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Symbiotic microbes and potential pathogens in the intestine of dead southern right whale (*Eubalaena australis*) calves

Carina F. Marón [a, b, *], Kevin D. Kohl [c], Andrea Chirife [d], Matías Di Martino [d], Mariola Penadés Fons [e], Mauricio A. Navarro [f], Juliann Beingesser [f], Denise McAloose [d, g], Francisco A. Uzal [f], M. Denise Dearing [h], Victoria J. Rowntree [b, d, h, i], Marcela Uhart [d, j]

[a] Facultad de Ciencias Exactas, Físicas y Naturales (FCEFyN), Universidad Nacional de Córdoba-CONICET, Córdoba, 5000, Argentina
[b] Instituto de Conservación de Ballenas, Ciudad Autónoma de Buenos Aires, 1429, Argentina
[c] Department of Biological Sciences, University of Pittsburgh, Pennsylvania, 15260, United States
[d] Southern Right Whale Health Monitoring Program, Chubut, 9120, Argentina
[e] Biomedical Research Institute (PASAPTA-Pathology Group), Facultad de Veterinaria, Universidad CEU Cardenal Herrera, CEU Universities, Valencia, Spain
[f] California Animal Health and Food Safety Laboratory, University of California, Davis, CA, 92408, United States
[g] Wildlife Conservation Society, Zoological Health Program, Bronx Zoo, Bronx, NY, 10464, USA
[h] School of Biological Sciences, University of Utah, Utah, 84112, United States
[i] Whale Conservation Institute/Ocean Alliance, Massachusetts, 01930, United States
[j] School of Veterinary Medicine, University of California Davis, California, 95616, United States

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ABSTRACT

Between 2003 and 2017, at least 706 southern right whale (*Eubalaena australis)* calves died at the Península Valdés calving ground in Argentina. Pathogenic microbes are often suggested to be the cause of stranding events in cetaceans; however, to date there is no evidence supporting bacterial infections as a leading cause of right whale calf deaths in Argentina. We used high-throughput sequencing and culture methods to characterize the bacterial communities and to detect potential pathogens from the intestine of stranded calves. We analyzed small and large intestinal contents from 44 dead calves that stranded at Península Valdés from 2005 to 2010 and found 108 bacterial genera, most identified as Firmicutes or Bacteroidetes, and 9 genera that have been previously implicated in diseases of marine mammals. Only one operational taxonomic unit was present in all samples and identified as *Clostridium perfringens* type A. PCR results showed that all *C. perfringens* isolates (n = 38) were positive for alpha, 50% for beta 2 (n = 19) and 47% for enterotoxin (CPE) genes (n = 18). The latter is associated with food-poisoning and gastrointestinal diseases in humans and possibly other animals. The prevalence of the *cpe* gene found in the Valdés' calves is unusually high compared with other mammals. However, insufficient histologic evidence of gastrointestinal inflammation or necrosis (the latter possibly masked by autolysis) in the gut of stranded calves, and absence of enterotoxin detection precludes conclusions about the role of *C. perfringens* in calf deaths. Further work is required to determine whether *C. perfringens* or other pathogens detected in this study are causative agents of calf deaths at Península Valdés.

1. Introduction

Animals maintain intimate associations with communities of microbes residing in their gastrointestinal tracts [1]. These microbes can influence energy balance [2], immune function [3–5], and even the behavior [6] of their hosts. While microbial communities are gaining considerable attention in many natural systems, they remain poorly characterized and understood in cetaceans, with most studies focusing on the taxonomic composition of the cetacean microbiota [7–9] or the functionality of these bacterial communities [10,11].

Over the past decade, southern right whale calves *(Eubalaena australis)* experienced unusually high mortality on their calving ground off Península Valdés, southern Argentina [12,13]. From 2003

* Corresponding author. Facultad de Ciencias Exactas, Físicas y Naturales (FCEFyN), Universidad Nacional de Córdoba-CONICET, 299 Avenida Vélez Sársfield, Córdoba Capital, Córdoba, 5000, Argentina.
E-mail addresses: carimaron@gmail.com, cari.maron@utah.edu (C.F. Marón).

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to 2017, we recorded 706 calf deaths with nearly half occurring between 2005 and 2010 (n = 331). Many potential causes for these calf mortalities have been investigated, but a common cause has yet to be identified.

Pathogenic microbes are often implicated in large stranding events in cetaceans [14–17]. However, to date no evidence has been found to support such hypotheses in the southern right whales off Argentina [18]. Our understanding of the bacterial communities that reside in stranded whales is also limited [10,11,14,19,20]. Sequencing-based approaches combined with culture-dependent methods of inventorying microbes have the potential to identify pathogenic microbes hosted by stranded dead whales.

The aim of this study was to characterize the bacterial communities in the intestines of dead southern right whale calves, and to investigate the presence of potential pathogens that could be associated to their deaths. We first characterized the microbiomes of southern right whale calves, and then we investigated several host factors (post-mortem decomposition, phylogenetic clades, sex, stranding location, year and age) that might influence gut bacterial communities. As these samples were collected from dead animals, investigations of bacterial community structure may be uninformative since relative abundances may not reflect those in living calves. Thus, we only investigated presence of species, and species richness. We then screened for potential pathogens, with a focus on bacterial genera that have previously been implicated in diseases of marine mammals. Additionally, we searched for microbes that were present in all samples, as these could be causative agents of repeated stranding events. Lastly, we conducted genetic testing to investigate the potential virulence of detected microbes and searched for lesions in the intestine and other organs of the stranded calves that could be caused by bacterial pathogens.

2. Materials and methods

2.1. Experimental design

Content from the small (n = 18) and large (n = 26) intestine was collected from a total of 44 stranded southern right whale calves that died in the two gulfs of Península Valdés (Golfo San José and Golfo Nuevo, 42°64′S 64°55′W) from 2005 to 2010. We only analyzed one sample (duodenum or distal third of large intestine) per whale. All samples were aseptically collected from the intestine as soon as the whale carcasses were opened; thus, we are confident that we inventoried the bacterial communities of the southern right whale intestine, and not of environmental sources. Approximately 1–5 gr of intestinal content was collected into sterile tubes and immediately preserved in liquid nitrogen then at −79 °C until analyzed. Individuals of both sexes in various states of postmortem decomposition (Table 1, [21]) were included in this study. Age ranged from newborn (1 day–2 weeks) to 3–4 months [18]. Given this age range, there is a strong likelihood that these animals were exclusively nursing at the time of death [22].

We investigated associations between the prevalence of bacterial genera and host factors (post-mortem decomposition, phylogenetic clade, sex, age, stranding location and year; Table 1) for bacterial genera that were detected in ≥10% of samples. Genetic influences were identified as belonging to whales in either phylogenetic Clade A or W using data provided by Valenzuela for 40 of the 44 individuals analyzed ([23], Valenzuela L.O. unpublished data, Table 1). The effect of geography was investigated by comparing whales found dead at either Golfo San José or Golfo Nuevo. The state of post-mortem decomposition was classified as fresh, moderate or advanced [21]. Additionally, calf total length (snout tip to fluke notch) was measured and used as a proxy for age, with small calves (up to 5.99 m, n = 34) being considered younger than larger calves (≥6 m, n = 11) [18,24].

2.2. Bacterial inventory

Bacterial DNA was extracted from all samples using a QIAamp DNA Stool Mini Kit (Qiagen, Germantown, MD) and sent to Argonne National Laboratories (Illinois, USA) for sequencing. Inventories of bacterial genera were conducted by amplifying the V4 region of the 16S rRNA gene using primers 515F and 806R, and paired-end sequencing was conducted on an Illumina MiSeq platform. Sequences were analyzed using the QIIME software package [25]. Sequences were grouped into operational taxonomic units (OTUs) if they shared greater than 97% sequence identity. OTUs were classified using the Ribosomal Database Project classifier with a minimum support threshold of 80%. We measured estimated species richness (Chao1) using 20 rarefactions of 19000 sequences per sample, thus controlling for different sequencing effort between samples. All sequences were deposited in NCBI's Sequence Read Archive under accession PRJNA421279.

2.3. Analysis of potential pathogens and Clostridium perfringens

We targeted potential bacterial pathogens belonging to genera that had been previously implicated in disease of marine mammals [16,17,26]. We then looked for OTUs that were shared by all samples as possible pathogens. The single OTU that was present in all intestinal samples was *Clostridium perfringens*. To investigate whether this was unique to dead whale calves, we obtained sequencing data of intestinal samples from healthy adult North Atlantic right whales (*Eubalaena glacialis*; [10]) and tested for the presence of this OTU in the data.

Clostridium perfringens is currently classified into seven toxinotypes based on the production of six typing toxins, i.e. alpha (CPA), beta (CPB), epsilon (ETX), iota (ITX), enterotoxin (CPE), and necrotic enteritis B-like (NetB) [27]. In addition, some *C. perfringens* isolates also produce several other toxins such as beta-2 toxin (CPB2), which are not used for toxinotyping [27]. We typed isolates

Table 1
Number of dead calves studied in each category/factor.

Factor/variables analyzed	Number per category	Total calves
Age	34 young: 10 old calves	44
Phylogenetic clade	19 clade A: 21 clade W	40
State of decomposition	18 fresh: 17 moderate: 9 advanced	44
Sex	16 females: 27 males	43
Intestinal content	18 small: 26 large intestine	44
Stranding location	33 Golfo Nuevo: 11 Golfo San José	44
Stranding year	4 in 2005:1 in 2006: 5 in 2007: 12 in 2008: 10 in 2009: 12 in 2010	44
Clostridium perfringens toxin genes	39 each α-toxin,β2-toxin, *cpe*	39
Histopathology	9 small intestine, 7 large intestine, 9 intestine (not further categorized due to autolysis)	20
	Additional tissues	34

of *C. perfringens* present in the gut of southern right whale calves (except for NetB, which, to our knowledge, has only been found in poultry and not in mammalian species) [28]. After homogenization, we cultured all intestinal samples (both with and without heat shock by plating them directly onto tryptose sulfite cycloserine (TSC) agar plates made of SFP agar base (Becton-Dickinson) with 0.04% D-cycloserine (Sigma-Aldrich), a selective medium for *C. perfringens*. Two different PCR reactions were run on *C. perfringens* isolates, one was used to detect the *cpa, cpb, etx, iap, and cpb2* genes, and the other to analyze the *cpe* gene, as previously described [27]. All oligonucleotide primers used in this study [27] were purchased from Integrated DNA Technologies (IDT). PCR reactions were performed using 1 μl of DNA in a final volume of 50 μl.

The multiplex PCR program was run in a Peltier Thermal Cycler PTC-100™ (MJ Research), with initial denaturation at 95 °C for 15 min, followed by 35 cycles of 30 s at 94 °C, 90 s at 55 °C and 90 s at 72 °C (denaturation, annealing and extension phases, respectively), followed by a final extension cycle for 10 min at 72 °C. Five microliters of the PCR products were separated by electrophoresis on a 1% (w/v) agarose gel (Agarose SFR™ Super Fine Resolution, AMRESCO®, code J234-25G), stained with 0.2 μg/ml of ethidium bromide (AMRESCO®, code X328-10 ML) for 20–30 min at 110V and visualized by UV transillumination. The length of the amplification product of the multiplex PCR could be easily discriminated in this gel because of a size difference of at least 52 bp and compared to a molecular weight marker (Amplisize® Molecular Ruler, 50–2000 bp Ladder, Cat. # 170–8200, Bio-Rad). DNA from two *C. perfringens* reference strains (types B and E, respectively) both *cpb2* and *cpe* positive, were used as controls.

To determine the presence of enterotoxin (CPE) in the gut of stranded calves, intestinal contents from all the animals were tested by a commercial ELISA, according to the instructions of the manufacturer (Techlab, Blacksburg, VA).

2.4. Histological analysis

Samples collected from the small or the large intestine of dead calves were fixed in 10% buffered (pH 7.2) formalin and processed using routine methods for histologic examination [18]. Briefly, they were embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin and eosin (HE). Additional samples collected from other organs were also examined histologically (Table 1).

2.5. Statistical analysis

To determine whether the prevalence of bacterial members differed among decomposition state, phylogenetic clades, sex, stranding location, year and age (aka whale body length), we used Chi-square analysis using the presence or absence of each genus. T-tests and linear regression were applied to test whether the estimated species richness varied between state of decomposition, phylogenetic clades, sex, stranding sites, and year. We also used linear regression to test whether species richness varied in relation to the age using state of decomposition as a covariate. These tests were conducted either for the small or the large intestine, but the bacterial composition and richness of both segments was not compared against each other. All statistical analyses were conducted in JMP 12.0.

3. Results

3.1. Presence of bacterial species

We analyzed 18 small intestine samples and 26 large intestine samples from 44 calves. Sequencing efforts resulted in an average of 44,836 ± 3518 sequences per sample.

These sequences were assigned to 22,106 OTUs at 97% sequence identity. Most bacterial sequences were identified as Firmicutes or Bacteroidetes. We documented the presence of 108 bacterial genera residing in the gastrointestinal tract of stranded right whale calves (Supplementary files: Table S1). In whales in advanced state of post-mortem decomposition, the prevalence of *Erysipelothrix* in the large intestine was higher ($P = 0.028$), while the prevalence of *Cetobacterium* in the small intestine was lower ($P = 0.032$). There were no host clade-specific genera from either small or large intestinal samples, suggesting that the two major phylogenetic clades of whales share similar microbiotas. There were also no differences in the bacterial genera hosted by males versus females.

We detected several possible geographic site-specific genera. *Allobaculum* was more prevalent in the large intestine samples collected from Golfo San José ($P = 0.004$). *Oscillospira* was specific to the small intestine of whales from Golfo Nuevo ($P = 0.018$), and *Sarcina* was more prevalent in the small intestines of whales from Golfo San José ($P = 0.022$).

Five genera decreased in prevalence over the duration of this study. *Dorea* and *Prevotella* were more prevalent in 2005–2007, *Bifidobacterium* and *Oscillospira* in 2005–2008, and *Erysipelothrix* in 2007. The genus *Sarcina* increased in prevalence in 2009–2010. The genera *Bifidobacterium, Desulfovibrio, Dorea, Eggerthella, Erysipelothrix, Oscillospira, Peptococcus, Prevotella, Proteus, Sutterella,* and *Treponema* were all more prevalent in the large intestine of older calves (those ≥6 m) compared to younger calves ($P < 0.05$ for all). Young calves did not exhibit higher prevalence of any microbes, and there were no age-related differences in small intestine prevalences.

3.2. Species richness

There were no associations between species richness and state of post-mortem decomposition, whale clade, sex, stranding location, or year. However, older calves hosted more bacterial species. There were significant correlations between calf age (length) and species richness in both the small intestine (Fig. 1A; $F_{1,16} = 5.89$, $P = 0.027$, $R^2 = 0.27$) and large intestine (Fig. 1B; $F_{1,25} = 7.98$, $P = 0.009$, $R^2 = 0.25$). We investigated decomposition state as a covariate, but it was not significant, so it was removed from the final models.

3.3. Pathogen identification and Clostridium perfringens

We identified 9 bacterial genera that have been previously implicated in marine mammal disease: *Erysipelothrix*, *Escherichia, Helicobacter*, *Pseudomonas*, *Mycoplasma, Clostridium*, *Streptococcus*, *Corynebacterium* and *Pasteurella* (Supplementary files: Table S1; [16,25]. *Clostridium perfringens* was the only OTU present in all samples. *C. perfringens* was isolated from most of the intestinal samples cultured (39 of 44). This OTU was also detected in sequencing data of intestinal samples from healthy adult North Atlantic right whales. All isolates were identified as *C. perfringens* type A and F. All intestinal samples were PCR positive for the *cpa* gene, 46% for *cpb2* ($n = 18$) and 44% for *cpe* ($n = 17$) (Supplementary files: Fig. S1). ELISA testing for CPE was negative in the intestinal content of all 44 calves.

3.4. Histopathology

Samples were available for histologic review from 34 of the 44 whales. These included: intestinal tissue ($n = 20$; 9 small intestine; 7 large intestine; 9 intestine [not further categorized due to autolysis]), and numerous other tissues ($n = 34$; 24 skeletal muscle;

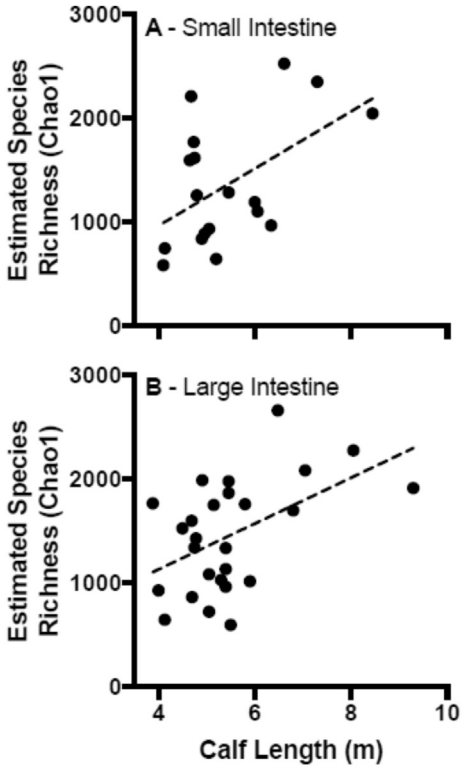

Fig. 1. Bacterial richness in small (A) and large (B) intestine as a function of calf length. Longer calves exhibit higher bacterial richness in both intestinal sections.

23 lung, kidney; 20 skin; 10 heart; 15 liver; 13 spleen; 12 brain, 12 testis; 10 lymph node, stomach; 9 bone marrow; 8 connective tissue; 7 urinary bladder, pancreas, artery, baleen; 6 tongue, esophagus; 5 thymus, ovary, penis, epididymis; 4 urethra; 3 cartilage; 2 trachea, adrenal gland, peripheral nerve, uterus, vagina, umbilicus; 1 gall bladder, bone, spinal cord, cervix). In 5 cases only skin (4) or skin, muscle and baleen (1) were available. Excluding these 5 cases, autolysis was mild to moderate in 10 whales, moderate to severe in 11, and severe in 8. Changes of varied significance were observed in the brain of 3 whales, the lung of 9, liver and/or spleen of 3, gastrointestinal tract (small intestine and esophagus) of 2, lymph node of 1, and other (umbilicus, artery thrombosis) in 3. One calf (4.1 m long, 2010 death) had mild multifocal neutrophilic and lymphoplasmacytic enteritis with multifocal large clear spaces suggestive of gas formation and/or edema. The small intestinal content of this calf was positive for the *C. perfringens cpe* toxin gene, as well as for *Streptococcus* and *E. coli*. A second calf (4.96 m long, 2010 death) had moderate, regionally extensive mural esophagitis. Autolysis in the small intestine of this calf was moderate to severe; large intestine was not available for histologic review. Mixed bacteria (*Corynebacterium, Pasteurella, Streptococcus, E. coli*) were found in its intestinal tract including *C. perfringens* that was positive for the *cpe* toxin gene. A third calf (5.05 m long, 2009 death) had mild to moderate multifocal non-suppurative meningitis. Intestinal samples were not available for histologic review, but *E. coli* and *C. perfringens* positive for the *cpe* toxin gene were found in its intestine. No other significant microscopic abnormalities were observed in any of the other samples examined.

4. Discussion

This is the first reported characterization of the bacterial communities that live within the intestines of baleen whale calves and one of the few to characterize potential pathogenic bacteria in stranded whale carcasses.

The bacterial communities in stranded southern right whale calves were represented by different genera that show similarities to the microbiomes described for other cetaceans. Most bacterial sequences were identified as Firmicutes or Bacteroidetes, which are the dominant phyla in other mammalian species [29–32], including cetaceans [10,11,33–35]. For example, the clade 5 *Verrucomicrobia* and the genus *Treponema* were found in the gut of both southern right and North Atlantic right whales. *Verrucomicrobia* is more abundant in mammals whose diets contain fermentable animal polysaccharides (such as chitinous zooplankton) [10].

Southern right whale calf microbiomes shared some similarities with the bacterial taxonomic groups found in a bottlenose dolphin calf (*Tursiops truncatus*). Both species shared several bacterial families, including *Clostridiaceae*, *Peptostreptococcaceae*, *Ruminococcaceae*, *Enterococcaceae*, *Streptococcaceae*, *Prevotellaceae* and *Sphingomonadaceae*. These bacterial families were also present in bottlenose dolphin maternal milk suggesting milk influences the calf's microbiota [35]. In southern right whale calves, maternal milk is most likely the only source of energy during the first three months of life at their calving ground in Península Valdés [22] and probably is an important source of bacteria. Accordingly, we found *Bifidobacterium* in 34% of the examined calves. This genus is known to play a role in digesting milk oligosaccharides [36], which are in especially high abundance in the milk of cetaceans [37,38].

Our inventories of bacterial genera also documented commensal or beneficial microbes, such as *Oscillospira*, in the guts of whale calves. The functional capabilities of *Oscillospira* are unknown, but it likely plays a role in fiber fermentation due to its presence in numerous rumen systems and its greater abundance when hosts are fed fiber [39]. The functional roles of these bacterial genera in southern right whales is currently unknown. Also unknown is whether their presence or absence influences host fitness.

Our data suggest that there is no association between host phylogenetic clade, location, sex and post-mortem decomposition, with the bacterial community structures of southern right whale calves. Evidence to date indicates that post-mortem decomposition is not an important structuring factor for mammal microbiomes, including studies on mice [40] and two species of kogiid whales [11]. Bacterial community structure remains largely unchanged, at least in the early stages of decomposition or before intestinal rupture occurs and the gut microbiota is exposed to aerobic conditions [40]. Other studies have also shown that sex has no significant effect on marine mammal microbiomes [8,11,30,32] if the species do not display sexual size dimorphism [41].

In contrast, calf microbiome varied with year of stranding. Non-pathogenic bacteria, such as *Dorea*, *Prevotella Bifidobacterium* and *Oscillospira* were more prevalent in early study years; however, the genus *Sarcina* were more prevalent in later years. *Bifidobacterium* perform important degradation of milk oligosaccharides [36]. Both *Oscillospira* and *Prevotella* are regularly found in ruminants [42,43], and are thought to degrade complex carbohydrates. High abundances of *Oscillospira* are associated with feeding on fresh forage [42], and so may play a role in fiber degradation. *Prevotella* are non-cellulolytic, and instead degrade xylans [44]. While it is unclear what carbohydrates these genera might be degrading in the guts of whale calves, previous studies have demonstrated that whales tend to have some similarities to herbivores in terms of bacterial community structure [10].

Calf age largely determined the bacterial community composition of right whale calves. Microbiota composition changed with growth in a breast-fed bottlenose dolphin calf from birth to 5–8 months of age and was probably due to nursing [35]. Southern right whale calves in the Valdés population are on average 5.5 m long at

birth and grow as much as 3 m during their first months of life at their calving ground [18,24]. Differences in the bacterial community composition between young (<6 m) and older calves (≥6 m) might be due to nursing, which has been demonstrated for terrestrial mammals [45]. In addition, infant humans and avian chicks exhibit increases in bacterial diversity during ontogeny, which converge in adult-like communities [46,47]. This shift is thought to be due to incidental exposure to environmental microbes that colonize the gastrointestinal tract [46]. Our study only investigated the bacterial communities of southern right whale calves; further studies are needed to characterize communities of adults as well.

The genera *Bilophila, Peptococcus*, and *Treponema* were only found in the large intestine of older calves (those ≥ 6 m). Small calves did not harbor any unique microbes. The abundance of *Bilophila* increases in response to dietary milk fats [48], and so may be more abundant in older calves due to greater milk intake. *Peptococcus* is rare in human children, but more abundant in adults [49]. Many other unidentified OTUs were present only in older calves, suggesting that the microbiota obtains new members as whales grow.

Several potential pathogens were detected in the intestine of stranded southern right whale calves including the genera *Mycoplasma*, *Streptococcus*, *Erysipelothrix* and *Clostridium. Mycoplasma* spp. have been associated with high mortality events in marine mammals, especially pinnipeds. Primary clinical diseases include pneumonia and septic polyarthritis [50–52]. In our study, *Mycoplasma* was present in the intestinal content of three calves. Several tissues (testis, skin, skeletal muscle, penis, kidney, liver and spleen) were available for histologic examination from only one and contained no lesions, suggesting this to be an incidental finding. Other cases, particularly those with pneumonia, should be analyzed to discard the role of this pathogen in right whale calf deaths at Valdés.

Streptococcus spp. are known to produce pneumonia and septicemia in pinnipeds [16], and two cases have been reported in odontocetes, the harbour porpoise (*Phocoena*, [53]) and the pilot whale (*Globicephala melaena*, [54]). An apparent cause of death related to infections by *Streptococcus* was not evident in the dead calves analyzed in our study. Some species of *Erysipelothrix* can produce infections in odontocetes and mysticetes. For instance, *Erysipelothrix rhusiopathiae* can cause lethal septicemia [16,55] and has been found in skin lesions of southern right whales [56]. In this study, *Erysipelothrix* was almost exclusively found in calves that died in 2007, a high mortality year when most calves showed unusually severe skin lesions [57]. However, few samples were available for histologic examination and the role of this potential pathogen remains to be evaluated. Kelp gulls (*Larus dominicanus*) at Península Valdés feed on skin and blubber of living right whales opening wounds on their backs of different size and severity [58]. Fiorito et al. [56] reported *E. rhusiopathiae* in one living and one dead calf with particular rhomboid shaped gull-inflicted lesions. Although the origin of the bacterium is unknown, it could potentially be directly transmitted by gulls, constitute normal skin microbiota, or be a direct or indirect (opportunistic infection in open wounds) pathogen. Our findings show that right whale guts are another potential source.

Clostridium perfringens was the only OTU identified in all samples. This bacterium is found at high prevalence in healthy individuals of multiple mammalian and avian species [59], and its presence in carcasses, especially those in advanced stages of decomposition, is relatively common. Detection in 100% of the stranded southern right whale calves examined was not unexpected. The class Clostridia is well represented among three species of baleen whales, the North Atlantic right whale, the humpback whale (*Megaptera novaeangliae*) and the sei whale (*Balaenoptera borealis*) [10]. *Clostridium perfringens* has been also found in stranded pygmy (*Kogia breviceps*) and dwarf (*K. sima*) sperm whales [11]) and has been detected in healthy North Atlantic right whales [10]. The presence of *C. perfringens* in all carcasses analyzed for this study may be also explained by changes in the gut environment under post-mortem conditions. Some microbes opportunistically dominate the gut microbiome after death due to a decreased intestinal blood flow and an increased digesta retention [60,61].

However, *C. perfringens* can also be a primary ante-mortem pathogen that causes disease in a broad variety of avian and mammalian species. This microorganism can produce a range of lethal toxins [62,63], and it has been associated with disease in several aquatic mammals including dolphins [64], sea otters [65], Weddell seals [66] and hooded seals [67]. Other *Clostridium* spp. can also affect marine mammals. These include *C. septicum*, which was associated with the death of two adult sperm whales (*Physeter macrocephalus*) in Denmark [20].

Pathogenicity of *C. perfringens* cannot be determined simply by its presence or detection [59]. In several mammalian species, pathogenicity must be confirmed based on gross and microscopic lesions, coupled with the presence of pre-formed toxins in the intestinal content. In several mammals, intestinal lesions caused by *C. perfringens* enterotoxin are characterized by necrosis and degeneration of the superficial epithelium, edema and congestion [68,69]. In whales, however, no diagnostic criteria for *C. perfringens* infections have been established. To date (2003–2017), enteritis or colitis has been identified histologically in only two stranded right whale calves at Península Valdés (n = 39, [18]). Intestinal content from the calf with enteritis in the current study tested positive for *C. perfringens cpe* toxin gene, but was negative for toxin production. In addition, toxic changes (such as mucosal necrosis that can occur secondary to clostridial toxins) were not observed in intestinal samples from this or other calves, and obvious inflammation was not seen in other calves. However, histology may not be the most sensitive indicator of toxin production, especially in animals in moderate to advanced stages of autolysis since decomposition is known to occur rapidly in gastrointestinal tissues of dead mammals [70,71] and can mask subtle lesions. Pre-formed toxins were not detected in any dead calf. This finding may be because for CPE production, *C. perfringens* must sporulate in the gastrointestinal tract of the host [72]. We did not detect *C. perfringens* sporulation in dead southern right whale calves.

We further investigated the presence of toxin genes in the microbiota samples from stranded calves. All cultured isolates of *C. perfringens* were identified as type A or type F. *Clostridium perfringens* type A is generally considered a commensal, non-pathogenic toxinotype in the intestine of most animals [73]. In contrast, *C. perfringens* type F producing enterotoxin, causes food-borne illness and gastrointestinal disease in humans [28,74,75], and has been associated with cases of enteritis or diarrhea in dogs and horses [58,76]. Nevertheless, its role in animal disease remains poorly understood [77]. The enterotoxin gene (*cpe*), was common in the gastrointestinal microbiota of southern right whale calves. The *cpe* gene was present in 44% of the cultured samples. This is a remarkable difference compared to only 5% prevalence of the global *C. perfringens* population [78,79]. Again, however, the low prevalence of gastrointestinal lesions and the absence of enterotoxin limits conclusions about the role of *C. perfringens* in calf deaths. Additional data from living healthy and sick whales will be necessary to evaluate the meaning of these findings.

While we lack the evidence to attribute a role to *C. perfringens* in calf deaths, the high prevalence of the *cpe* gene is unusual when compared to other mammals. Moreover, some microbes that have been classified as 'pathobionts', or microbes that are normal

members of the gut microbiota could induce disease under certain conditions, such as when hosts are stressed or immunocompromised [80]. Further work is required to determine whether *C. perfringens* or other possible pathogens reported in this study might be contributors to calf mortality at Península Valdés.

5. Conclusions

Our findings provide the first culture-independent inventory of bacterial genera in the gut of stranded baleen whale calves. We identified many commensal and beneficial bacterial species. We also identified several potential pathogens such as *C. perfringens*. Further work is required to determine whether *C. perfringens* or other pathogens detected in this study are causative agents of calf deaths at Península Valdés. Our inventory also provides insight into the bacterial ecology of baleen whale calves. Further research related to the functions of various microbes within the calf gut is warranted.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2019.04.003.

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