (*P. litorale, P. acidaminophilum, P. paradoxum* and *P. thermoalcaliphilum*). The remaining clade members were classified as uncultured bacteria. It should be noted that depending on the taxonomic reference database used, the taxonomic classification regarding members of the genus *Peptoclostridium* may differ between studies. For instance, some studies may refer to species such as *Clostridoides difficile* (previously known as *Clostridium*) as *Peptoclostridium difficile (Pereira et al., 2016)*. All four species included in the SILVA 132 database have been

isolated from environments with little or no oxygen (Galperin *et al.*, 2016). Despite these species being linked to environmental samples, *Peptoclostridium* was found in at least 90 % of the samples. The particular condition required for this bacterial species to thrive makes it unlikely that the *Peptoclostridium* members found in JFFS faeces originated from sample contamination by surrounding environmental bacteria. Such high prevalence may be a sign of a deeper relationship between these uncharacterised bacteria and the host.

The microbiome is constantly reshaping through an individual's lifetime. Most of the changes occur within symbiotic margins responding to factors such as diet, reproductive state and age, but some changes may also result in dysbiosis and disease (Ley, *et al.*, 2008b; Nicholson *et al.*, 2012). Despite the limited information available on free-range pinnipeds, a few hypotheses may be suggested to explain the significant changes observed between the two clusters reported in our study.

There is evidence that the mammalian gut microbiota changes over time. This difference is particularly evident between suckling and post-weaning stages, possibly due to dietary changes (milk vs solids). As discussed earlier, *Firmicutes* are known for their capacity to regulate lipid absortion (Semova *et al.*, 2012). Juan Fernandez fur seal milk composition contains a higher proportion of lipids in comparison to many pinnipeds (~ 41 %) (Ochoa-Acuña, Francis and Oftedal, 1999). Thus, if the faecal samples from Cluster 2 were collected from pre-weaning pups (7-10 months old), it may be expected to that a higher relative abundance of members of the phylum *Firmicutes* would be found. Similar to the microbial pattern observed in Cluster 2, samples analysed from Australian fur seal were dominated by the class *Clostridia* in six and nine months old pups (Smith *et al.*, 2013). In the same study, the families *Lachnospiraceae* and *Ruminococcaceae* were the most

dominant family within this Class, while the overall relative abundance of *Peptostreptococcaceae*, was less than 4 %. Despite age (preweaning diet) being a reasonable explanation for the difference observed in our dataset, this hypothesis arrives with a critical bias. Samples were collected between February and March, and at this point, pups would be no older than four months. At this stage, pup faeces are still distinguishable from older individuals in color and consistency. Individuals from the previous reproductive season would be older than a year and milk would no longer form a part of their diet. This suggests that pre-weaning diet is not the explanation for the abundance of *Peptoclostridium*.

Differences between genders may also be an explanation of the difference in samples. Otarids and Phocids such as northern and southern elephant seals exhibit an important degree of sexual size dimorphism (Ralls and Mesnick, 2009). Gender differences in foraging behaviour and prey selection have also been reported (Ochoa Acuña and Francis, 1995; Lewis *et al.*, 2006; Andersen *et al.*, 2013). Based on the differences in diets, it is not surprising to find studies in gut microbial composition also showing gender-based differences. Samples collected from adult Southern elephant seals evidenced significant differences between adult males and females (Nelson *et al.*, 2013; Kim, Cho and Lee, 2020). The same studies did not find differences in leopard or Weddel seals, less sexually dimorphic phocids. Adult southern elephant seal females showed a significantly higher relative abundance of *Firmicutes* and less *Fusobacteria* and *Bacteriodetes* than males (Nelson *et al.*, 2013; Kim, Cho and Lee, 2020). The proportional changes are very similar to the one observed between clustera 1 and 2 here. Cluster 2 shows patterns similar to those observed in females. It seems that the microbial community diverges early in life based on

gender as reported in northern elephant seal pups under naturally controlled diet (Stoffel *et al.*, 2020). Sexual dimorphism is a common mating strategy in otariids. Thus, it is possible that otariids such as JFFS, show similar differences as the ones observed in elephant seals. This hypothesis could be confirmed by using molecular methods for gender identification.

A commonality between the gender and age hypotheses is their relationship to the diet. Differences in diet have been identified as one of the main drivers of gut microbiome diversity (Ley, Hamady, *et al.*, 2008; Nelson *et al.*, 2013; Nishida and Ochman, 2018). While pups rely on lipid-rich milk, fish from the family *Myctophidae* are the most important prey of adult female JFFS (Francis, Boness and Ochoa-Acuña, 1998). Myctophids are known to be rich in fatty acids (Lea, Nichols and Wilson, 2002; Baby, Sankar and Anandan, 2014). Pacheco-Sandoval *et al.* (2019) showed that harbour seal faecal samples containing more lipid-rich preys had a much higher abundance of *Firmicutes* and lower *Fusobacteria* and *Bacteriodetes (Pacheco-Sandoval et al., 2019).* Molecular identification of prey species in faecal samples, may therefore help to determine whether diet is the driving factor behind the microbial differences observed here.

Conclusion:

This study characterised the faecal microbiome of the Juan Fernandez fur seal for the first time, including colonies from two of the three islands of the Juan Fernandez archipelago to which the species is endemic. Our findings showed that the overall microbiome composition was similar to compositions described for other pinnipeds. However, some of the samples showed a very different microbial composition pattern. This difference was mostly explained by an inverse relationship between *Peptoclostridium* and *Fusobacterium*

abundance. Gender, and its relationship to foraging behaviour, seems to be the most likely explanation of this phenomenon. However, additional studies investigating the relationship between gender, age and prey are required to test this hypothesis. Overall, the results of this study provide a good baseline from which future hypothesis-based studies can be carried out and it contributes to the understanding of host-microbial interaction in free-ranging, wild populations of pinnipeds. We highlight the need to expand knowledge in this field, particularly on microbial functionality, to understand its different members' roles and compare microbial patterns between and within species.

Data availability

Raw reads data are publicly available in the European Nucleotide Archive (ENA) under the study accession PRJEB36555. All the scripts used in this study can be accessed in https://github.com/Cotissima/JFFS_microbiome_first_characterisation.

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Conflict of interest

The authors confirm that they have no conflicts of interest related to the content of this article.

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729 # Tables

Table 1. Amplicon sequence variants present in at least 90% of the samples. Only three were present in all the samples. Unrarefied data was used to build this table. Abundances (abun) was calculated based on the total ASVs counts.

ASV	Phylum	Family	Genus	Abun (%)
Present in all the samples				
57729b2b058d8d5253d3e56e4f6386ca	*Fusobacteria*	*Fusobacteriaceae*	*Fusobacterium*	14.93
e8b1922518029c50c69add839142db03	*Fusobacteria*	*Fusobacteriaceae*	*Fusobacterium*	6.52
c0dc53aad260a1b951b7f99966251c7c	*Fusobacteria*	*Fusobacteriaceae*	*Fusobacterium*	3.73
Present in at least 90% of the samples				
f347c63fc5e4aeb97531e656e3765e2a	*Firmicutes*	*Peptostreptococcaceae*	*Peptoclostridium*	8.29
57f9edc6542ce6b78ff352942d6774c6	*Bacteroidetes*	*Bacteroidaceae*	*Bacteroides*	4.28
31984a302fdfe46b5e852fa473e682a4	*Bacteroidetes*	*Bacteroidaceae*	*Bacteroides*	4.26
1153942c5cc40d6ba5609222ded586fe	*Firmicutes*	*Lachnospiraceae*	*Coprococcus 3*	2.98
65dd9f625700a97a1cce9f5eefe4e6cb	*Firmicutes*	*Lachnospiraceae*	*Blautia*	2.18
435975b6d032d4b05233d8b94193b2ad	*Firmicutes*	*Lachnospiraceae*	*[Ruminococcus] gauvreauii group*	1.93
03f74c0ea1f0654719b21d2701e9fa30	*Proteobacteria*	*Burkholderiaceae*	*Sutterella*	1.30
8e10797dedc288dbc0be61fe4b5a5dfb	*Actinobacteria*	*Coriobacteriaceae*	*Collinsella*	1.16

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Table 2. SIMPER analysis comparing the faecal microbiota composition of Juan Fernadez fu seal at the genus level. The table showing up to a cumulative contribution of 70%. Cluster averages were calculated based on total counts. Kruskall-Wallis results are only shown when reaching significant difference.

Genus	Mean cluster 1	Mean cluster 2	Mean Diss	Contrib (%)	Cum (%)	w	P-value
Peptoclostridium	3%	29%	17%	25	25	3	<0.001
Fusobacterium	34%	8%	17%	24	49	456	<0.001
Bacteroides	14%	6%	7%	10	59	365.5	0.006
Ruminococcaceae UCG-005	4%	7%	4%	6	65		No sig
[Ruminococcus] gauvreauii group	1%	6%	4%	5	70	124	0.06

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Figure legends

Figure 1. Juan Fernandez fur seal (*Arctophoca philippii philippii*).

Figure 2. Simplified map of Robinson Crusoe and Santa Clara islands. The plane indicates the airfield and the dotted line the access route from the airfield to San Juan Bautista Village (the only settlement on the island). Fur seal icons show the sampling locations. El Arenal (EA) (n = 9), Bahia El Padre (BP) (n = 23), Piedra Carvajal (PC) (n = 1), Punta Trueno (PT) (n = 1), Santa Clara (SC) (n = 12), Tierras Blancas (TB) (n = 10) and Vaqueria (V) (n = 1). 57 samples in total.

Figure 3. Composition of the Juan Fernandez fur seal faecal microbiome at the family level. Only families with >1% relative abundance are shown. A) Average relative abundance across all samples with standard deviations. B) Relative abundance per sample grouped by location: EA= El Arenal, BP= Bahia El Padre, PC = Piedra Carvajal, PT= Punta Truenos, SC= Santa Clara, TB= Tierras Blancas, V= Vaqueria.

Figure 4. Comparison of three different alpha diversity indices between the four reproductive colonies in the Juan Fernandez archipelago. Samples collected from Tierras Blancas show a tendency to have higher levels of alpha diversity. Filtered rarefied data was used to calculate the diversity estimates.

Figure 5. PCoA using Bray-Curtis dissimilarity distance matrix using the filtered rarefied core dataset. Samples clustered in two groups. (circles = cluster 1, triangles = cluster 2). Location is not driving the clustering.

Figure 6. Relative average abundance of the dominant phyla according to the clusters identified with Bray Curtis dissimilarity. Showing only phyla with an average relative abundance ≥ 1 %. The differences in microbial patterns can be identified from high taxonomic levels.

Figure 7. Spearman rank correlation correlogram between bacterial genera and the first two principal components generated from Unifraq WU_PC1 and WU_PC2 and Bray-Curtis (BC_PC1 and BC_PC2) distances. The plot shows the direction (blue = positive, red = negative) and the strength (larger = stronger) of the correlation between each pair combination. Only significant correlations ($p \leq 0.05$) are represented with circles.