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RESEARCH ARTICLE

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Rookery through rehabilitation: Microbial community assembly in newborn harbour seals after maternal separation

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Abstract

Microbial community assembly remains largely unexplored in marine mammals, despite its potential importance for conservation and management. Here, neonatal microbiota assembly was studied in harbour seals (*Phoca vitulina richardii*) at a rehabilitation facility soon after maternal separation, through weaning, to the time of release back to their native environment. We found that the gingival and rectal communities of rehabilitated harbour seals were distinct from the microbiotas of formula and pool water, and became increasingly diverse and dissimilar over time, ultimately resembling the gingival and rectal communities of local wild harbour seals. Harbour seal microbiota assembly was compared to that of human infants, revealing the rapid emergence of host specificity and evidence of phylosymbiosis even though these harbour seals had been raised by humans. Early life prophylactic antibiotics were associated with changes in the composition of the harbour seal gingival and rectal communities and surprisingly, with transient increases in alpha diversity, perhaps because of microbiota sharing during close cohabitation with other harbour seals. Antibiotic-associated effects dissipated over time. These results suggest that while early life maternal contact may provide seeding for microbial assembly, co-housing of conspecifics during rehabilitation may help neonatal mammals achieve a healthy host-specific microbiota with features of resilience.

INTRODUCTION

Harbour seals are important indicator species for coastal ecosystems; they are long-lived, widely distributed and feed at a high trophic level (Anderson et al., 2015; Glad et al., 2010; Ross et al., 2004). In California, the springtime harbour seal pupping season coincides with peak beach use by humans; consequently, mother-pup pairs often become separated (Colegrove et al., 2005). Abandoned pups along the

central California coast are transported to a dedicated nursery at The Marine Mammal Center rehabilitation facility and marine mammal hospital (Sausalito, CA). These harbour seals are often only days old when they begin rehabilitation. The rescue of nursing pups after maternal separation necessitates an abrupt transition from maternal care to that of human handlers, from milk to formula and from the rookery to man-made enclosures. The majority of rehabilitated pups are successfully released back into the wild post-weaning (Murillo-Cisneros et al., 2022); however, it is unclear to what extent early life human stewardship influences the development of their nascent microbiota and if their microbiota ultimately achieves a structure similar to that observed in nature.

Considering that rehabilitated harbour seal pups have a formula diet and are raised by humans in a man-made environment, their upbringing shares features with that of human infants despite clear differences in host ancestry and provenance. Humans, another monogastric mammal, are the foundation for our current understanding of microbial assembly in mammals. Beginning at the time of birth, commensal microbes rapidly colonise the human gut (Palmer et al., 2007). The resulting communities convey essential nutritional, immunological and metabolic functions (Gerritsen et al., 2011; Koenig et al., 2011; Turnbaugh et al., 2007). Within several years, the dynamic gut communities of the infant develop adult-like features: higher diversity, relative stability and individualised structure (Backhed et al., 2015; Costello et al., 2009; de Muinck & Trosvik, 2018; Yatsunenko et al., 2012). The gut microbiota of humans, and of other adult mammals also typically demonstrates a strong signal of phylosymbiosis; community structure is host-species specific and more similar among closely related hosts (Ley et al., 2008).

Given the young age at which harbour seals are admitted to the nursery, it is likely that their rehabilitation coincides with the early assembly of commensal microbial communities. However, distal gut microbiota assembly has yet to be described in detail in marine mammals. Adult marine mammals have been shown to harbour host-specific (Dudek et al., 2022; Nelson et al., 2013a) microbial communities with high diversity, novelty (Bik et al., 2016) and functional potential (Dudek et al., 2017); however, it is unclear how these communities are established and to what extent vertical transfer contributes to acquired diversity and the development of a mature, host-specific structure. A few studies have examined age-related differences between broad age classes of pinnipeds and have detected differences in the richness and composition of faecal communities, suggesting maturation (Nelson et al., 2013b; Smith et al., 2013). Oral microbial community assembly, only recently characterised in mice, remains unexplored in other non-human mammals (Koren et al., 2021).

Neonatal pups arriving at the rehabilitation facility often present with an open umbilicus prone to infection; omphalitis can be life-threatening (Dierauf & Gulland, 2001; Frouin et al., 2013). Consequently, healthy newborn pups are commonly given antibiotics to prevent omphalitis. Ciprofloxacin is often administered due to its broad spectrum activity against known pathogens and convenient once-a-day dosing regimen (Barbosa et al., 2015). While prophylactic antibiotic use is common, it is not the rule, since facility veterinarians have been concerned that early life exposure might coincide with, and potentially disrupt, microbiota assembly. In human adults, microbial communities demonstrate resilience to perturbations such as antibiotic treatment, but severe or repeated disturbances can lead to alternative stable states (David et al., 2014; Dethlefsen & Relman, 2010; Nakayama et al., 2015). In high-income countries, nearly half of infants receive antibiotics, despite evidence that off-target effects on commensals may impair microbiota maturation (Anderson et al., 2017). Infant gut communities display reduced diversity and delayed maturation in association with antibiotic treatment (Bokulich et al., 2016; Yassour et al., 2016). Early life antibiotic exposure has also been correlated with subsequent development of chronic diseases, such as asthma and diabetes (Arrieta et al., 2014; Kostic et al., 2015). Compared to humans, much less is known about how antibiotics affect microbiota maturation trajectories in companion animals and wildlife, despite widespread use in veterinary medicine. Optimal wildlife rehabilitation depends upon maintenance of a health-promoting microbiome and avoidance of unnecessary harm through human interventions.

Here, we provide the first description, to our knowledge, of early life gingival and rectal bacterial community assembly in a marine mammal species from the first days of life through weaning, and the effects of antibiotics. We characterised microbial community assembly in healthy neonatal harbour seals rescued while <2 weeks old. Gingival and rectal swabs were collected longitudinally over several months, a period that encompassed a dietary change from formula to fish at the age seals are typically weaned in the wild, and the microbiota structure was characterised. We compared harbour seal-associated communities to those within the formula and pool water to identify sources of bacterial seeding at the facility. We compared the microbiota of the oldest seals just prior to release to the microbiota of wild local harbour seals of a comparable age to determine if assembly during rehabilitation approximated the trajectory predicted to have occurred in these animals in the wild. In tandem, we analysed a similar set of 16S rRNA sequence data from longitudinal human infant stool collections (Palmer et al., 2007) to compare distal gut microbiota assembly between neonatal mammalian species with different ancestries

and determine whether and when evidence of phylosymbiosis became evident in this harbour seal cohort, given their young age, proximity to humans, and limited exposure to their natural environment. To evaluate the impact of early life antibiotic exposure on microbial community assembly, we also looked specifically at the effects of these drugs on the harbour seal microbiota to understand better the potential risks of prophylactic treatment.

EXPERIMENTAL PROCEDURES

Rehabilitation facility protocols and sample collection

We studied 15 Pacific harbour seals (*Phoca vitulina richardii*) undergoing rehabilitation at The Marine Mammal Center (TMMC) in Sausalito, CA. Recently born and abandoned at nearby rookeries, pups were captured without sedation after maternal separation. Age was estimated by examining tooth development and the umbilical stump (Dierauf & Gulland, 2001). On the basis of veterinary examination, the 15 study seals were assessed to be systemically healthy; nonetheless, oral antibiotic treatment was initiated for some to prevent potential infection (Table S1). Although the decision to give prophylactic antibiotics was not randomized among the animals, health status was comparable between treated and untreated animals. Those that became ill or received other antibiotics or anti-inflammatory medications after the initiation of sample collection were excluded from subsequent analysis. Staff provided formula feedings (multi-milk replacement formula; Pet-Ag, Inc., Hampshire, IL) with herring and fish oil via gavage (Colegrove et al., 2005; Greig et al., 2014). When pups reached 2 months of age (or weighed ~15 kg) they were fed herring exclusively in preparation for release from the facility (Trumble et al., 2013).

Illumina Epicentre Catch-All foam swabs (Madison, WI) were used to sample the left mandibular gingival sulcus and rectum every other day while pups were physically restrained for gavage of formula. Once free feeding on fish, sampling occurred biweekly for 2 weeks, then weekly, to reduce human exposure in anticipation of the seal's eventual release. Harbour seals were housed in dynamic age-matched groups, first in smaller enclosures with shallow pools that allowed bathing but not swimming, then larger enclosures with deeper pools that allowed swimming. Pools contained artificial seawater that originated from a common source and was delivered to each pool separately (i.e., no direct mixing of water occurred between pools). Pool water was drained, filtered and sterilised before recirculation into the system and a portion (~20%) was replaced weekly with new artificial seawater. Weekly surface pool water samples were collected using sterile 50 mL conical vials. Water samples and swabs were stored without preservative at −80°C, then transported to Palo Alto on dry ice for processing.

Wild harbour seal sample collection

Wild harbour seals in the same age range (~30–100 days old) as the oldest rehabilitated seals were sampled at Castro Rocks (Richmond, CA), the largest rookery in the northern San Francisco Bay (Kopec & Harvey, 1995). Wild seals of approximately the desired age based on gross appearance were captured by hoop net and physically restrained without sedation under NOAA-NMFS Permit No. 18786. Seals were examined by veterinarians; no clinical signs of disease, injury or emaciation were observed in those from whom samples were subsequently collected. During examination, age classification was refined based on tooth development, pelage and morphometrics. Although exact age could not be ascertained, this population has a predictable pupping season of March–May (Grigg et al., 2012); wild seals were sampled mid-May and had permanent dentition typical of weaned seals (>4 weeks old; Meyer & Matzke, 2004). One larger harbour seal was estimated to be in the 'yearling' age class (12–24 months old; Lambourn et al., 2013). Gingival and rectal swab samples were obtained, transported on dry ice, then stored at −80°C. Wild seals were immediately released after sampling.

DNA extraction

Pool water was filtered through Nalgene™ 150 mL 0.2 μm analytical filter units (Thermo Fisher Scientific, Waltham, MA). Filters were cut into strips and transferred to 2-mL screwcap vials for DNA extraction. Filter strips and swab samples were extracted with QIAamp DNA Mini Kits (QIAgen, Hilden, Germany) using the manufacturer's tissue protocol with several modifications: 200 μL of buffer ATL was used per sample (instead of 180 μL), the optional RNAse A step was omitted, and samples were incubated at 95°C for an additional 5 min (for additional lysis). During every extraction round, at least one empty tube per batch (15–17 samples) was processed in parallel as a negative extraction control. DNA was eluted in 200 μL AE buffer and stored at −80°C. Aliquots of DNA were frozen to avoid multiple freeze–thaw cycles.

PCR amplification and pyrosequencing

The hypervariable V3-V5 region (~589 bp) of the bacterial 16S rRNA gene was amplified using barcoded,

broad-range primers (338F and 906R) and methods previously described (Bik et al., 2016; Costello et al., 2013). As an additional negative control, at least one PCR reaction without added DNA template was performed per 96 well plate. To reduce PCR bias, samples were amplified in triplicate (Polz & Cavanaugh, 1998) and pooled before sequencing. Concentrations of DNA were measured (Invitrogen Quant-iT High Sensitivity DNA Assay kit, Carlsbad, CA) and PCR products were pooled in equimolar amounts, precipitated in ethanol, and gel purified (QIAgen QIAquick Gel Extraction kit, Hilden, Germany). Purified pools were sequenced using unidirectional 454 pyrosequencing (Roche Life Sciences Genome Sequencer FLX Titanium platform). Pyrosequencing was performed at the University of Illinois (UIUC) Roy J. Carver Biotechnology Center, Urbana, IL and at the University of California San Diego's Translational Virology Core.

Human infant stool dataset

A set of bacterial 16S rRNA gene sequences (V3-V5) from the stool of 14 normal-birthweight infants/children (0–215 days old) was analysed together with the harbour seal microbiota data. Stool samples were collected from infants by their parent(s). Each mother also collected at least one of their own stool samples, as did some fathers. DNA was extracted using a QIAamp stool DNA minikit (Qiagen), as described in Palmer et al. (2007). Using the archived DNA as template, 16S rRNA genes were amplified and pyrosequenced at UIUC using the methods described above (Costello et al., 2013). The raw pyrosequencing data were integrated into the current study and analysed in parallel with the raw pyrosequencing data from the harbour seals. The Stanford University Administrative Panel on Human Subjects in Medical Research approved this work, and the infants' parents provided written informed consent.

Raw sequence read processing

Read quality was assessed for each run individually. Primer removal, quality filtering and demultiplexing were performed (maximum error = 1.5, quality score = 50, maximum ambiguous bases = 1, UIUC I = 375, UIUC L = 600, UCSD I = 500, UCSD L = 700) using QIIME2 v.2021.22 (Caporaso et al., 2010). Demultiplexed reads were processed with DADA2 using parameters recommended for pyrosequencing data (Code S1) (Callahan et al., 2016; McMurdie & Holmes, 2013). Sequences from all pyrosequencing runs were merged prior to chimera removal. Taxonomy was assigned using a naïve Bayesian classifier ('assignTaxonomy', DADA2) and the SILVA v132 reference database (formatted for DADA2; Cole et al., 2009). The 'addSpecies' DADA2 function was used to make species-level assignments for amplicon sequence variants (ASVs) with exact matches to sequences in the reference database. ASVs were inserted into the SILVA v132 reference alignment using SATé-enabled phylogenetic placement ('qiime fragment-insertion sepp', QIIME2) (Janssen et al., 2018). Singletons, eukaryotic reads, and ASVs with unclassified phylum-level taxonomy were removed. Sequences were recovered from 21 of 40 extraction and amplification negative controls (median = 5 reads/control, range = 1–47) representing 20 ASVs (median = 4 reads/ASV, range = 1–52). These ASVs were not identified as contaminants via decontam v1.12.0 ('isContaminant', method = 'prevalence') (Davis et al., 2018). After evaluating negative controls, samples with <200 reads were excluded.

Combined dataset

After quality filtering, 922 samples were retained in the dataset: 41 pool water samples (Code S2: 119,833 reads, mean = 2923 reads per sample, range = 594–5290 reads), 3 formula samples (5686 reads, mean = 1895, range = 1372–2157), 199 gingival swabs from rehabilitated seals (468,912 reads, mean = 2356, range = 538–5217), 199 rectal swabs from rehabilitated seals (719,170 reads, mean = 3614, range = 234–7490), five gingival swabs from wild seals (21,216 reads, mean = 4243, range = 3354–5278), five rectal swabs from wild seals (31,131 reads, mean = 6226, range = 5671–6657), 432 infant stool samples (Code S3: 276,106 reads, mean = 639.1 reads, range = 216–1501), and 38 stool samples from the parents (30 samples from 15 mothers, 8 samples from 8 fathers: 16,553 reads, mean = 435.6 reads, range = 200–949). Sample and subject metadata are provided in Data S1A. ASV tables are provided in Data S1B,C. Sequences are available at NCBI SRA BioProject PRJNA577962.

Ecological and general statistical analyses

Sequence novelty was assessed using the vsearch package v2.21.1 (Rognes et al., 2016) in QIIME2; ASVs with <97% similarity to any sequence in the reference database (SILVA v132) were classified as 'novel'. All other analyses were performed using Phyloseq v1.36.0 (McMurdie & Holmes, 2013) in R (v4.1.1). The Shannon diversity index was calculated (phyloseq) using raw reads and rarefaction was used (phyloseq) to control for library size prior to richness analyses; both Shannon index and observed richness were examined for each analysis of alpha diversity (Data S1E). Venn diagrams were made using VennDiagram v1.6.20 (Chen & Boutros, 2011) and MicEco v0.9.19

(Russel, 2021). Wilcoxon rank-sum tests ('wilcox.test', stats v4.1.1) and linear mixed-effects (LME) models fit by restricted maximum likelihood (REML 'lme', nlme v3.1–152) were used for alpha diversity comparisons. Subject ID was used to account for repeated measures. Autocorrelation was factored into models when appropriate. Tukey post hoc tests ('glht', multcomp, v1.4–19) were used to evaluate pairwise differences and adjust for multiple testing. For other analyses, data were further filtered to include only ASVs present in ≥2 samples with ≥10 reads/sample (study findings for these ASVs are summarised in Data S1D). Bray-Curtis, weighted Unifrac and unweighted Unifrac (UWU) dissimilarity/distance metrics were used for unsupervised analyses of the data. UWU revealed the strongest clustering based on study covariates and was used moving forward (Lozupone et al., 2006). Beta diversity analyses were repeated using rarified data to verify the robustness of results (Data S1E) (Weiss et al., 2017).

Supervised classification and feature selection

Random Forest (RF) regression models (Breiman, 2001) were built ('train' (method = 'rf', controlMethod = 'cv'), caret v6.0–88; Kuhn, 2008) to identify age-discriminatory taxa. A five-fold cross-validation was performed on age versus ASV relative abundance (RA) in each swab. Predictor ASVs with high RF importance scores (scaled to a maximum of 100) better reduced the mean square error and improved age-classification accuracy when added to the model ('varImp', caret). VennDiagram was used on non-normalised data to identify ASVs that were unique to subgroups (at RA >0.01%) even when all reads were considered (Data S1D, Code S4). DESeq2 (v1.32.0) was used to identify discriminatory taxa between subgroups (Love et al., 2014). The treeDA package (v0.0.5) was used to run sparse linear discriminant analyses (sparse LDA) and identify clades of taxa or individual ASVs that discriminated treatment groups (Fukuyama et al., 2017).

Analysis of variance

Canonical correspondence analysis (CCA) and ANOVA permutations of resulting models ('anova.cca') were performed using vegan v2.5–7 (Oksanan et al., 2022) to determine the relative contributions of explanatory covariates (considered as factors) to variability in the data. Group dispersions (average UWU distances to the centroid) were determined using 'betadisper' (vegan). Homogeneity between group dispersions was assessed with ANOVA. Differences in community composition between groups were identified with multivariate ANOVA (MANOVA, 'adonis', vegan; Oksanan et al., 2022). Subject ID was used in the 'adonis' permutation block (nperm = 999) to account for repeated measures.

Longitudinal diversity analyses

Diversity was regressed against age using general LME models fit by REML (nlme) with subject as the random effect and the temporal autocorrelation structure specified via the 'lme' corAR1 argument. ANOVA permutations of the models evaluated the relative influence of age on diversity. Conditional R^2 values, the variation explained by fixed and random effects, were extracted using piecewiseSEM ('rsquared', v2.1.2). SplinectomeR's (v0.1.0) 'permuspliner' function tested for differences in overall diversity or ASV abundance trajectories between two groups throughout the entire study, while 'slidingspliner' tested for transient differences (significant longitudinal *p*-values are displayed in Data SF; Shields-Cutler et al., 2018). Core taxa were identified with the microbiome package (v1.14.0).

Bacterial source tracking

Sourcetracker R (v1.0) was used to determine the proportion of bacterial taxa 'acquired by' antibiotic-treated harbour seals ('sinks') from other sites sampled at the rehabilitation facility ('sources') over time (Code S5; Knights et al., 2011). Full results are displayed in Data S1G.

RESULTS AND DISCUSSION

Rehabilitation facility dataset

A series of gingival ($n = 199$ swabs, range = 4–20 swabs/seal, median = 14 swabs/seal) and rectal ($n = 199$, range = 4–21, median = 15) swabs were collected from 15 harbour seals (Figure S1A) along with formula ($n = 3$) and pool water ($n = 48$) at the facility. Pups ranged from 0 to 11 days old (average = 6.7 - days) when admitted (Table S1). Feeding on solid food started on average at 19.6 days old (range = 14–33 days). Excluding controls, 2795 bacterial ASVs were identified. Samples had a mean of 3022 reads (range = 234–7490). Gingival swabs contained 1044 ASVs belonging to 11 bacterial phyla. Rectal swabs contained 1294 ASVs from 13 bacterial phyla. Water contained 688 ASVs from 22 phyla. High novelty was observed in harbour seal-associated swabs; 92.2% of gingival ASVs and 91.0% of rectal ASVs were considered 'novel' (<97% identity match against SILVA v132) versus 26.0% of ASVs in pool water (Code S2)

Harbour seal gingival and rectal microbiotas were distinct from each other and from those of pool water and formula

Harbour seal gingival and rectal community structures were structurally distinct from each other (Figure 1A, 'adonis'; R2 = 0.22, Pr(>F) < 0.001, Data S1E). Similarly, previous studies have found biogeography to influence the indigenous microbiotas of managed dolphins; rectal communities were distinct from those in the mouth (Bik et al., 2016) and those on periumbilical skin (Cardona et al., 2018). Whereas dolphins have been shown to have more diverse gingival than rectal microbiota (Bik et al., 2016), alpha diversity did not differ significantly between harbour seal body sites in our study (Figure S2A).

In the gingival microbiota, the most abundant phyla were Bacteroidetes (37.4% RA), Proteobacteria (30.0%), Fusobacteriota (23.2%) Firmicutes (5.3%) and Actinobacteria (2.4%) (Table S2). Gracilibacteria, a candidate phylum previously detected at deep-sea hydrothermal vents (Rinke et al., 2013) and in the mouths of fur seal pups (Emami-Khoyi et al., 2020), was also detected in the rehabilitated harbour seal gingival microbiota (0.8%, Code S2) but not the rectal microbiota. At the level of genus (Figure S2B), the gingival microbiota was composed primarily of *Oceanivirga* (15.6%), *Neisseria* (10.0%) and *Ornithobacterium* (10.0%), all undetected at the other facility sample sites, as well as *Fusobacterium* (11.6%) (Table S3). *Oceanivirga*, also unique to the gingival microbiota, has previously been isolated from the oral cavity of adult

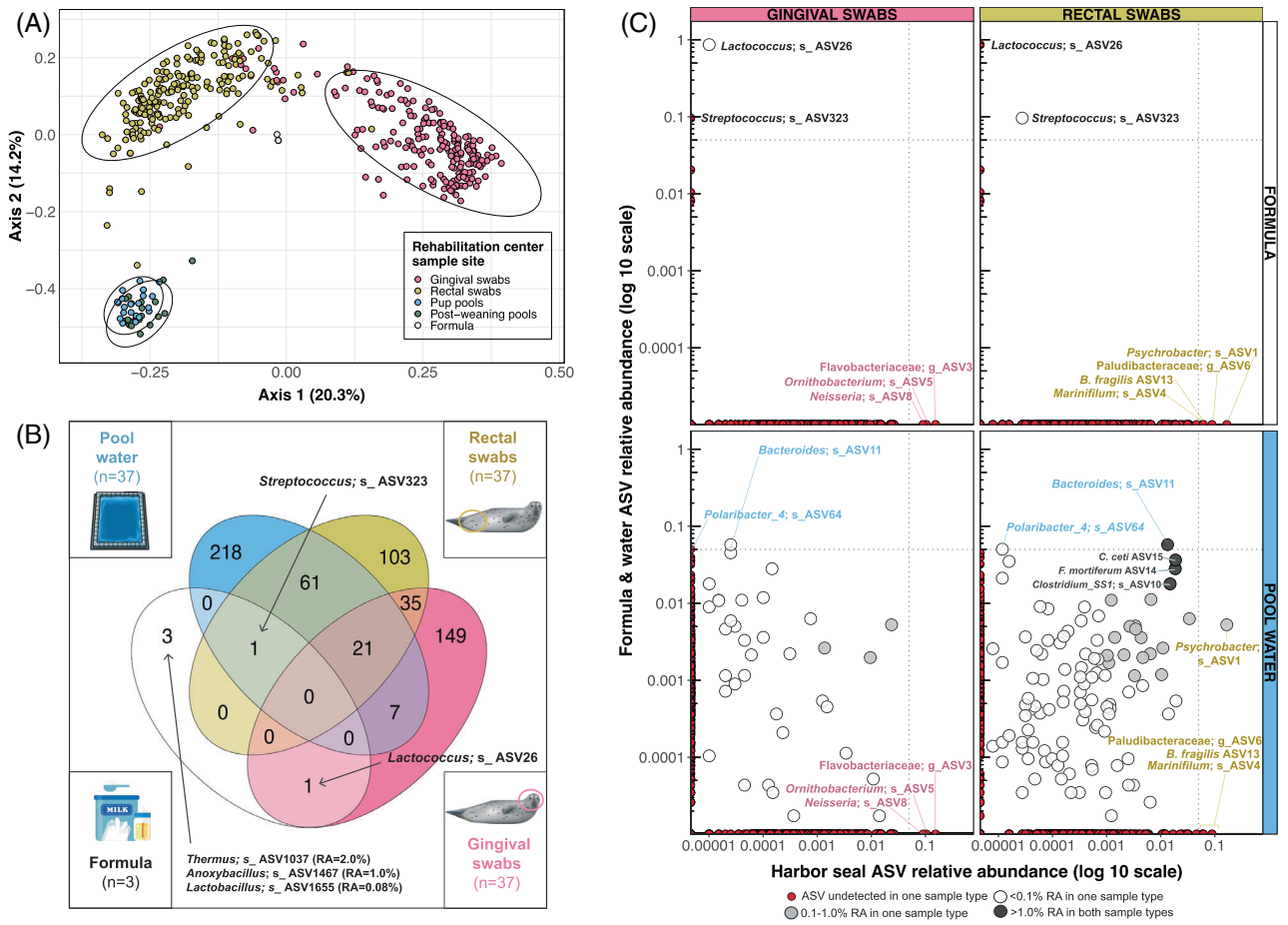


FIGURE 1 Rehabilitated harbour seals' gingival and rectal bacterial communities were distinct from one another, and from those in formula and pool water. (A) Principal coordinate analysis ordination based on an unweighted UniFrac distance matrix of the proportion-transformed data from all samples collected at the rehabilitation facility: harbour seal gingival swabs, rectal swabs, formula, water from small 'pup pools' and larger 'post-weaning pools'. Data ellipsoids were drawn at a level of 95%. Pairwise comparisons are listed in Data S1E ('pairwise.adonis2'). (B) A Venn diagram (MicEco package, 'ps_venn') comparing the observed richness in the gingival and rectal swabs of antibiotic-untreated rehabilitated harbour seals to that of their water and formula. Only amplicon sequence variants (ASVs) present in ≥2 samples with ≥10 reads/sample were considered. Data were normalised and rarefied to 1303 reads to match the sequencing depth of the formula. (C) Unrarefied data from B were used to plot the relative abundance (RA) of ASVs detected in all formula (top) or water (bottom) samples against the RA of ASVs found in all harbour seal gingival (left) or rectal (right) swabs. Labels of ASVs with >5% RA in one dataset are coloured according to the sample type in which they were more abundant. ASVs with a RA 1%–5% in both sample types are coloured and labelled in dark grey. Light grey, white and red points denote ASVs that had 0.1–1.0% RA, 0–0.1% RA or were undetected in either sample type, respectively.

elephant seals at the same facility (Volokhov et al., 2018) and healthy wild harbour seals (Palmer et al., 2020). In the rectal microbiota, Bacteroidetes was also the most abundant (32.3%) phylum, followed by Proteobacteria (29.2%), Fusobacteriota (16.8%), Firmicutes (16.5%), Epsilonbacteraeota (2.6%) and Actinobacteria (2.5%). The high RA of Fusobacteriota is characteristic of the marine carnivore distal gut, unlike most terrestrial mammals regardless of diet (Nelson et al., 2013a). The most abundant genera in the rehabilitated seal rectal microbiota (Figure S2C) were *Psychrobacter* (21.4%) and *Fusobacterium* (14.3%)

Both harbour seal-associated microbiotas were structurally distinct from the bacterial communities in pool water (Figure 1A, R^2gingival = 0.29, R^2_{rectal} = 0.21, Pr(>F) < 0.001). The pools at the rehabilitation facility contained filtered artificial seawater that had higher diversity than seal-associated communities (Figure S2A, 'lme', Pr(>|z|) <0.001). In a previously published study, the microbiota in artificial seawater at an aquarium was less diverse than that associated with the resident dolphins (Cardona et al., 2018); this could reflect differences in pool volumes, filtration systems, diet or the host species. The most abundant genera characteristic of shallow (Figure S2D) and deep (Figure S2E) pools were *Polaribacter_4* (9.8%) and SM1A02 (11.6%), respectively. Although pool water shared 28 ASVs with the harbour seal gingival swabs (Figure 1B), none were abundant (>1% RA) in both sample types (Figure 1C). Rectal communities shared a great number of bacterial taxa with water (84 ASVs), including four taxa that were abundant in both sample types (RArectal = 1.4–1.9% RA, RAwater=1.1–5.8% RA). The greater overlap of shared taxa between rectal swabs and pool water was expected since harbour seals defecate in their enclosures throughout rehabilitation. However, considering that most abundant pool water taxa were rare or undetected in rectal communities (Figure 1C), water did not appear to exert a strong influence on the harbour seal-associated microbiota.

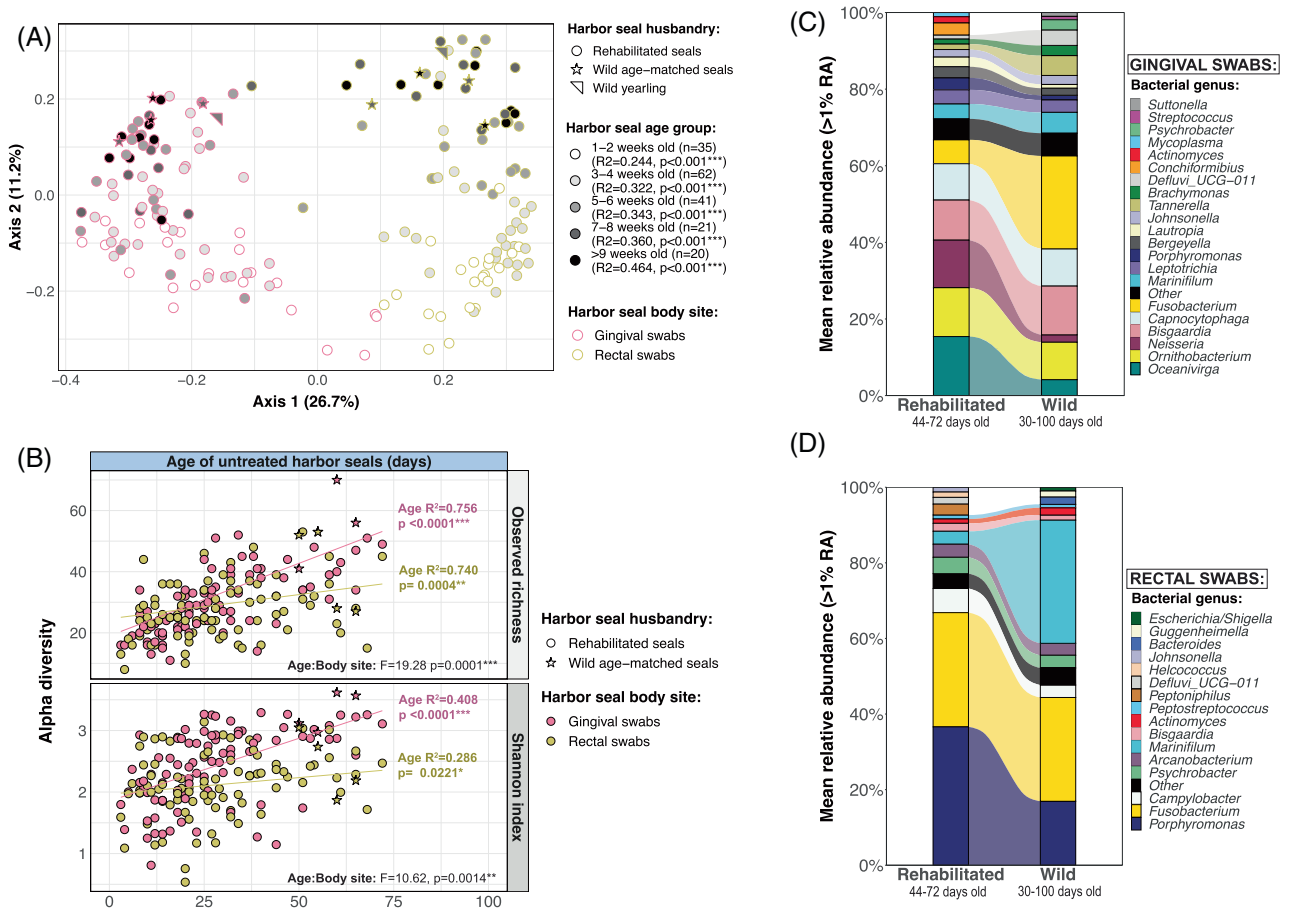
Only five bacterial genera were detected in formula (Figure S2F), each represented by a single ASV. Of these, only one ASV was shared with each harbour seal body site (Figure 1B) and both were present in the seal microbiota at extremely low abundance (Figure 1C). Formula was dominated by *Lactococcus* ASV26 (RA = 84.1%, Table S3); however, *Lactococcus* was undetected in the harbour seal rectal microbiota and *Lactococcus* ASV26 was only detected in one gingival swab at RA = 0.001%. (Code S2). The second most abundant taxon in formula, *Streptococcus* ASV323 (10.0%), was undetected in gingival swabs and only detected in two rectal swabs at RA <0.003%. *Thermus* ASV1037 and *Anoxybacillus* ASV1467 were identified in formula, but neither genus was detected in harbour seals. These results suggest that formula was not a major source of acquired bacterial diversity for harbour seals during rehabilitation.

Lactobacillus was detected at low abundance in formula and was represented by ASV1655 (0.08% RA), whereas *Lactobacillus* ASV102 was the sole representative in the harbour seal microbiota. Previous research has demonstrated an abundance of *Lactobacillus* in the faeces of puppies soon after parturition, as well as in their mothers' milk (Ge et al., 2021). BLAST analysis (Boratyn et al., 2012) against the NCBI database (full BLAST results in Data S1D) revealed that *Lactobacillus* ASV102 had 100% sequence similarity to *Lactobacillus murinus*, which has been isolated from canine milk (Martín et al., 2009) and has been shown to protect against sepsis in neonatal mice (Singer et al., 2019). Vertical transmission of *Lactobacillus* implies that this genus may play an important role in neonatal health. It is possible that *Lactobacillus* ASV102 was transferred to the harbour seal pups during the brief amount of time they spent with their mothers prior to separation. Previous research has shown that the faecal microbiota of neonatal dairy calves (which are separated from their mothers after birth) arrives at a similar composition to that of beef calves (which have continued maternal care), suggesting that limited early life maternal contact may be sufficient for successful vertical transfer of host-associated taxa (Barden et al., 2020). At the harbour seal rehabilitation facility, the strong distinction between the harbour seal-associated microbiota and that of the formula suggests that early life exposures had a stronger influence on microbial composition than did the initial diet.

By the end of rehabilitation, harbour seal pup indigenous communities resembled those of local wild harbour seals of comparable age

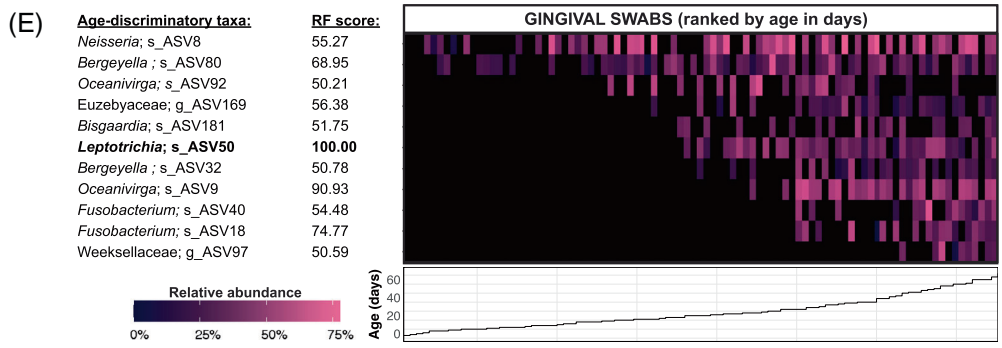
Rehabilitation afforded a rare opportunity to sample healthy, wild-born marine mammals longitudinally under semi-controlled conditions. However, rehabilitation is an unnatural process, with seals handled regularly and fed an artificial diet (initially formula by gavage and later frozen whole herring placed in the pool). Therefore, we asked whether at the end of rehabilitation the harbour seal indigenous microbiota resembled that of age-matched wild harbour seals. Swabs were collected from four wild harbour seals of the same age range at which rehabilitated harbour seals are typically released (50–80 days old), and from one older 'yearling' (Table S1).

As observed in rehabilitated seals, Bacteroidetes was dominant in both body sites of wild age-matched seals (Table S2, $RA_{gingival}$ 40.7%, RA_{rectal} 61.8%) and the yearling ($RA_{gingival}$ 61.0%, RA_{rectal} 53.9%). This is consistent with data from wild elephant seal weaners



(E)

Age-discriminatory taxa:	RF score:
Neisseria; s_ASV8	55.27
Bergeyella ; s_ASV80	68.95
Oceanivirga; s_ASV92	50.21
Euzebyaceae; g_ASV169	56.38
Bisgaardia; s_ASV181	51.75
***Leptotrichia*; s_ASV50**	**100.00**
Bergeyella ; s_ASV32	50.78
Oceanivirga; s_ASV9	90.93
Fusobacterium; s_ASV40	54.48
Fusobacterium; s_ASV18	74.77
Weeksellaceae; g_ASV97	50.59



(F)

Age-discriminatory taxa:	RF score:
Psychrobacter; s_ASV1	58.43
Streptococcus; s_ASV42	57.95
Peptostreptococcus russellii ASV57	65.99
Campylobacter; s_ASV17	99.24
Marinifilum; s_ASV4	76.11
Fusobacterium; s_ASV2	99.99
***Peptostreptococcus;* s_ASV113**	**100.00**

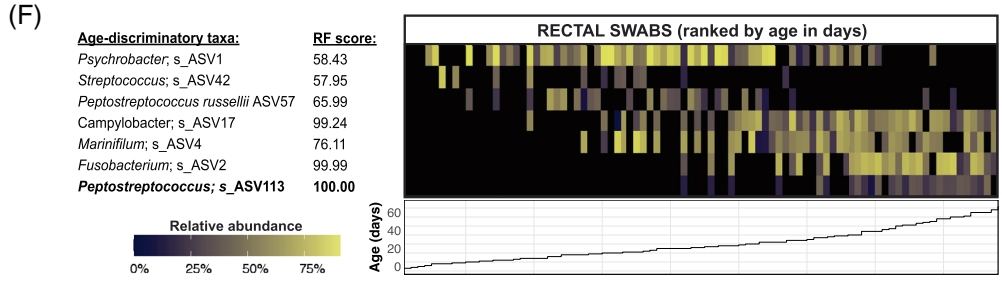


FIGURE 2 Legend on next page.

(*Mirounga angustirostris*) (Stoffel et al., 2020), although in a review of adult pinniped distal gut microbiotas, Firmicutes was dominant in 10 of 12 studies (Acquarone et al., 2020). Older rehabilitation facility seals had similar gingival (Figure S3A) and rectal (Figure S3B) community structure to that of the wild harbour seals (Figure 2A). Shannon diversity of gingival (Figure S3C) and rectal (Figure S3D) communities in rehabilitated seals ultimately attained similar values to those observed in wild seals (Figure 2B). Husbandry (rearing at the facility versus in the wild) did not influence beta diversity at either body site (Table S4; 'adonis2'), even when just the samples from the oldest age-matched seals were considered (Code S2). Differences in alpha diversity were not detected between husbandry groups normalised to the same sample size (Figure S3E, Wilcoxon rank-sum). Husbandry groups had similar genus-level diversity in their gingival (Figure 2C) and rectal (Figure 2D) microbiota. Among the genera detected at >1% RA in the wild cohort, 94.1% (16/17) of gingival genera and 76.9% (10/13) of rectal genera (Table S5) were also abundant in the oldest untreated rehabilitated seals and the four that were less abundant in the rehabilitated cohort (undetected-0.6% RA) only had modest abundance in the wild harbour seal microbiota (1.0–2.7% RA). Our findings suggested that captivity had little influence on the overall levels of alpha and beta diversity in the marine mammal distal gut microbiota, as has been reported by others for terrestrial wildlife (Ley et al., 2008; Youngblut et al., 2019) and dolphins (Bik et al., 2016); the same was true for the harbour seal gingival microbiota.

Other research suggests that captivity may affect the pinniped faecal microbiome (Delport et al., 2016; Nelson et al., 2013b). In general, rehabilitated pups have less maternal investment than their wild counterparts, so some differences might be expected (Bokulich et al., 2016). In our study, more unique taxa were identified in wild than in rehabilitated harbour seal gingival (Figure S3F) and rectal (Figure S3G) microbiotas, but only four wild individuals were available to us. Furthermore, whereas umbilical healing status allowed the age of most rehabilitated pups to be classified within a window of days, broader age ranges could only be estimated for wild seals; it is possible some wild seals were older than the rehabilitated seals. Unlike in our study, greater faecal richness has been observed in zoo-housed versus wild seals; this has been suggested to reflect microbial species acquisition, in part from keepers and the public (Nelson et al., 2013b; Numberger et al., 2016). Access to the harbour seal nursery in our study was restricted to trained staff wearing protective gear, minimising this potential route of exposure.

The differences in gingival microbial diversity between the oldest rehabilitated harbour seals and the age-matched wild seals were primarily explained by the relatively high abundance of *Oceanivirga* and *Neisseria* in the rehabilitated cohort (Figure 2C). Rehabilitated seal gingival samples were distinguished specifically by *Oceanivirga* ASV9 (Figure S3H, DESeq2, padj $= 6.43e^{-12}$) and *Neisseria* ASV8 (padj $= 9.53e^{-11}$), neither of which was detected in the pool water (Figure 1C, Code S2). BLAST analysis revealed that ASV8 was 99.7% identical to *Neisseria zalophi* previously isolated from sea lion gingival swabs (Yassin & Busse, 2009) at the same facility (Data S1D). Compared to the oldest rehabilitated seals, the wild harbour seal gingival microbiota had a higher abundance of *Fusobacterium* (Figure 2C) and was best differentiated by the taxon *Johnsonella* ASV212 (Figure S3H, padj $= 1.06e^{-06}$). Wild harbour seal rectal swab samples were characterised by a high abundance of *Marinifilum* (Figure 2D), specifically *Marinifilum* ASV85 (Figure S3I, RA $= 10.04\%$, padj $=6.72e^{-15}$), which was also abundant in the wild yearling (Table S2, RA $= 5.67\%$), but undetected in the harbour seals at the rehabilitation facility. These distinctions likely reflect the differences in environment, maternal contact, and exposure to humans. However, the broader findings suggest that rehabilitated harbour seals were able to achieve microbial community diversity and structure similar to that observed in nature, even though they were raised largely in the absence of maternal care. This implies that early life exposures, environmental filtering and/or bacterial

FIGURE 2 Gingival and rectal communities of rehabilitated harbour seals become more diverse and distinct as pups age, ultimately resembling those of local wild harbour seals of similar age. (A) Principal coordinate analysis based on unweighted UniFrac distances and proportion-normalised data. The colour (pink or gold) indicates harbour seal body site. Fill shading denotes age at time of sampling. Age-matched wild harbour seals were estimated to be in a similar age range (30–100 days old) as the oldest rehabilitated seals (42–79 days old). A wild yearling (1–2 years old) was also sampled. Multivariate ANOVA (vegan package, 'adonis2') was performed for all age groups; R2 values indicate the proportion of variation attributed to body site. (B) Alpha diversity was regressed against age using general mixed-effect linear models and fit by restricted maximum likelihood (nlme package, 'lme'). ANOVA permutations of the models were used to identify interactions between covariates. Wild harbour seal swabs are staggered in order of estimated age rank. (C,D) Stacked relative abundance (RA) bar plot comparing the abundant bacterial genera (≥1% RA) in (C) gingival and (D) rectal swabs collect from the four wild age-matched harbour seals (30–100 days old) and the last swabs taken from the four untreated harbour seals that were the oldest at the end of rehabilitation (60–72 days old). (E,F) Heat maps of the (E) gingival and (F) rectal data display the most age-discriminatory bacterial taxa identified by Random Forests (RF) regression. High RF importance scores indicate age-predictive taxa that substantially reduced the model's mean square error. Only taxa with RF scores >50 are shown. Columns represent individual swabs arranged left to right in increasing ranked age. Colour gradient indicates the RA of each amplicon sequence variant in that swab. Only rehabilitated seals that did not receive antibiotics are included in all panels. The wild yearling is excluded in panels B–F.

seeding between co-housed conspecifics during rehabilitation were sufficient to promote natural features of microbiota assembly.

Gingival and rectal communities became more diverse and distinct as harbour seal pups aged

Data from wild and rehabilitated seals were used to identify age-associated patterns of diversity. Ordination of UWU distances separated the samples by body site along Axis 1 and by age along Axis 2 (Figure 2A). Gingival and rectal communities became more distinct as pups aged ('adonis2'; $R^2_{BodySite} = 0.24$ to 0.46, $p < 0.001$), in a similar manner to that demonstrated in previous studies of human infants (Costello et al., 2013). We found positive correlations between microbiota richness and age, indicating diversification of gingival (Figure 2B, $R^2_{observed} = 0.756$, $p < 0.0001$) and rectal communities ($R^2_{observed} = 0.740$, $p = 0.0004$) over time. Age had a stronger influence on gingival (Table S6, LME, Age:BodySite $p_{observed} < 0.0001$, $p_{Shannon} = 0.0014$, gingival $R^2_{Shannon} = 0.408$, $p < 0.0001$) microbiota diversity than on rectal microbiota diversity (Figure 2B, $R^2_{Shannon} = 0.286$, $p = 0.0221$), with the highest gingival microbiota richness in harbour seals post-weaning (Figure S3C).

Early life microbiota assembly in non-human species is under-researched (Jakobsson et al., 2014; Sulyanto et al., 2019). In piglets, faecal diversity increased up to the time of weaning then subsequently plateaued (Frese et al., 2015). Accordingly, we expected alpha diversity in the harbour seal pup rectal microbiota to correlate with age before the dietary shift to fish, but no significant pre-weaning trends were observed (Table S6). A previous study demonstrated that the rectal communities of adult elephant seals were more diverse than those of pups (Nelson et al., 2013b), yet a more recent study (Stoffel et al., 2020) showed that alpha diversity in the rectal microbiota of newly weaned elephants seals (~28 days old) was stable over two 15-day intervals. Considering that our results did identify an increase in harbour seal rectal microbiota diversity across the entire study period overall, this change may have been gradual and difficult to identify at finer timescales. Earlier trends were potentially also obscured by high intra- and inter-individual variability. Alternatively, since pups ranged from 2 to 11 days old when admitted, varying degrees of microbial community assembly may have occurred in the wild before sampling. The transition from mother's milk to formula may have also impacted diversity; formula is associated with reduced diversity in infant stool, as compared with breast-milk dominated diets (Bokulich et al., 2016). Relative to the rectal microbiota, even less is known regarding gingival microbiota assembly in non-human species. Mice were recently demonstrated to have mid-weaning loss of gingival diversity (Koren et al., 2021). In contrast, when harbour seal gingival communities were examined over the first month of life, a positive correlation was observed between alpha diversity and age (Table S6, $p_{Shannon} < 0.0001$, $p_{observed} = 0.0004$).

As the harbour seals grew older, the RA of Fusobacteriota increased while Firmicutes declined at both body sites (Figure S4A,B). Over time, *Neisseria*, *Oceanivirga* and *Ornithobacterium* became the dominant genera in gingival communities (Figure S5A). We built regression models using data from untreated seals (excluding the wild yearling) to predict age as a function of ASV RA. In gingival communities, *Leptotrichia* ASV50 (RF = 100.0) and *Oceanivirga* ASV9 (RF = 90.9) were most age-predictive (Figure 2E); both became abundant after the pups ate only fish (Figure S5B). *Neisseria* ASV8 appeared early in gingival community-assembly and ultimately became the dominant taxon in older seals ($RA_{>1mo} = 15.1\%$). Similarly, *Neisseria*, a 'late colonizer' of the human mouth, has low abundance in infants but is a dominant genus in children by 7 years of age (Dzidic et al., 2018). In rectal communities, *Fusobacterium* and *Porphyromonas* became the dominant genera over time (Figure S5A). *Fusobacterium* ASV2 was age-predictive for older rehabilitated seals (Figure 2F, RF = 99.9); its abundance increased post-weaning (Figure S5C) and it was the most abundant taxon in the yearling rectal swab (Table S2, 32.6%). Low abundance *Peptostreptococcus* ASV113 (97.9% identical to northern fur seal skin isolates) was most distinctive of the rectal swab samples from harbour seals over 1 month old (RF = 100.0, Data S1D: $RA_{<1mo} = 0.02\%$, $RA_{>1mo} = 0.43\%$).

The dense sampling frequency at the beginning of rehabilitation enabled us to capture nuanced patterns of assembly within the neonatal harbour seal microbiota such as extinction and colonisation events (Figure S5D). A taxon was said to have undergone an 'extinction event' if it were abundant (>1% RA) in younger harbour seals (<1 month old) and then subsequently became undetectable. Although no taxa met these criteria in the gingival microbiota of untreated harbour seals, *Proteus*_ASV121 (2.0%), *Psychrobacter*_-ASV93 (1.2%) and *Lactobacillus* ASV102 (1.8%) were all abundant in the seal pup rectal microbiota but undetected post-weaning (Table S7). In piglets, *Lactobacillus* abundance has also been shown to decrease in conjunction with the introduction of solid food (Shi et al., 2018). Conversely, a 'colonisation event' was used to describe the emergence of taxa that were undetected in harbour seal pups but became abundant in older harbour seals. Specifically, we were interested in taxa acquired during rehabilitation (versus from early life exposures in the wild) so we identified the first appearance of ASVs in harbour seals that had already been at the facility for at least 1 week. In accordance

with our previous finding that harbour seal-associated communities diversified over time, colonisation events were more frequently identified (relative to extinctions) in both the gingival (7 ASVs) and rectal (8 ASVs) microbiota of older seals. Given that these taxa emerged over a week since the harbour seals last had contact with their mothers and natural habitat, these colonisation events support the conclusion that the harbour seals acquired at least some of their microbial diversity from environmental filtering at the rehabilitation facility. Alternatively, these taxa may have been pre-existing but present at undetectable levels at the beginning of rehabilitation and the harbour seal pups' developing immune system may have influenced microbial succession (Zheng et al., 2020).

Despite high temporal variability in the nascent harbour seal microbiota, core community members were identified

The early life harbour seal microbiota was characterised by dramatic taxonomic changes over time, including apparent extinction events, colonisation events, and post-weaning blooms. The dynamic nature of these communities raised the question—are the taxa that emerge in the pup nascent microbiota conserved among different harbour seals? Temporally persistent or predictably dynamic taxa may represent a 'temporal core microbiome' (Caporaso et al., 2011), thought to contribute to overall community stability (Kokou et al., 2019). Identification of 'common core' taxa (Turnbaugh et al., 2007) prevalent among harbour seal populations, could help elucidate ecological and evolutionary drivers of host-microbial interactions (Bik et al., 2016; Risely, 2020). One study of adult harbour seals identified 21 abundant bacterial groups shared among the faecal samples of five co-housed individuals (Numberger et al., 2016). Another study identified three taxa shared among adults of two phocid species, captive and wild (Nelson et al., 2013b). Here, 'core taxa' were defined as those with ≥50% prevalence at ≥0.1% RA among samples from untreated rehabilitated seals, that were also detected in the wild age-matched seals and yearling. Four taxa met these criteria in gingival communities: Flavobacteriaceae ASV3 (in 5/5 untreated seals; RA = 8.1%–18.6%), *Ornithobacterium* ASV5 (4/5; 1.5%–21.7%), *Fusobacterium* ASV22 (5/5; 2.0%–3.3%) and Cardiobacteriaceae ASV58 (5/5; 0.3%–1.3%; Figure S5B, Code S2). Among these four, ASV3 was most prevalent (68/87 gingival swabs) and identical to a gastric microbiota sequence from adult sea lions (Bik et al., 2016). In the rectal microbiota (Figure S5C), the only core taxon identified, ASV16 (55.7% of swabs; 5/5 seals; <0.1%–8.5%), was identical to the sequence of *Psychrobacter lutiphocae* strain IMMIB L-1110 isolated from seal faeces (Yassin & Busse, 2009). The presence of conserved taxa amidst the dynamic landscape of early life community assembly may speak to their importance for natural development.

Distal gut community composition in infants and neonatal harbour seals rapidly diversifies in early life and becomes host-specific with age

Literature on the human microbiome provides the foundation for our understanding of microbiota assembly in mammals. We compared the harbour seal dataset to sequences from a longitudinal dataset of human infant stool samples that had previously been collected by our laboratory (Palmer et al., 2007). Both studies utilised the same methods, had similar sampling schedules that encompassed weaning (Figure S1B and S6A and Table S8), and had variable antibiotic exposure. We were interested to learn whether similar patterns of microbiota assembly and resilience were shared between these disparate hosts. Considering the variable nature of early life microbiota assembly, we questioned whether evidence of phylosymbiosis would be detected between such young hosts—especially since both were raised under human care. We also identified shared features between the harbour seal and human adult distal gut microbiota to help estimate the likelihood of inter-host species bacterial transfer at the rehabilitation facility.

The harbour seal rectal microbiota had higher richness (Figure S6B) and more novel bacterial taxa (Code S2) than the infant stool microbiota. Only 14 bacterial taxa were shared between the two host species. *Clostridium perfringens* ASV10 was the only abundant taxon (RA >1%) in both (Figure S6B). Alpha diversity increased with age in infants and harbour seals (Figure S6C, LME, $p_{seal} = 0.032$, $p_{infant} = 0.040$), but arrived at a higher Shannon index in the latter group. The community structure of the harbour seal microbiota was distinct from that of infants (Figure S6D,'adonis2', $R^2_{species} = 0.182$, $p < 0.001$). The microbiota of neonatal infants and seals initially exhibited high variability, but as both cohorts aged, the microbiotas became rapidly host-specific (Figure S6E). This was largely driven by the rise and eventual dominance of Fusobacteriota (undetected in infant stool) in the harbour seal microbiota (Figure S4B), whereas infant stool became dominated by Firmicutes (Figures S4C and S5E). Detailed results of this comparison are discussed in the Supporting Text.

Phylosymbiosis has been previously demonstrated for adult humans (Groussin et al., 2017; Ley et al., 2008) and pinnipeds (Song et al., 2020), but the timeline in which this signal emerges in the developing microbiota of young mammals is largely unexplored. Our finding of a strong phylosymbiotic signal, despite

high temporal variability, and the speed at which it emerged was compelling—especially considering that harbour seals had limited contact with their mothers and natural habitat. The rapid acquisition of a harbour seal-specific microbial signature suggests that continued vertical transmission was not necessary for the establishment of host-specific structure. Early life contact with the mothers and rookery environment prior to rescue may have allowed the colonisation of core harbour seal taxa. Co-housing of harbour seals during rehabilitation may have also promoted transfer of essential taxa between seals that had experienced different degrees of natural exposure. The detection of rectal-associated taxa in pool water (Figure 1C) offers one potential avenue for this transfer to occur. Harbour seals could have also shared taxa among their cohoused group via direct physical contact with one another since these social animals commonly interact. These results suggest that early life exposures and microbial sharing between conspecifics during rehabilitation were sufficient to achieve a host species-specific microbiota in the absence of continued maternal care.

The human handlers at the rehabilitation facility, another potential source of bacterial seeding, were not sampled due to logistics. Instead, we analysed the stool microbiota of the parents in the infant cohort to help identify, which acquired taxa in the harbour seal microbiota were characteristic of the adult human gut. Only six taxa were shared between adult humans and harbour seals (Figures S6F and S6G). *Bacteroides fragilis* ASV7 was the only shared taxon found in relatively higher abundance (4.4%) in the harbour seal rectal microbiota and it declined with age. Considering *Bacteroides fragilis* ASV7 was also detected in the age-matched wild seals it likely represents a common mammalian gut commensal rather than an acquisition from human handlers. Exposure to the handler's skin was the most logical route for potential bacterial transfer; however, examination of the most common members of the human skin microbiota revealed little overlap with harbour seal communities at either body site. These results suggest that humans were not an important source of bacterial seeding. This is notable considering recent studies that have found captive canids (Trevelline & Moeller, 2022), as well as wild canids living near urban areas (Dillard et al., 2022), to have adopted a more human-like microbiota structure relative to their counterparts living in nature. Canids and harbour seals are members of suborder Caniformia that eat meat and possess simple monogastric guts, so the apparent robustness of the harbour seal microbiota to humanization is interesting and could speak to selective pressures in the gut of diving mammals. That said, handlers at this rehabilitation facility wore sterile gloves and restricted contact with the harbour seals as much as possible, so this finding could also simply underscore the effectiveness of local husbandry protocols.

Transient periods of higher alpha diversity and altered community composition were observed in the gingival microbiota of antibiotic-treated seals

Gingival bacterial diversity was compared between untreated harbour seals and those that received early life antibiotic treatment. Interestingly, the Shannon diversity index was higher (Data S1F 'slidingspliner' $p_{min} = 0.028$) in antibiotic-treated seals beginning at about days 38–40 of age, over a 2 week-period after treatment had concluded for all study seals (Figure 3A). Richness was also greater post-treatment ($p_{min} = 0.049$) (Figure 3B). A comparison of treated and untreated groups revealed 31.1% more unique taxa in the treated harbour seals (Figure S7A). Alpha diversity trajectories for the overall study period did not differ significantly between groups ('permuspliner') using either metric. Gingival microbiota richness plotted against age for each treated seal versus the baseline of all untreated seals, did not reveal clear associations with antibiotic class, seal age at intake, or age at first treatment (Figure S7B). Treatment explained 3.6% of the variation in UWU distances ('adonis2', $Pr(>F) = 0.001$) and separated groups along the PCoA Axis 2 (Figure S8A). The overall trajectories of Axis 2 loadings differed between treatment groups (Figure 3C, $p = 0.024$) and significant divergence was detected during the first week post-treatment ($p_{min} = 0.004$).

Ciprofloxacin, doxycycline and clindamycin all demonstrate activity in canine and feline saliva (Greene, 2006) and were expected to influence gingival bacterial communities in harbour seals. Nonetheless, the observation of higher diversity in treated seals was unexpected; it contradicts findings in humans that suggest antibiotics (e.g., combinations of amoxicillin and metronidazole) have a negligible to negative influence on gingival diversity (Bizzarro et al., 2016; Hagenfeld et al., 2018). However, those findings occurred in adults receiving concurrent periodontal cleanings (another form of perturbation) and sampling occurred months post-intervention. Research on rat oral microbiota has demonstrated stable or increased alpha diversity following antibiotic treatment (Cheng et al., 2018; Wu et al., 2020).

To identify sources of acquired diversity, treated seal gingival communities were compared to communities from other facility habitats. The majority of acquired diversity was not attributed to any habitat (Figure S9A). Of those habitats that did serve as a possible source, the largest contributors were the gingival microbiota of cohabitating seals, followed by their own rectal microbiota and that of cohabitating seals (Data S1G). Two seals acquired substantial fractions of their own rectal taxa in their gingiva microbiota mid-treatment. The degree of bacterial seeding from cohabitating seals did not appear to correlate with the number of seals co-

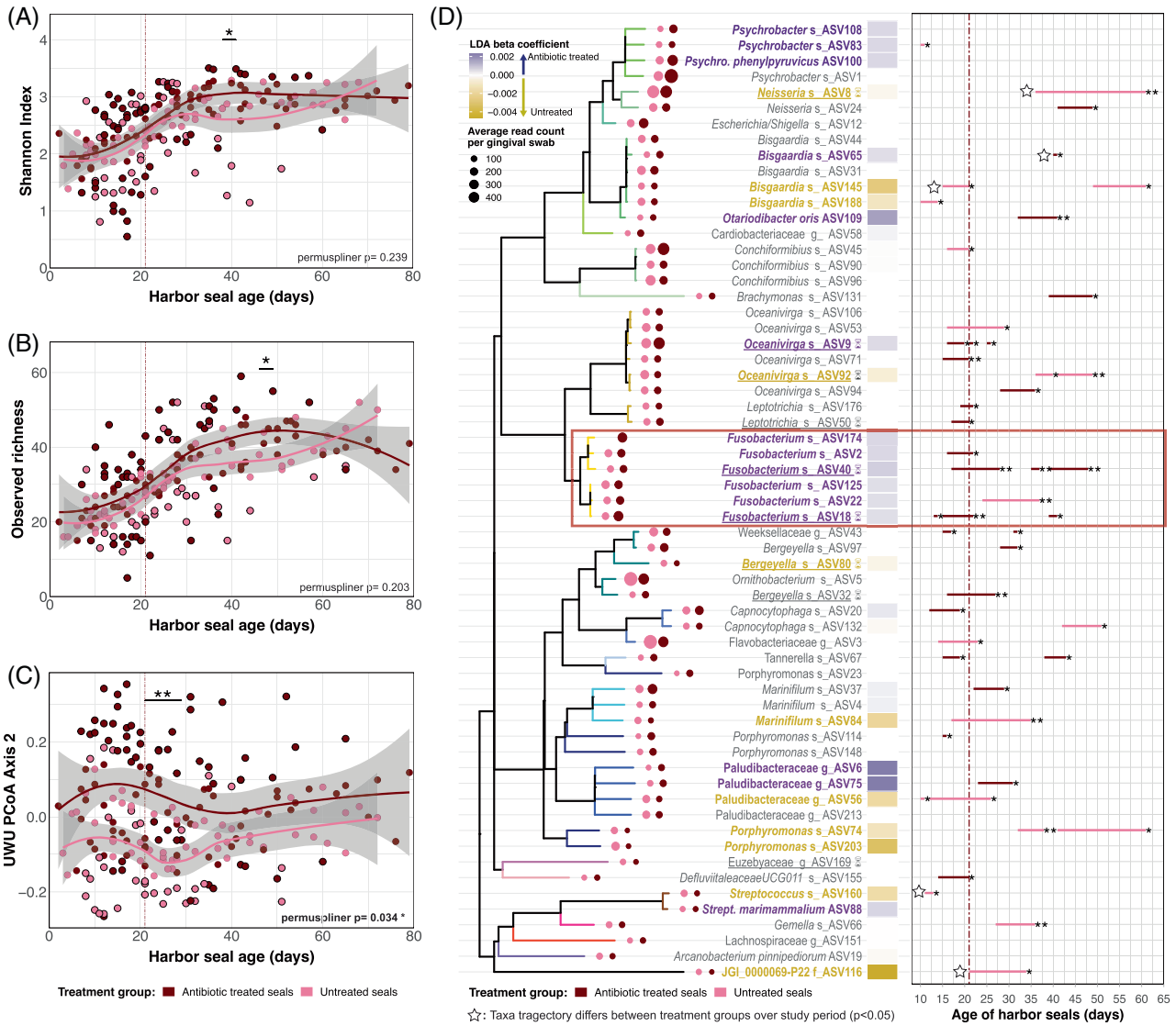


FIGURE 3 Transient increases in alpha diversity and the differential abundance of *Fusobacterium* were observed in the gingival microbiota of antibiotic-treated versus untreated seals. (A,B) Gingival microbiota alpha diversity was plotted against age. Raw reads were used to determine (A) Shannon index and data were rarefied (957 reads/sample) for analysis of (B) richness. (C) Axis 2 loadings of the PCoA ordination based on unweighted Unifrac pair-wise distances in Figure S8A (which best separated the data by treatment group) were plotted against age. For (A–C), Loess smoothing lines and data-points (individual swab samples) are coloured by treatment group (maroon, antibiotic-treated; pink, −untreated). Grey regions show 95% confidence intervals. Dot-dash lines indicate when antibiotic treatment had concluded for all seals. Overall diversity trajectories were compared between treatment groups with 'permuspliner' (SplinectomeR); *p*-values are indicated. Transient windows of differential diversity between groups were identified with 'slidingspliner' (horizontal black lines) (* = p_{min} <0.05, ** = p_{min} <0.01). Longitudinal *p*-values are shown in Data S1E,F. (D) Left: Phylogeny of amplicon sequence variants (ASVs) prevalent in ≥3 gingival swabs with ≥90 reads/sample and ≥1250 reads total. Branches are coloured to denote ASVs in the same family. The size of tip-circles represents mean ASV abundance in each treatment group. Middle: Sparse linear discriminant analysis (LDA, 'treeda') was performed using 28 predictors (determined by cross-validation). The heatmap displays ASV LDA loadings, coloured to indicate discrimination strength (beta coefficient) and direction. The colour of the text indicates the direction of the LDA beta coefficient for taxa with coefficients >0.0035 (purple) or < −0.0035 (gold). The red box highlights the *Fusobacterium* clade found to discriminate the treatment group. RIGHT: Bars denote age ranges with differential ASV abundance identified with 'slidingspliner'; colour indicates the treatment group with higher abundance. Large stars signify ASVs that had different trajectories overall ('permuspliner'). Underlined taxa with hourglass symbols were identified as age-discriminatory in Figure 2E.

housed. Pool water contribution to acquired gingival diversity was negligible.

Differences in alpha and beta diversity between treated and untreated animals dissipated with age, suggesting resilience of gingival communities in response to antibiotic-induced perturbations. Resilience has been observed in the gingival microbiota of rats treated with ciprofloxacin (Manrique et al., 2013). Human-associated bacterial communities have shown resilience to antibiotics used in conjunction with periodontal cleaning (Bizzarro et al., 2016). The canine gingival microbiota has demonstrated resilience following dental

prophylaxis (Flancman et al., 2018). One should note however, that the reduced sampling frequency of older harbour seals—necessary to prepare them for return to the wild, reduced our power to detect more subtle, persistent antibiotic-associated changes.

Antibiotic treatment was associated with higher RAs of Fusobacteriota (Table S9), specifically ASVs within the genus *Fusobacterium* (Figure S10A,B) in the gingival microbiota (see Supporting Text). Paludibacteraceae ASVs were identified as most distinctive to the gingival communities of treated seals (Figure 3D). Actinobacteria and Bacteroidetes had higher RAs in untreated seals. Gracilibacteria member JGI_0000069-P22 ASV116 was most distinctive to the untreated group and was more abundant in untreated seals post-treatment (Data S1F). Notably, eight of the nine gingival taxa most predictive of harbour seal age (Figure 2E) also discriminated between treated and untreated groups. Antibiotic-associated changes in age-discriminatory gingival taxa could explain why transient differences were observed between treatment group loadings along Axis 1 (Figure S8B, Data S1E, $p_{min} = 0.008$), even though the Axis primarily separated samples by age alone. However, all of the taxa that experienced colonisation events in the untreated harbour seal microbiota during rehabilitation (Figure S5D) were also found to be increasingly abundant in the antibiotic-treated cohort, and both treatment groups experienced the same solitary extinction event (*Escherichia/Shigella*_ ASV12, Table S7). These results suggest that antibiotic treatment did not have a lasting effect on acquired diversity in the gingival microbiota.

Higher alpha diversity was also observed in the antibiotic-treated harbour seal distal gut microbiota, whereas lower diversity was observed in infants

Shannon diversity was higher in the rectal communities of the antibiotic-treated seals (Figure 4A, $p_{min} = 0.028$) post-treatment (days 38–42 of age). Richness was also intermittently higher between days 34 and 45 (Figure 4B, $p_{min} = 0.008$). However, alpha diversity trajectories for the entire study period did not differ significantly between treated and untreated animal groups using either metric. Antibiotic-treated seals had 42.2% more unique taxa (Figure S11A). When each treated seal's richness trajectory was compared to the untreated baseline, no associations were apparent with antibiotic class or seal age at intake (Figure S11B). Unlike the gingival microbiota where most seals returned to baseline before release, half of the treatment group concluded the study with higher-than-baseline richness. However, the sparse sampling of older seals limited our power to draw conclusions about possible prolonged or delayed effects of antibiotics on the distal gut microbiota. Treatment had less of an effect on the overall structure of the rectal communities ($R^2_{tx} = 2.0\%$) relative to gingival communities ($R^2_{tx} = 3.4\%$; Table S4, 'adonis2,' Pr(>F) = 0.001) and separated samples along PCoA Axis 2 (Figure S8C). Axis 2 loading trajectories did not differ significantly between treatment groups throughout the overall study but a significant divergence was observed post-treatment (Figure 4C, days 35–44 of age, $p_{min} = 0.019$). Axis 1 loading trajectories were similar between treatment groups over time (Figure S8B).

The higher distal gut alpha diversity observed in antibiotic-treated seals was surprising: multiple studies of antibiotic-treated mice and humans have demonstrated reduced diversity in the distal gut (Azad et al., 2016; Bokulich et al., 2016; Nobel et al., 2015; Yassour et al., 2016). In fact, in our study, antibiotic treated infants had lower bacterial richness (Data S1E, $p_{min} = 0.01$) from days 115 to 144 of age compared to infants who were never treated (treatments occurred between 0 and 179 days of age; Figure S12A,B). The one study to date that addressed antibiotic-associated changes in the marine mammal gut microbiota found reduced diversity in hooded seal small intestines, but was limited to single-timepoints from four dead adults (Acquarone et al., 2020). In the dynamic harbour seal pup microbiota, antibiotics may have suppressed established bacterial clades or influenced colonisation, allowing foreign taxa to compete favourably for newly exposed or yet uncolonized niches. More recently, increased diversity was described in the faecal microbiota of antibiotic-treated mink (*Neovision vision*; Marker et al., 2017). Faecal communities changed to resemble those in feed, presumably due to its high bacterial load and the rapid mink gastrointestinal transit time (GTT, 3–5 h). Harbour seals have a similar GTT (~5 h; Martensson et al., 1998). However, *Lactococcus*, which dominated pup formula (84.1% RA), was hardly detected in the rectal swabs of either treatment group (≤0.01%, Data S1D and Figure 1C); formula did not appear to be a source of bacterial seeding (Figure S9B). The older harbour seal diet, herring, was sampled but DNA amplification was unsuccessful, likely due to PCR inhibitors in the herring gut (Larsen et al., 2015).

Our laboratory has demonstrated that the microbiota of sea lions and their food, mackerel are distinct (Bik et al., 2016), but the harbour seal pup microbiota lacks the stability characteristic of adult marine mammals and perhaps was more readily colonised by herring-associated taxa following antibiotic treatment. Diet is known to influence the dolphin rectal microbiota, especially during probiotic administration (Cardona et al., 2018). However, our comparison of young pup (6–12 days old) rectal communities from treated and untreated animals also revealed 42.8% more unique taxa in treated pups (Figure S11C), implying contributions from sources other than herring. Pool water

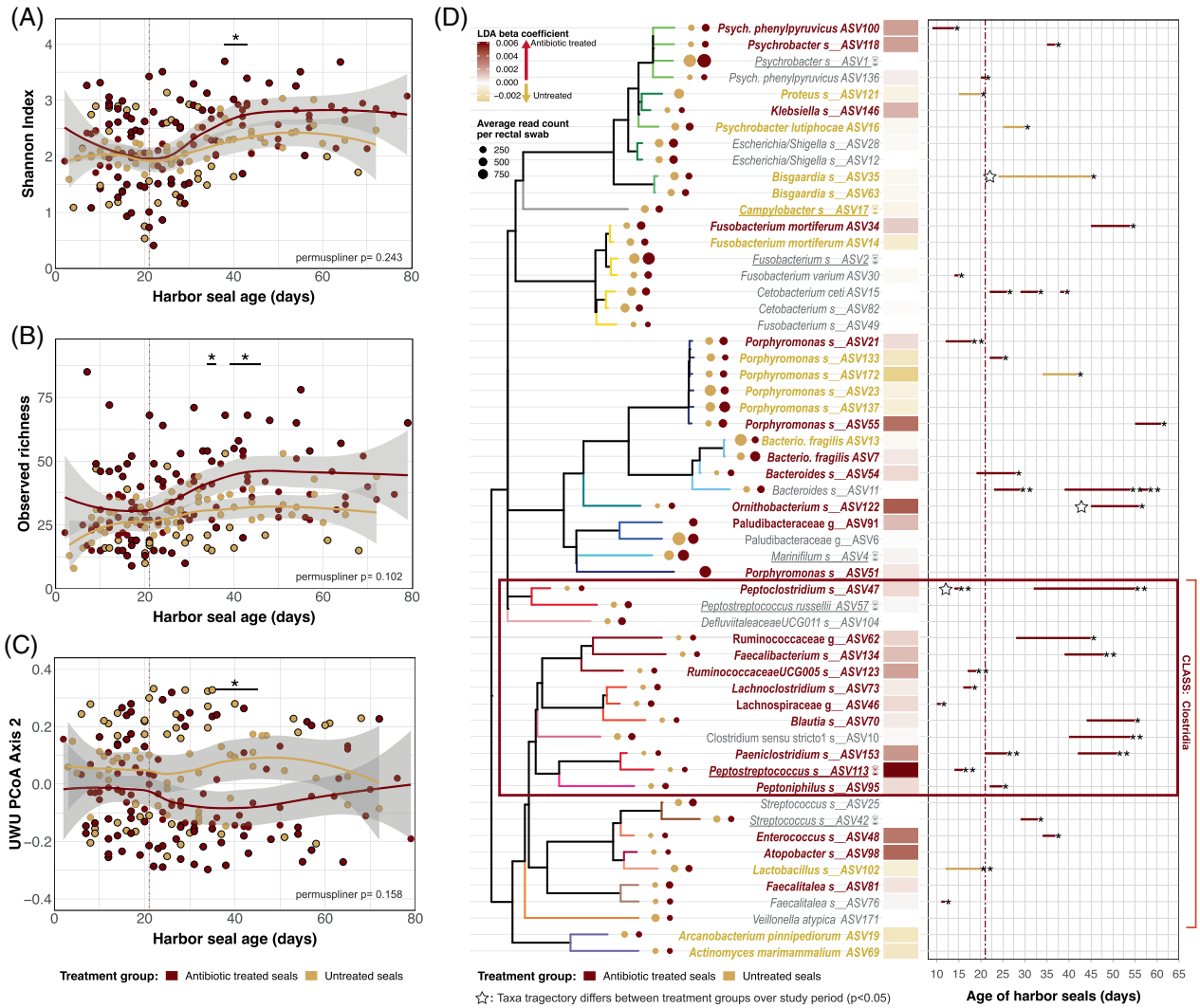


FIGURE 4 Transient increases in alpha diversity and the differential abundance of Firmicutes, particularly of Class Clostridia, were observed in the rectal microbiota of antibiotic-treated versus untreated seals. (A,B) Rectal microbiota alpha diversity was plotted against age. Raw reads were used to determine (A) Shannon index and data were rarefied (1134 reads/sample) for analysis of (B) richness. (C) Axis 2 loadings of the PCoA ordinations based on unweighted unifrac pair-wise distances in Figure S8C (which best separated the data by treatment group) were plotted against age. For (A–C), Loess smoothing lines and data-points (individual swab samples) are coloured by treatment group (brown, antibiotic-treated; yellow, −untreated). Grey regions show 95% confidence intervals. Dot-dash lines indicate when antibiotics were discontinued. Overall, diversity trajectories were compared between treatment groups with 'permuspliner' (SplinectomeR); *p*-values are indicated. Transient windows of differential diversity between groups were identified with 'slidingspliner' (horizontal black lines) (* = p_{min} <0.05, ** = p_{min} <0.01). Longitudinal *p*-values are in Data S1E,F. (D) Left: Phylogeny of amplicon sequence variants (ASVs) prevalent in ≥4 rectal swabs with ≥100 reads/sample and ≥1650 reads total. Branches are coloured to denote ASVs in the same family. The size of tip-circles represents mean ASV abundance in each treatment group. Middle: Sparse linear discriminant analysis (LDA, 'treeda') was performed using 49 predictors (determined by cross-validation). The heatmap displays ASV LDA loadings, coloured to indicate discrimination strength (beta coefficient) and direction. The colour of the text indicates the direction of the LDA beta coefficient for taxa with coefficients >0.0035 (red) or < −0.0035 (gold). The red box highlights the class Clostridia and the red bracket highlights ASVs affiliated with Firmicutes. Right: Bars denote age ranges with differential ASV abundance identified with 'slidingspliner'; colour indicates the treatment group with higher abundance. Large stars signify ASVs that had different trajectories overall ('permuspliner'). Underlined taxa with hourglass symbols were identified as age-discriminatory in Figure 2F.

contributed a higher proportion of bacteria to the rectal versus gingival microbiota, and while this was only a small amount (Figure S9), it could be one conduit for bacterial transfer between animals at the facility. Of the facility habitats sampled, the rectal microbiota of cohabitating seals contributed the most bacterial seeding to treated-seal rectal communities (Supporting Text), which suggests that cohousing with other harbour seals may have contributed to the resilience exhibited by these communities.

Antibiotic-treated harbour seal rectal microbiotas had higher abundance of Fusobacteriota (Table S9) and Firmicutes, particularly class *Clostridia* (Figure 4D), relative to untreated seals (Supporting Text). The most

differentially abundant ASV in the treated seal rectal microbiota was *Peptostreptococcus* ASV113, which had higher abundance in young treated seals during the treatment period (Figure S13A,B; Data S1F). The untreated harbour seal rectal microbiotas had higher RAs of Actinobacteria, Epsilonbactereota and Proteobacteria. *Porphyromonas* ASV172, *Actinomyces marimammalium* ASV69 and *Arcanobacterium pinnipediorum* ASV19 best differentiated the rectal microbiota of untreated seals. Of the seven age-discriminatory rectal taxa identified, only three appeared to be impacted by antibiotics. Unlike gingival communities, rectal swabs from both treatment groups had similar Axis 1 loading trajectories associated with host aging, suggesting antibiotic therapy had less influence on the maturation of rectal communities (Figure S8B).

Interestingly, clindamycin-treated Seal 9, whose gingival community was seeded by its own rectal microbiota mid-treatment, also had evidence of seeding of its rectal community by its own gingival microbiota post-treatment (Figure S9B). The distal gut could potentially be seeded by proximal regions of the same GI tract. Acquarone et al. (2020) has reported that the hooded seal small intestine has a higher RA of Firmicutes relative to pinniped faeces. Distal translocation along the gut could help explain why many Firmicutes differentiated the treated from the untreated-harbour seal rectal microbiotas. Considering that these harbour seals were fed by gavage prior to weaning (and during treatment) it is possible that oral taxa were physically transferred to the stomach by the feeding tube and later excreted in the faeces. Interestingly, the only two ASVs to undergo colonisation events in the rectal microbiota of the treatment group that were undetected in untreated harbour seals (Paludibacteraceae ASV91 and *Porphyromonas* ASV51, Table S7) both had >98% similarity to sequences from the sea lion gingiva (Data S1D). Other potential unsampled sources, such as respiratory exhalates, the medical staff, and fomites (e.g., feeding supplies), could transmit taxa among harbour seals, or between different marine mammal species at the facility. However, in this study, cross-contamination was minimised using sterile gloves, shoe baths, and equipment sterilisation protocols, and the scarcity of human-associated taxa in the harbour seal microbiotas suggest little cross-species transfer. All the taxa, which experienced colonisation events in the untreated harbour seal microbiota during rehabilitation, also became abundant in the antibiotic-treated group. These taxa often emerged at an earlier age in the treatment group, which suggests that one component of the antibiotic-treated seals' transiently higher diversity could be earlier acquisition of commensals. These results, taken with the finding that no unique extinction events were detected among the antibiotic-treated seals (Figure S5D and Table S7), suggest that antibiotic treatment had limited lasting effects on the rectal microbiota.

Sexual dimorphism and sex-dependent effects of antibiotic-treatment were observed in the indigenous microbiota of neonatal harbour seals and humans

Despite small numbers of available animals, evidence of sexual dimorphism in the harbour seal microbiota was observed (Supporting Text). Analysis of the study covariates and community dispersion (Table S10) revealed that sex had a weaker influence than antibiotics on gingival communities (Figure 5A), but a stronger influence than antibiotics on rectal communities (Figure 5B). Gingival communities from untreated females had higher richness than males (Figure S14A). Sex influenced UWU distances at both body sites (Table S4) especially during weaning (Figure S14B,C). Sex-discriminatory taxa were identified in both body sites during the first 6 weeks of life, but afterwards only in the rectal microbiota (Figure S15A,B). Further discussion of the influence of sex on microbial assembly, including a similar analysis of the human dataset (Figure S12C–F), is available in Supporting Text.

Antibiotic treatment had sex-dependent effects on diversity that were more pronounced in gingival communities (Table S4). No differences in overall alpha or beta diversity trajectories (over the entire study period) were detected between antibiotic-treated harbour seal sexes at either body site (Figure S16A,B). However, comparisons of treatment groups within each sex (Figures S16C–F) only revealed antibiotic-associated effects among the males; study-wide differences in alpha and beta diversity trajectories were observed between the gingival communities of treated versus untreated male seals. These findings must be interpreted cautiously given that opportunistic subject recruitment led to an underrepresentation of female seals. That said, these preliminary results warrant further exploration and highlight the importance of gender-based analyses in evaluating the potential impact of clinical therapies on the developing microbiome of neonatal mammals.

SUMMARY

Rehabilitation provided a unique opportunity to sample wild-born neonatal harbour seals longitudinally at a fine timescale and observe the influence of antibiotics in otherwise healthy individuals. Harbour seal-associated bacterial communities contained high novelty and were distinct from those in surrounding water and food. Gingival and rectal communities became more diverse and distinct as pups aged, arriving at a composition like that of wild harbour seals of roughly similar age by the end of rehabilitation. This suggests that microbiota assembly at the facility paralleled that which occurs in nature.

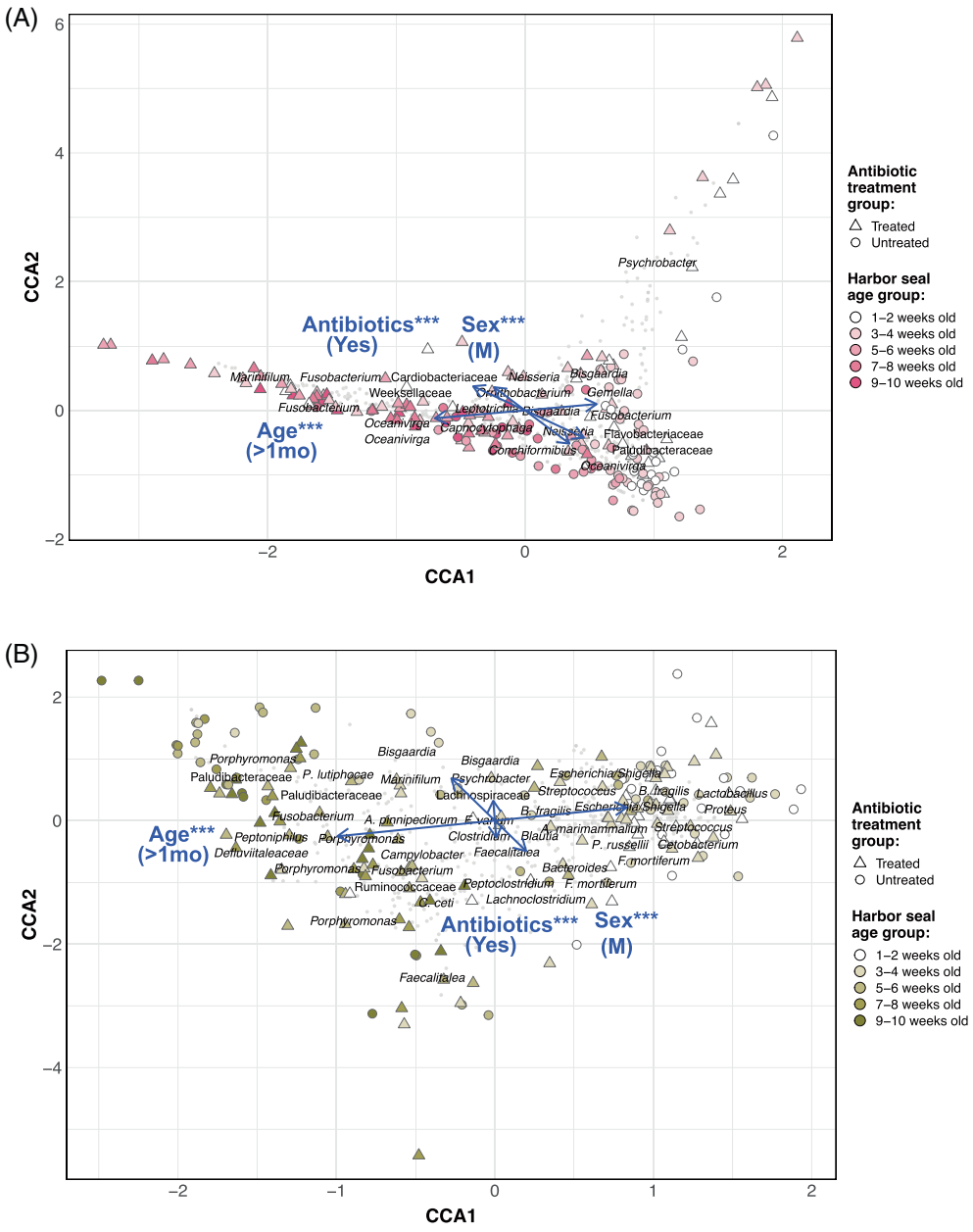


FIGURE 5 Individuality and age best explained bacterial community variation at both harbour seal body sites, followed by sex for gingival communities and antibiotic treatment for rectal communities. Canonical correspondence analysis triplots of the rehabilitated seals' (A) gingival and (B) rectal bacterial communities with harbour seal ID treated as a random effect. Shapes represent treatment groups. Shades of colour denote age. Blue arrows and text indicate study covariates that explain increasing proportions of the dispersion observed among the data in the direction of the arrow. Arrow length corresponds with the strength of the association. Light grey dots denote the bacterial taxa that characterise the sample communities in that region of the triplot; only taxa with ≥0.5% relative abundance in each dataset are labelled (dark grey text). Significance is noted for each covariate based on ANOVA permutations of the models ('anova.cca,' *** = Pr(>*F*) <0.001). Full results are provided in Table S10.

Relative to harbour seals, human infant stool communities harboured less novelty and diversity. Despite sharing similarities in phylum-level composition, the microbiotas of the two host species quickly diverged with age even though harbour seals had limited maternal contact and were raised by humans, implying that host species-specific features assume importance early in life and may be influenced by factors other than vertical transmission and exposure to the marine environment. The observation of emergent taxa in the microbiota of harbour seals during rehabilitation over a week after they had been removed from their natural habitat could be explained by immune-mediated control of bacterial succession within the host, environmental filtering at the facility, or transfer between co-housed seals with different histories of environmental exposures.

Community structure differed between rehabilitated harbour seals that received early life antibiotic treatment, and untreated harbour seals. Gingival and rectal communities displayed higher alpha diversity in association with antibiotic treatment, in contrast to the decreased richness observed in the stool of treated infants. Antibiotic-treated seals acquired a portion of their diversity from the corresponding body sites of cohoused seals. This evidence for bacterial transfer between harbour seals, the demonstration of new colonisation events at the facility, and the ultimate acquisition by the rehabilitated harbour seal microbiota of a host-specific signature all strongly suggest that bacterial filtering between cohoused harbour seals may promote normal microbiota assembly in a rehabilitation setting. This conclusion may highlight an underappreciated benefit of cohousing neonatal conspecifics after maternal separation and/or antibiotic treatment; the transfer of core taxa between individuals may help the group achieve and maintain a host-specific community structure.

Although antibiotic-induced changes in the abundances of some specific taxa may have persisted after their release, the overall community structure associated with antibiotic-treated seals appeared to return towards baseline (as defined by untreated seals) by the end of rehabilitation, implying resilience to this form of perturbation. This conclusion is bolstered by evidence that rehabilitated harbour seal pups, despite frequent antibiotic-exposure, have similar survivorship to wild seals once released back into nature (Lander et al., 2002).

AUTHOR CONTRIBUTIONS

Alexandra Switzer: Conceptualization (lead); data curation (lead); formal analysis (lead); investigation (supporting); methodology (lead); writing – original draft (lead). **Benjamin Callahan:** Data curation (supporting); formal analysis (supporting); methodology (supporting). **Elizabeth Costello:** Data curation (supporting); formal analysis (supporting); methodology (supporting); writing – review and editing (supporting). **Elisabeth Bik:** Data curation (supporting); investigation (supporting); writing – review and editing (supporting). **Christine Fontaine:** Investigation (supporting); methodology (supporting). **Frances Gulland:** Conceptualization (supporting); methodology (supporting); project administration (supporting); resources (supporting); supervision (supporting); writing – review and editing (supporting). **David A. Relman:** Conceptualization (equal); formal analysis (supporting); investigation (supporting); methodology (supporting); project administration (lead); resources (lead); supervision (lead); writing – review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

DNA sequences of the 16S rRNA gene amplicons reported in this paper are available at the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under BioProject PRJNA577962 (accession numbers SAMN13044504-SAMN13044918).

ETHICS STATEMENT

The rehabilitated harbour seals in this study were sampled during veterinary examinations, feedings and/or other handling required for routine animal care at the rehabilitation facility; therefore, no IACUC approval was necessary. Wild harbour seals were sampled by veterinarians under NOAA-NMFS MMPA Permit No. 18786.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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