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Patterns of the fecal microbiota in the Juan Fernández fur seal (*Arctocephalus philippii*)

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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 characterize the fecal 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 fecal samples were collected from seven different JFFS colonies within the Juan Fernandez Archipelago, Chile. Bacterial composition and abundance were characterized 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 fecal microbiome, these results contribute towards our understanding of the natural microbial diversity and composition in free-ranging pinnipeds.

KEYWORDS
Arctophoca philippii, marine mammals, microbiome, pinnipeds, scatology

1 | 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 recognized as one of the largest microbial reservoirs (O'Hara & Shanahan, 2006). This microbial community fulfills 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, 2008b; Nicholson et al., 2012). Similar factors shaping the gut microbiome in terrestrial mammals influence that of marine mammals (Nelson et al., 2013; Pacheco-Sandoval et al., 2019; Smith et al., 2013; 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) (Bik et al., 2016; Nelson et al., 2013). 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 characterization of the core microbiome of these populations is therefore required as a fundamental baseline before we can attempt to understand its functions, roles, interactions, and possible uses (Shade & Handelsman, 2012).

The fecal microbiome has been characterized 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*, 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 (Figure 1). The archipelago is a hotspot for biodiversity with a high number of endemic marine species, including the JFFS (Aguayo et al., 1971; Friedlander et al., 2016; Pompa et al., 2011). 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 & Francis, 1995; Trites, 2019). Their feeding behavior, 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 characterize the JFFS fecal 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.

FIGURE 1 Juan Fernandez fur seal (*Arctophoca philippii philippii*)

2 | METHODS

2.1 | Sample collection

Fecal 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) (Figure 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 color to reduce the variability caused by the delay between the defecation and collection. A disposable wooden spatula was used to expose the center of the feces to avoid collecting material in direct contact with the surrounding elements. Using a sterile Copan FLOQSwab®, a sample from the core of the feces was placed into RNAlater® (Sigma-Aldrich) (Blekhman et al., 2016; Vlčková et al., 2012). 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 h post collection for 1–2 months until arrival in the laboratory, where they were transferred to −80°C until further analysis.

2.2 | 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 the collection will not be explored in this study.

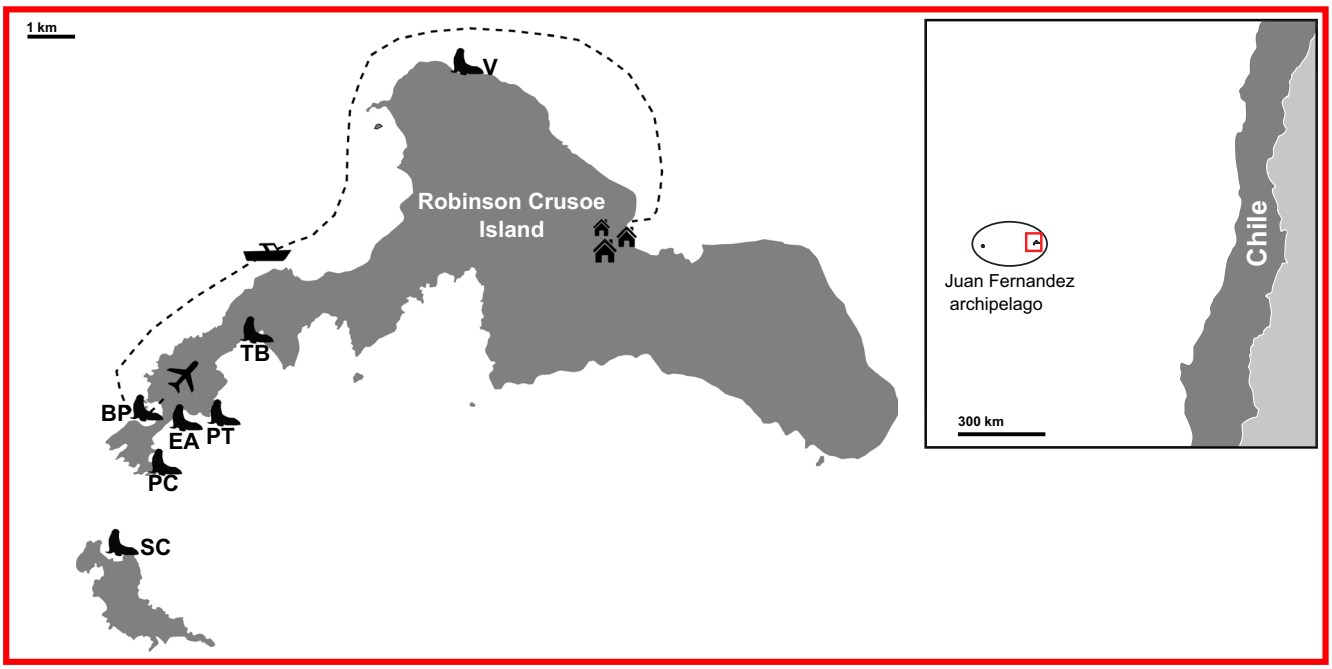

FIGURE 2 Simplified map of Robinson Crusoe and Santa Clara Islands. The plane indicates the airfield and the dotted line the access route from the airfield to San Juan Bautista Village (the only settlement on the island). Fur seal icons show the sampling locations. El Arenal (EA) (n = 9), Bahia El Padre (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

Samples were thawed on ice and centrifuged at 10,000 *g* for 15 min to pellet the sample out of RNAlater®. Genomic DNA was extracted from each pelleted sample (approximately 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 polymerase chain reaction (PCR) amplified targeting a 250 bp region covering the V4 variable region. PCR amplification, barcode tagging, and library preparation were performed according to 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 2 × 250 bp paired-end reads in the 2017 samples while the 2018 sequences were 2× 150 bp paired-end reads.

2.3 | 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). Demultiplexed raw sequences were imported into QIIME2-2019.10 (Bolyen et al., 2019) where quality control, de-replication, read truncation, and paired read merging were 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 modeling and correcting Illumina sequencing errors. This step was carried out separately according to the year of collection. However, to normalize 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 & 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 et al., 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).

2.4 | 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 an insufficient depth of sampling prior to analysis, multiple filtering steps were applied to the data using the phyloseq package (McMurdie & 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 (Figure A1), three samples were identified as having an 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 were rarefied using the same threshold used for filtering samples (Table A1) (McKnight et al., 2019).

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

2.4.1 | 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 and 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 were used to explore the alpha diversity. To compare locations, a one-way analysis of variance test (ANOVA) or a non-parametric Kruskal–Wallis test was performed for each estimate. ANOVA assumptions were tested by visualization of the data and statistical testing. A Shapiro–Wilk test was used to confirm normality and 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 visualization 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).

2.4.2 | 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 visualized 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 Percentage 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's 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 visualized in a correlation matrix plot, and only those significantly and strongly correlated (Rho (ρ) ≥ |0.6|) were explored further. For this method, only the core microbiome dataset was used at the genus level.

3 | RESULTS

Following the 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 (Table A1). Three samples were removed from the analysis due to rarefaction analysis indicating the insufficient depth of sequencing. The rarefied dataset ended up with 518 ASVs and a total of 754,974 reads.

3.1 | Composition of the Juan Fernandez fur seal fecal microbiome

A total of 10 bacterial phyla were detected in the feces 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) (Table A2). 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* (Figure 3a,b) (Table A3). Forty-six ASVs were present in at least 50% of the samples (Table A4). 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).

3.2 | Alpha diversity

Three alpha diversity indices (Chao1, Shannon-Weiner, and Simpson) were used to compare within-sample diversity between locations (Table A5). 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 Chao1 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 (Figure 4, Figure A2).

3.3 | 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 the 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) was separated from the main cluster (cluster 1) along PC1 (Figure 5, Figure A3).

Based on the relative average abundance of the dominant phyla, evident differences in the overall microbial composition were visualized between the two clusters (Figure 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, R^2 = 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.

3.4 | 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 (Figure 7, Table A6). 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, $\rho = -0.75$, $p \leq 0.001$).

4 | 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 fecal microbiome in free-ranging pinnipeds and the first of JFFS. Our approach focused on characterizing the core members of the JFFS fecal 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 fecal microbiome: *Firmicutes*, *Fusobacteria*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* (Bik et al., 2016; Kim et al., 2020; Nelson, Rogers, & Brown, 2013; Numberger et al., 2016; Pacheco-Sandoval et al., 2019; Stoffel et al., 2020). When comparing our results to other southern pinnipeds, different microbial patterns were found in feces from other fur seal species (Medeiros et al., 2016; Smith et al., 2013). The fecal 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) characterized the fecal 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

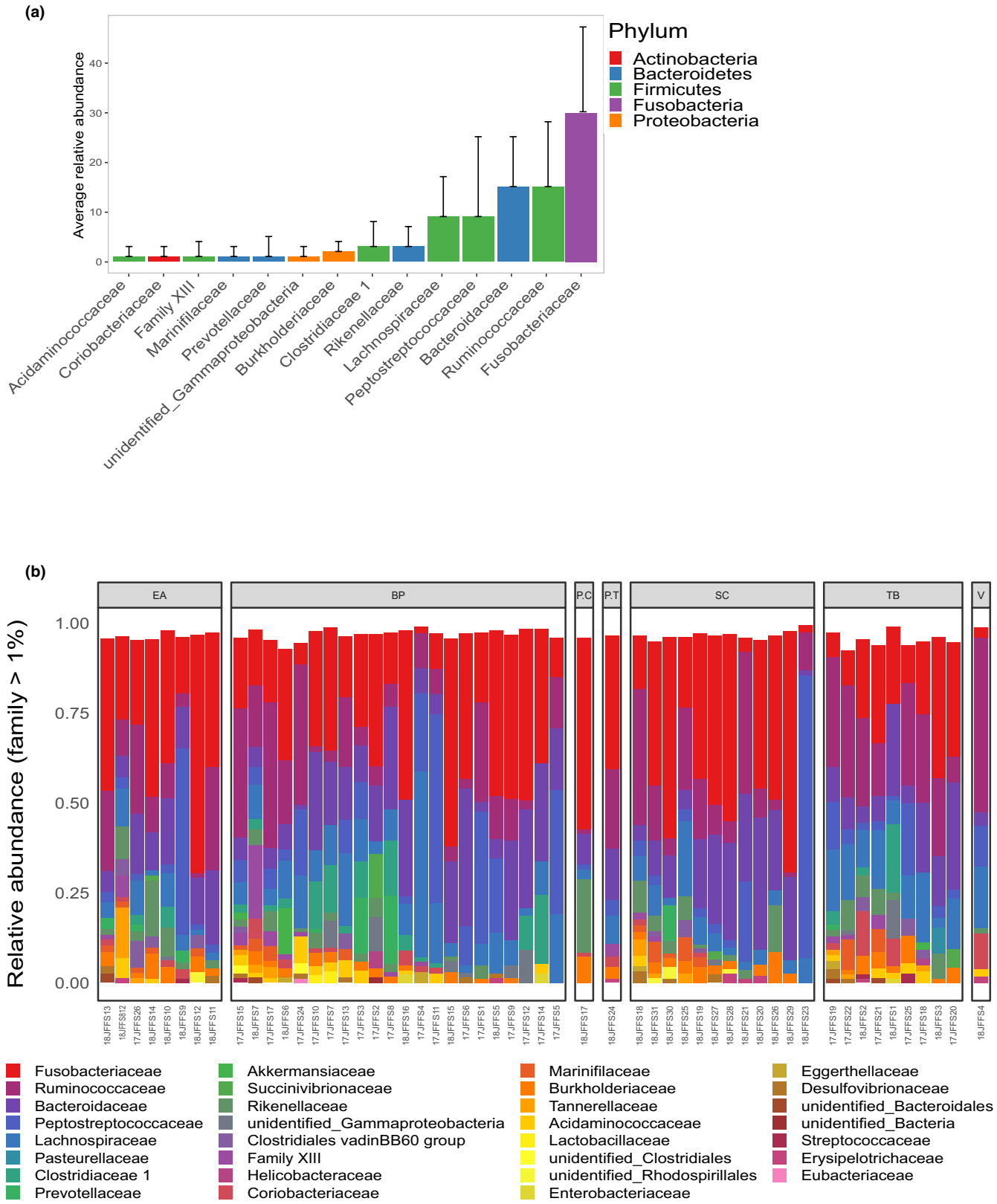

FIGURE 3 Composition of the Juan Fernandez fur seal fecal microbiome at the family level. Only families with >1% relative abundance are shown. (a) Average relative abundance across all samples with standard deviations. (b) Relative abundance per sample grouped by location: EA = El Arenal, BP = Bahia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

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

ASV	Phylum	Family	Genus	Abun (%)
Present in all 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

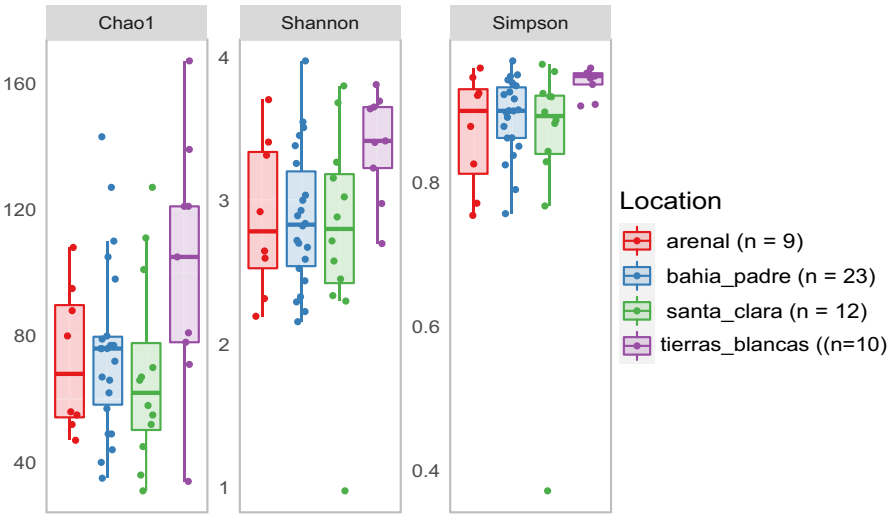

FIGURE 4 Comparison of three different alpha diversity indices between the four reproductive colonies in the Juan Fernandez archipelago. Samples collected from Tierras Blancas show a tendency to have higher levels of alpha diversity. Filtered rarefied data were used to calculate the diversity estimates

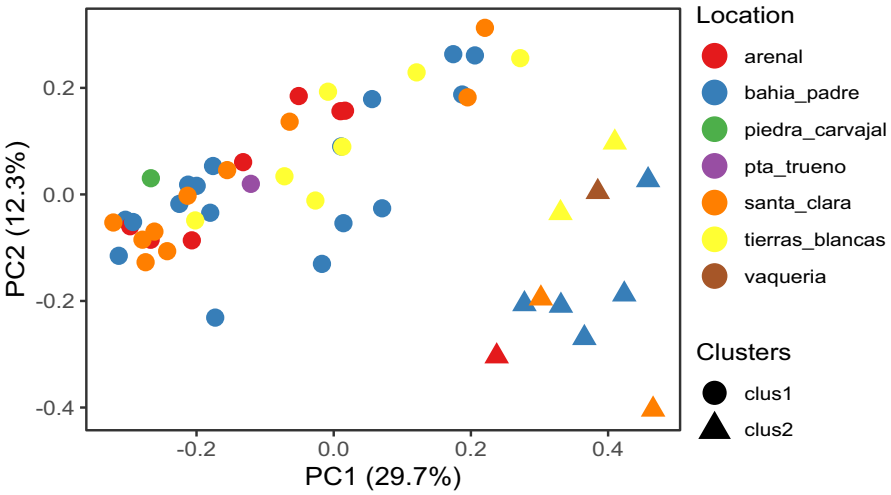

FIGURE 5 PCoA using Bray-Curtis dissimilarity distance matrix using the filtered rarefied core dataset. Samples clustered in two groups. (circles = cluster 1, triangles = cluster 2). Location is not driving the clustering

of the adults. However, the authors only relied on fluorescent *in situ* hybridization (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 harbor seals) have been found to consistently dominate the overall microbial composition of pinnipeds, followed by *Proteobacteria* and *Actinobacteria* (Nelson, Rogers, & Brown, 2013; Pacheco-Sandoval et al., 2019). The latter two are usually at the 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 humans 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 (Hildebrandt et al., 2009; Pu et al., 2016; Turnbaugh et al., 2006). We also observed changes in the relative abundance of *Fusobacteria* were similar to those observed in *Bacteroidetes*. This suggests some functionally redundant roles.

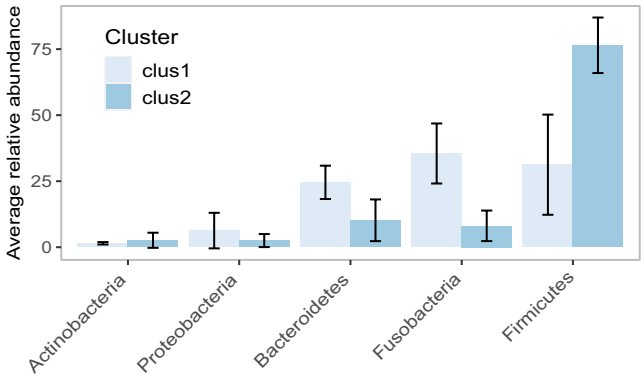

FIGURE 6 Relative average abundance of the dominant phyla according to the clusters identified with Bray Curtis dissimilarity. Showing only phyla with an average relative abundance ≥1%. The differences in microbial patterns can be identified from high taxonomic levels

The phylum *Firmicutes* is common in mammalian gut microbiomes (Ley et al., 2008a, 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* (Hildebrandt et al., 2009; Pu et al., 2016; Turnbaugh et al., 2006). 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 & 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 the high relative abundance in the gut microbiomes of strict carnivores adapted to diets rich in proteins, purines, and polyunsaturated fatty acids (Guo et al., 2020; Zhu et al., 2018). 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-centered 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 (Garrett & Onderdonk, 2014; Huh & Roh, 2020; Potrykus et al., 2008). 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 et al., 2008).

Similar to *Fusobacteria*, the phylum *Bacteroidetes*, especially members of the genus *Bacteroides*, are associated with diets high in animal proteins (Guo et al., 2020; Zhu et al., 2018). 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

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

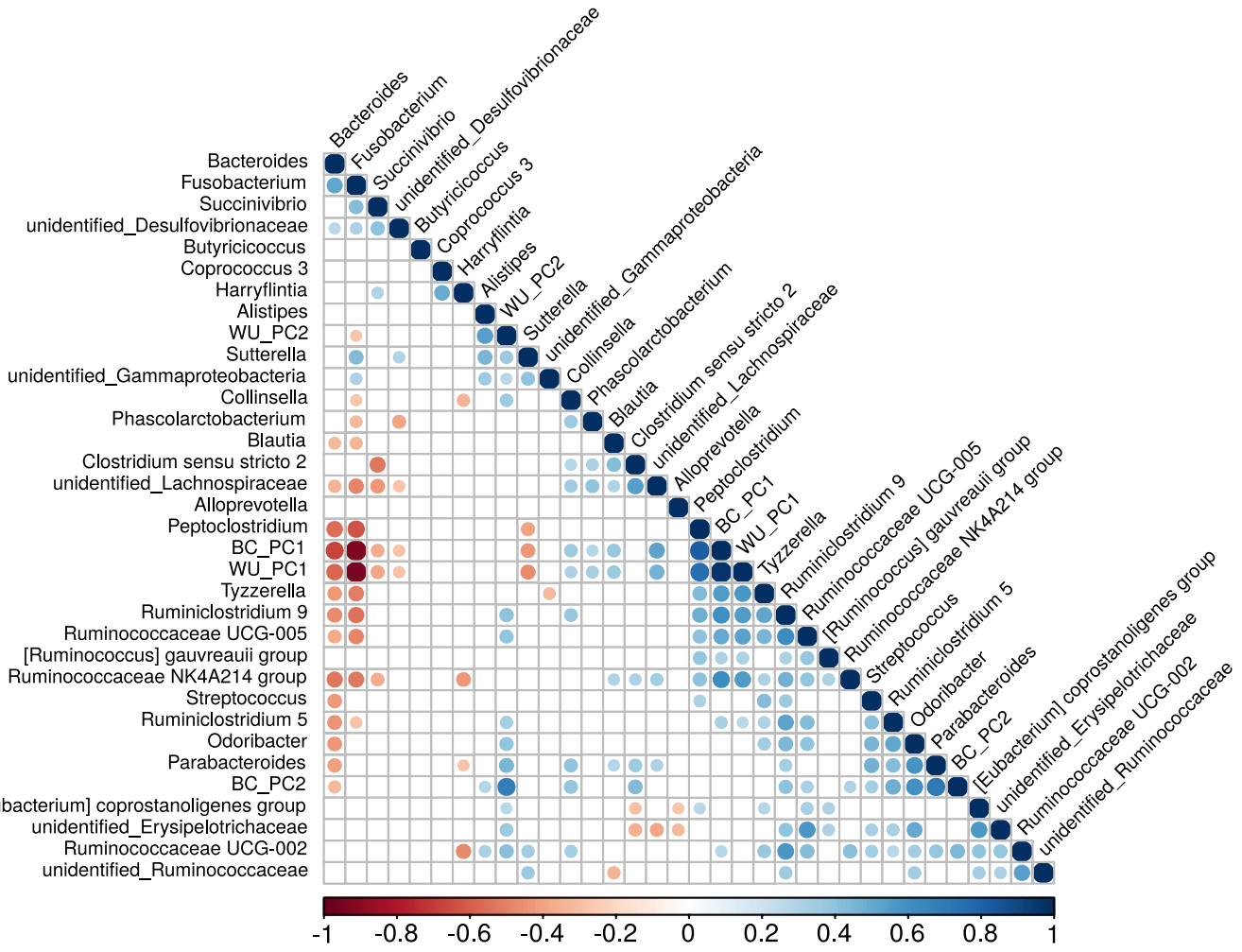

FIGURE 7 Spearman rank correlation correlogram between bacterial genera and the first two principal components generated from Unifraq WU_PC1 and WU_PC2 and Bray-Curtis (BC_PC1 and BC_PC2) distances. The plot shows the direction (blue = positive, red = negative) and the strength (larger = stronger) of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

suggests differences in nutritional needs and will be discussed later in the text.

4.1 | Within sample diversity

Initially, we hypothesized 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 (Figure 1). We found instead that BP did not differ from other less-visited locations such as EA and SC. Therefore, this finding is different from a previous report showing an association between exposure to anthropogenic stressors and reduced alpha diversity in harbor 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 (Li 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). The population density of JFFS and its effects on the microbiome have 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 feces (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 from the other locations. The other two diversity estimates (Chao1 richness and Shannon-Weiner) did not reach our significance cut-off. Both these estimates are affected by the detection of rare taxa, and larger libraries and sample sizes are more likely to input rare taxa into the data set. ANOVA was also used to compare locations with these diversity estimates. ANOVA is sensitive to differences in sample size, and therefore small group sizes may have affected statistical power.

4.2 | Variation between samples

The Bray-Curtis dissimilarity PCoA revealed two distinct clusters. Seventy-five percent 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% (Delport et al., 2016; Nelson, Rogers, & Brown, 2013; 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 characterized 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 feces originated from sample contamination by surrounding environmental bacteria. Such high prevalence may be a sign of a deeper relationship between these uncharacterized 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 absorption (Semova et al., 2012). Juan Fernandez fur seal milk composition contains a higher proportion of lipids in comparison to many pinnipeds (~ 41%) (Ochoa-Acuña et al., 1999). Thus, if the fecal samples from Cluster 2 were collected from preweaning pups (7–10 months old), it may be expected that a higher relative abundance of members of the phylum *Firmicutes* would be found. Similar to the microbial pattern observed in Cluster 2, samples analyzed from Australian fur seals 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 feces 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 a pre-weaning diet is not the explanation for the abundance of *Peptoclostridium*.

Differences between sexes 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 & Mesnick, 2009). Sex differences in foraging behavior and prey selection have also been reported (Andersen et al., 2013; Lewis et al., 2006; Ochoa Acuña & Francis, 1995). Based on the differences in diets, it is not surprising to find studies in gut microbial composition also showing sex-based differences. Samples collected from adult Southern elephant seals evidenced significant differences between adult males and females (Kim et al., 2020; Nelson, Rogers, & Brown, 2013). 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 (Kim et al., 2020; Nelson, Rogers, & Brown, 2013). The proportional changes are very similar to the one observed between clusters 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 sex as reported in northern elephant seal pups under a naturally controlled diet (Stoffel et al., 2020). Sexual dimorphism is a common mating strategy in otariids. Thus, otariids such as JFFS may show similar differences as the ones observed in elephant seals. This hypothesis could be confirmed using molecular methods for sex identification.

A commonality between the sex 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 et al., 2008a; Nelson, Rogers, & Brown, 2013; Nishida & Ochman, 2018). While pups rely on lipid-rich milk, fish from the family *Myctophidae* are the most important prey of adult female JFFS (Francis et al., 1998). Myctophids are known to be rich in fatty acids (Baby et al., 2014; Lea et al., 2002). Pacheco-Sandoval et al. (2019) showed that harbor seal fecal 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 fecal samples may therefore help to determine whether the diet is the driving factor behind the microbial differences observed here.

5 | CONCLUSION

This study characterized the fecal 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. Sex and its relationship to foraging behavior seem to be the most likely explanation of this phenomenon. However, additional studies investigating the relationship between sex, 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.

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CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

Constanza Toro-Valdivieso: Conceptualization (equal); Formal analysis (lead); Funding acquisition (equal); Investigation (lead); Methodology (equal); Visualization (lead); Writing-original draft (lead); Writing-review & editing (equal). **Frederick Toro:** Formal analysis (supporting); Writing-review & editing (supporting). **Samuel Stubbs:** Formal analysis (supporting); Writing-original draft (supporting); Writing-review & editing (equal). **Eduardo Castro-Nallar:** Formal analysis (supporting); Funding acquisition (equal); Writing-review & editing (equal). **Barbara Blacklaws:** Conceptualization (equal); Formal analysis (supporting); Funding acquisition (equal); Methodology (equal); Supervision (lead); Writing-original draft (supporting); Writing-review & editing (equal).

ETHICS STATEMENT

All fecal 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 the importation of samples into the United Kingdom was also obtained (ITIMP16.1158).

DATA AVAILABILITY STATEMENT

Raw reads data are publicly available in the European Nucleotide Archive (ENA) under the study accession PRJEB36555: https://www.ebi.ac.uk/ena/browser/view/PRJEB36555. All scripts used in this study can be accessed in GitHub at https://github.com/Cotissima/JFFS_microbiome_first_characterisation.

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APPENDIX

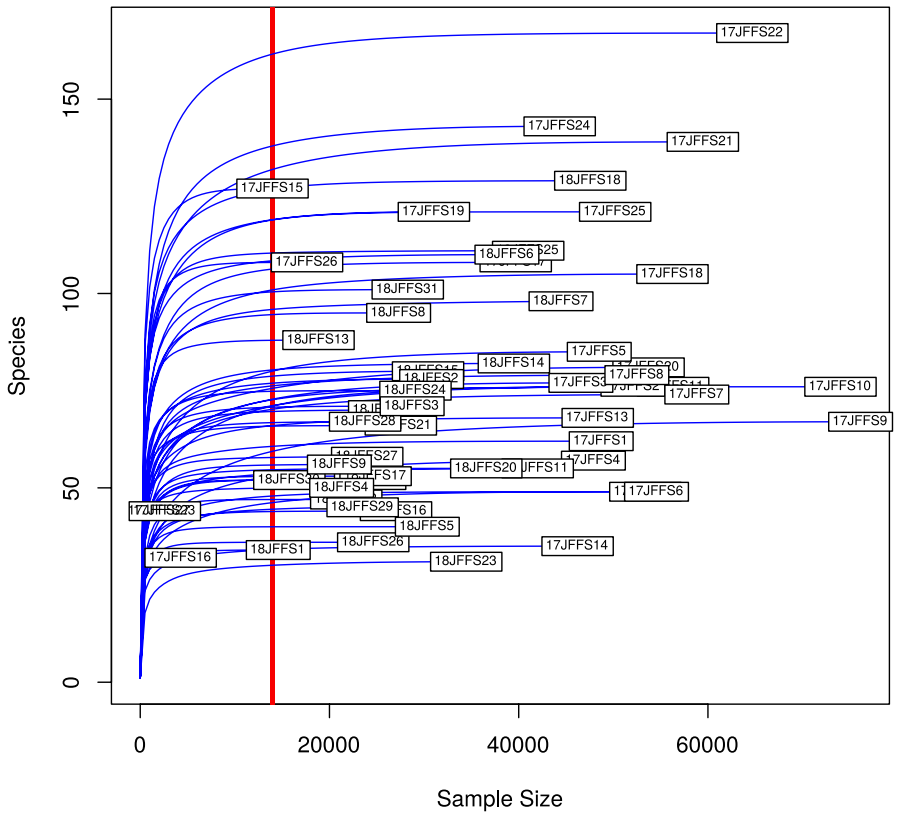

FIGURE A1 Rarefaction curve estimating the number of ASVs (y-axis) for a given read count (x-axis). The vertical line indicates the cutoff at which samples were retained and rarefied

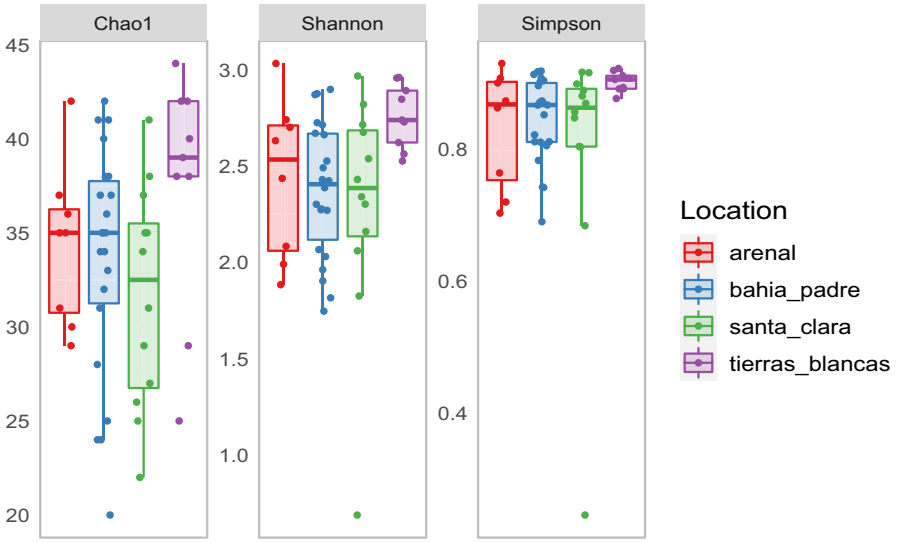

FIGURE A2 Comparison of three different alpha diversity indices between four reproductive colonies in the Juan Fernandez archipelago. Samples collected from Tierras Blancas show a tendency to have higher levels of alpha diversity. Core rarefied data were used to calculate the diversity estimates

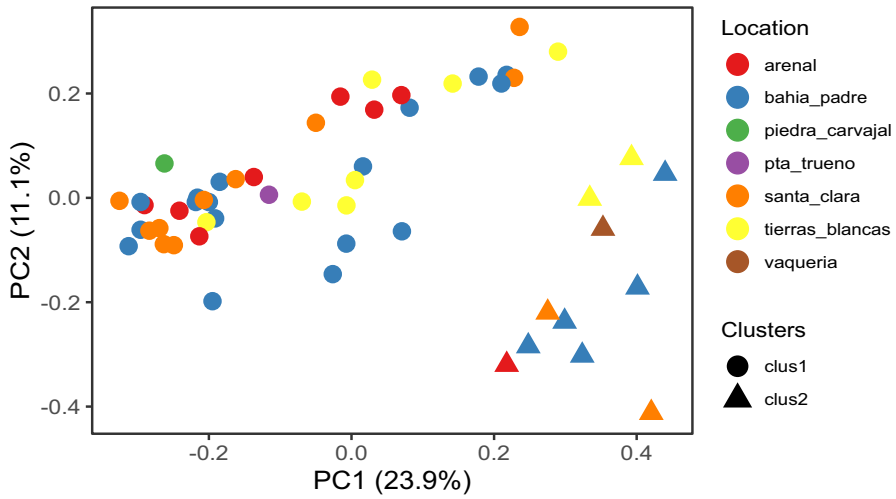

FIGURE A3 PCoA using Bray-Curtis dissimilarity distance matrix using the filtered rarefied full dataset. Samples clustered in two groups (circles = cluster 1, triangles = cluster 2). Location is not driving the clustering

TABLE A1 Inputs and outputs of each preprocessing step

Preprocessing steps	Number of samples	Number of ASVs	Min. number of reads per sample	Max. number of read per sample	Filtered reads	Total
Raw	57	595	2042	76,134	0	2,074,038
Filter ASVs (non-bacterial and ambiguous)	57	577	2042	76,134	2081	2,071,957
Filter samples	54	577	13,981	76,134	8916	2,063,041
Filter Contaminants	54	558	13,981	76,134	278	2,062,763
Rarefaction	54	518	13,981	13,981	1,307,789	754,974

TABLE A2 Bacterial phyla detected in Juan Fernandez fur seal feces

Family counts	Total	Total counts rel. ab (%)	Mean rel. ab (%)	Rel. ab SD	Total ASV
Firmicutes	863,365	41.85	40	24	296
Fusobacteria	582,406	28.23	30	17	46
Bacteroidetes	455,251	22.07	22	10	94
Proteobacteria	113,805	5.52	6	4	74
Actinobacteria	30,597	1.48	2	3	21
Verrucomicrobia	6653	0.32	0	2	3
Epsilonbacteraeota	6554	0.32	0	1	10
Unidentified	2204	0.11	0	0	2
Tenericutes	1005	0.05	0	0	8
Lentisphaerae	900	0.04	0	0	3
Spirochaetes	34	0.00	0	0	3

TABLE A3 Summary of the bacterial family detected in feces of Juan Fernandez fur seal. Data are arranged in decreasing order based on counts mean

Family	Total counts	Counts rel. ab (%)	Counts mean	Counts SD	Mean rel.ab (%)	Re. ab SD	Total ASV
Fusobacteriaceae	582,404	28.23	10,785.26	6958.72	30	17	45
Bacteroidaceae	320,047	15.52	5926.8	5319.43	15	10	28
Ruminococcaceae	310,109	15.03	5742.76	5206.41	15	13	139
Lachnospiraceae	213,725	10.36	3957.87	4195.97	9	8	61
Peptostreptococcaceae	193,151	9.36	3576.87	6353.37	9	16	16
Rikenellaceae	65,548	3.18	1213.85	1543.63	3	4	20
Clostridiaceae 1	60,276	2.92	1116.22	2385.25	3	5	16
Burkholderiaceae	47,544	2.3	880.44	849.59	2	2	8
unidentified_ Gammaproteobacteria	27,169	1.32	503.13	1116.46	1	2	9
Acidaminococcaceae	27,237	1.32	504.39	734.56	1	2	3
Marinifilaceae	25,673	1.24	475.43	1022.21	1	2	13
Prevotellaceae	24,111	1.17	446.5	1666.42	1	4	4
Coriobacteriaceae	23,956	1.16	443.63	688.99	1	2	1
Family XIII	22,734	1.1	421	1294.81	1	3	11
Clostridiales vadinBB60 group	16,935	0.82	313.61	586.95	1	2	2
Tannerellaceae	15,153	0.73	280.61	647.81	1	2	8
Succinivibrionaceae	14,801	0.72	274.09	931.79	1	2	7
Desulfovibrionaceae	12,759	0.62	236.28	296.88	1	1	10
Erysipelotrichaceae	6926	0.34	128.26	161.05	0	1	7
Akkermansiaceae	6644	0.32	123.04	696.94	0	2	2
Eggerthellaceae	5951	0.29	110.2	241.78	0	1	4
Helicobacteraceae	5185	0.25	96.02	371.85	0	1	7
Streptococcaceae	4000	0.19	74.07	192.29	0	0	6
unidentified_Rhodospirillales	3691	0.18	68.35	153.19	0	1	4
Lactobacillaceae	3649	0.18	67.57	336.59	0	1	3
unidentified_Bacteroidales	3395	0.16	62.87	168.66	0	0	5
Enterobacteriaceae	3289	0.16	60.91	195.2	0	1	6
unidentified_Clostridiales	2650	0.13	49.07	186.76	0	1	10
unidentified_Bacteria	2204	0.11	40.81	129.68	0	0	2
Pasteurellaceae	2192	0.11	40.59	285.04	0	1	6
Campylobacteraceae	1369	0.07	25.35	92.27	0	0	3

(Continues)

TABLE A3 (Continued)

Family	Total counts	Counts rel. ab (%)	Counts mean	Counts SD	Mean rel.ab (%)	Re. ab SD	Total ASV
Spongiibacteraceae	1064	0.05	19.7	79.73	0	0	1
Nitrosomonadaceae	888	0.04	16.44	53.79	0	0	1
Mycoplasmataceae	881	0.04	16.31	92.11	0	0	7
Eubacteriaceae	806	0.04	14.93	70.4	0	0	1
Victivallaceae	662	0.03	12.26	71.52	0	0	2
Flavobacteriaceae	568	0.03	10.52	38.49	0	0	4
Barnesiellaceae	639	0.03	11.83	41.46	0	0	2
Peptococcaceae	438	0.02	8.11	22.97	0	0	2
Enterococcaceae	325	0.02	6.02	26.47	0	0	4
Vibrionaceae	113	0.01	2.09	9.78	0	0	2
vadinBE97	238	0.01	4.41	28.16	0	0	1
unidentified_Mollicutes RF39	124	0.01	2.3	16.87	0	0	1
Shewanellaceae	108	0.01	2	14.7	0	0	1
Corynebacteriaceae	246	0.01	4.56	14.72	0	0	3
Coriobacteriales Incertae Sedis	200	0.01	3.7	16.27	0	0	1
Christensenellaceae	180	0.01	3.33	8.89	0	0	2
Actinomycetaceae	188	0.01	3.48	9.79	0	0	6
Veillonellaceae	96	0	1.78	10.53	0	0	2
unidentified_Verrucomicrobiae	9	0	0.17	0.86	0	0	1
unidentified_Firmicutes	8	0	0.15	1.09	0	0	1
unidentified_Bacteroidia	2	0	0.04	0.27	0	0	1
unidentified_Actinobacteria	13	0	0.24	1.18	0	0	1
Thioalkalispiraceae	2	0	0.04	0.27	0	0	1
Staphylococcaceae	35	0	0.65	2.84	0	0	1
SC-I–84	3	0	0.06	0.41	0	0	1
Saprospiraceae	2	0	0.04	0.27	0	0	1
Rhodobacteraceae	8	0	0.15	0.79	0	0	2
Rhodanobacteraceae	9	0	0.17	1.22	0	0	1
Rhizobiales Incertae Sedis	4	0	0.07	0.54	0	0	1
Pseudomonadaceae	16	0	0.3	1.24	0	0	3
Porphyromonadaceae	2	0	0.04	0.27	0	0	1
OCS116 clade	2	0	0.04	0.27	0	0	1
Nocardioidaceae	5	0	0.09	0.68	0	0	1
Neisseriaceae	80	0	1.48	9.69	0	0	2
Muribaculaceae	2	0	0.04	0.27	0	0	1
Moraxellaceae	12	0	0.22	1.16	0	0	3
Micrococcaceae	32	0	0.59	2.26	0	0	1
Leptotrichiaceae	2	0	0.04	0.27	0	0	1
Halomonadaceae	36	0	0.67	3.62	0	0	2
Gracilibacteraceae	29	0	0.54	2.96	0	0	2
Family XI	6	0	0.11	0.57	0	0	2
Dietziaceae	4	0	0.07	0.38	0	0	2
Desulfobulbaceae	3	0	0.06	0.41	0	0	1
Crocinitomicaceae	6	0	0.11	0.82	0	0	1
Chitinophagaceae	101	0	1.87	8.26	0	0	4
Carnobacteriaceae	10	0	0.19	0.97	0	0	2
Cardiobacteriaceae	12	0	0.22	1.21	0	0	1
Brachyspiraceae	34	0	0.63	3.02	0	0	3
Bacillaceae	40	0	0.74	3.6	0	0	3

TABLE A4 Amplicon sequence variants present in at least 27 of the samples (50%). Relative abundance was calculated from the unrarefied data

ASV	Phylum	Family	Genus	Abundance (%)
57729b2b058d8d5253d3e56e4f6386ca	Fusobacteria	Fusobacteriaceae	Fusobacterium	14.93
f347c63fc5e4aeb97531e656e3765e2a	Firmicutes	Peptostreptococcaceae	Peptoclostridium	8.29
e8b1922518029c50c69add839142db03	Fusobacteria	Fusobacteriaceae	Fusobacterium	6.52
57f9edc6542ce6b78ff352942d6774c6	Bacteroidetes	Bacteroidaceae	Bacteroides	4.28
31984a302fdfe46b5e852fa473e682a4	Bacteroidetes	Bacteroidaceae	Bacteroides	4.26
b8d6a5a80d025861f2afccb79e0a1aaf	Bacteroidetes	Bacteroidaceae	Bacteroides	3.80
c0dc53aad260a1b951b7f99966251c7c	Fusobacteria	Fusobacteriaceae	Fusobacterium	3.73
1153942c5cc40d6ba5609222ded586fe	Firmicutes	Lachnospiraceae	Coprococcus 3	2.98
65dd9f625700a97a1cce9f5eefe4e6cb	Firmicutes	Lachnospiraceae	Blautia	2.18
e176cb3e4c2f33cee5d529c21ff5534e	Firmicutes	Clostridiaceae 1	Clostridium sensu stricto 2	1.95
435975b6d032d4b05233d8b94193b2ad	Firmicutes	Lachnospiraceae	[Ruminococcus] gauvreauii group	1.93
1a73c668a4bb92b74a18b79f9ae63460	Firmicutes	Ruminococcaceae	Ruminococcaceae UCG−005	1.75
5b87f47a447ef9a905807d2abed5b638	Bacteroidetes	Rikenellaceae	Alistipes	1.68
bf4112a100b11b4cbe9a25bdc591ea52	Fusobacteria	Fusobacteriaceae	Fusobacterium	1.38
03f74c0ea1f0654719b21d2701e9fa30	Proteobacteria	Burkholderiaceae	Sutterella	1.30
1188ef0238977f665e179642f287aead	Firmicutes	Ruminococcaceae	Ruminococcaceae UCG−005	1.29
25699f81befd34e0c9d81cfa84f4e751	Firmicutes	Lachnospiraceae	unidentified_Lachnospiraceae	1.27
8e10797dedc288dbc0be61fe4b5a5dfb	Actinobacteria	Coriobacteriaceae	Collinsella	1.16
2553bcb6afcdea16b173909555484369	Firmicutes	Ruminococcaceae	[Eubacterium] coprostanoligenes group	1.15
b15e41c7f20b8dcd4b0ed9f6c526885d	Bacteroidetes	Prevotellaceae	Alloprevotella	1.14
ca28c391514fb33d2d2df1c3c8e12317	Firmicutes	Ruminococcaceae	Ruminococcaceae UCG−005	1.12
76ded93fadbc4155db4e9dcba2012c81	Firmicutes	Ruminococcaceae	Ruminococcaceae UCG−002	1.07
c45b2a8ebeca2fca503c6312e8611416	Bacteroidetes	Marinifilaceae	Odoribacter	1.07
ce3476a906008973a3ab56de06817d56	Proteobacteria	Burkholderiaceae	Sutterella	0.87
975258836de3a001cb4d91cf6cf7de06	Firmicutes	Acidaminococcaceae	Phascolarctobacterium	0.72
1cde608d0a8b17d6fed116653581f050	Proteobacteria	Succinivibrionaceae	Succinivibrio	0.68
6c4c9e8ad2f56316cfffac9587c173ec	Firmicutes	Ruminococcaceae	Ruminococcaceae UCG−005	0.58
58514f20ebf4be2b13d619ba3bd2cf83	Bacteroidetes	Bacteroidaceae	Bacteroides	0.55
a0eee6d353d432299b53c9663cf05597	Bacteroidetes Bacteroidaceae	Bacteroides	0.54	
0ac8214c377877609cd0f88567086b2e	Firmicutes	Lachnospiraceae	Tyzzerella	0.44
420f3edebd00de18846a5941b55a6d5e	Bacteroidetes	Rikenellaceae	Alistipes	0.44
0e7fdaa233c333cb8363b63a41bbfc32	Firmicutes	Ruminococcaceae	Ruminococcaceae NK4A214 group	0.43
df408056297f20c5ce5cc68907e39cc8	Firmicutes	Lachnospiraceae	Tyzzerella	0.41
c00129ca877cb31776ad4e4e03a9091d	Fusobacteria	Fusobacteriaceae	Fusobacterium	0.41
93623ff4fe3615ce4aa4f0a9554fd4de	Proteobacteria	Desulfovibrionaceae	unidentified_ Desulfovibrionaceae	0.35
a672e8b3efeb3a28e5beabe661606ad2	Firmicutes	Ruminococcaceae	unidentified_ Ruminococcaceae	0.33
0c3d2038714f019f70fdc3b6f4b40419	Firmicutes	Ruminococcaceae	Ruminiclostridium 9	0.30
b6578d861d1c0e923087c8a5a81c8501	Proteobacteria	unidentified_ Gammaproteobacteria	unidentified_ Gammaproteobacteria	0.25
57f0c2ba2627cebfea197aa991777cb0	Bacteroidetes	Tannerellaceae	Parabacteroides	0.24

(Continues)

TABLE A4 (Continued)

ASV	Phylum	Family	Genus	Abundance (%)
305caa259fb99e3e9aa1eb5dac615002	Firmicutes	Erysipelotrichaceae	unidentified_ Erysipelotrichaceae	0.19
cbeb8d4b3d3f4b0bfa328178582220a5	Firmicutes	Streptococcaceae	Streptococcus	0.17
66c7c850483807f63638f7e03975cf27	Proteobacteria	unidentified_ Gammaproteobacteria	unidentified_ Gammaproteobacteria	0.15
78990f6a6e53bd64b9371e316ad97362	Firmicutes	Ruminococcaceae	Butyricicoccus	0.14
e8f48023e5081f948df1291acd8d356a	Firmicutes	Lachnospiraceae	unidentified_Lachnospiraceae	0.09
1c4ff74a77a35261b972eb21737647e9	Firmicutes	Ruminococcaceae	Ruminiclostridium 5	0.07
99ea1bdfe0e8b83616c6178b8fdbf1e4	Firmicutes	Ruminococcaceae	Harryflintia	0.05

TABLE A5 Table reporting the mean values of Chao-1, Shannon-Weiner and Simpson (D) indexes and their standard deviation for each location. Tierras Blancas consistently show higher values than the other three locations. Simpson here is used as 1-D. Thus, the higher the number, the more diverse. Non-normalized data were used to build this table

Location	Chao1	Shannon-Weiner	Simpson
Arenal	72.6 ± 23.0	2.9 ± 0.5	0.87 ± 0.08
Bahia El Padres	75.7 ± 27.4	2.9 ± 0.5	0.89 ± 0.05
Santa Clara	68.3 ± 30.0	2.8 ± 0.7	0.85 ± 0.16
Tierras Blancas	101.9 ± 40.1	3.4 ± 0.4	0.94 ± 0.02

TABLE A6 The selected value of the Spearman's rank correlation performed on the rarefied core data including PC1 and 2 for each dissimilarity distance. The table reporting only the correlation that showed to be strong ($0.6 \leq |\rho| \leq 0.79$) and very strong, ($0.8 \leq |\rho| \leq 1$)

	Correlation pair	ρ	Strength	p
Bacteroides	Bray-Curtis PC1	−0.67	Strong	<0.001
Fusobacterium	Bray-Curtis PC1	−0.92	Very strong	<0.001
Peptoclostridium	Bray-Curtis PC1	0.81	Very strong	<0.001
Ruminiclostridium 9	Bray-Curtis PC1	0.63	Strong	<0.001
Ruminococcaceae NK4A214 group	Bray-Curtis PC1	0.61	Strong	<0.001
Odoribacter	Bray-Curtis PC2	0.62	Strong	<0.001
Parabacteroides	Bray-Curtis PC2	0.71	Strong	<0.001
Fusobacterium	Peptoclostridium	−0.63	Strong	<0.001
Ruminiclostridium 9	Ruminococcaceae UCG−005	0.61	Strong	<0.001
Fusobacterium	Weighted Unifrac PC1	−0.94	Very strong	<0.001
Peptoclostridium	Weighted Unifrac PC1	0.75	Strong	<0.001