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

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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 2005-2010 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 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)

81 that might influence gut bacterial communities. As these samples were collected from
82 dead animals, investigations of bacterial community structure may be uninformative
83 since relative abundances may not reflect those in living calves. Thus, we only
84 investigated presence of species, and species richness. We then screened for potential
85 pathogens, with a focus on bacterial genera that have previously been implicated in
86 diseases of marine mammals. Additionally, we searched for microbes that were present in
87 all samples, as these could be causative agents of repeated stranding events. Lastly, we
88 conducted genetic testing to investigate the potential virulence of detected microbes and
89 searched for lesions in the intestine and other organs of the stranded calves that could be
90 caused by bacterial pathogens.
91
92 **2. Materials and methods**
93 **Experimental design**
94 Content from the small (n=18) and large (n=26) intestine was collected from a
95 total of 44 stranded southern right whale calves that died in the two gulfs of Península
96 Valdés (Golfo San José and Golfo Nuevo, 42°64′S 64°55′W) from 2005-2010. We only
97 analyzed one sample (duodenum or distal third of large intestine) per whale. All samples
98 were aseptically collected from the intestine as soon as the whale carcasses were opened;
99 thus, we are confident that we inventoried the bacterial communities of the southern right
100 whale intestine, and not of environmental sources. Approximately 1-5 gr of intestinal
101 content was collected into sterile tubes and immediately preserved in liquid nitrogen then
102 at -79 °C until analyzed. Individuals of both sexes in various states of postmortem
103 decomposition (Table 1, [21]) were included in this study. Age ranged from newborn (1

104 day to 2 weeks) to 3-4 month [18]. Given this age range, there is a strong likelihood that
105 these animals were exclusively nursing at the time of death [22].
106 We investigated associations between the prevalence of bacterial genera and host
107 factors (post-mortem decomposition, phylogenetic clade, sex, age, stranding location and
108 year; Table 1) for bacterial genera that were detected in ≥10% of samples. Genetic
109 influences were identified as belonging to whales in either phylogenetic Clade A or W
110 using data provided by Valenzuela for 40 of the 44 individuals analyzed ([23],
111 Valenzuela L.O. unpublished data, Table 1). The effect of geography was investigated by
112 comparing whales found dead at either Golfo San José or Golfo Nuevo. The state of post-
113 mortem decomposition was classified as fresh, moderate or advanced [21]. Additionally,
114 calf total length (snout tip to fluke notch) was measured and used as a proxy for age, with
115 small calves (up to 5.99 m, n=34) being considered younger than larger calves (≥6 m,
116 n=11) [18,24].
117 **Bacterial inventory**
118 Bacterial DNA was extracted from all samples using a QIAamp DNA Stool Mini
119 Kit (Qiagen, Germantown, MD) and sent to Argonne National Laboratories (Illinois,
120 USA) for sequencing. Inventories of bacterial genera were conducted by amplifying the
121 V4 region of the 16S rRNA gene using primers 515F and 806R, and paired-end
122 sequencing was conducted on an Illumina MiSeq platform. Sequences were analyzed
123 using the QIIME software package [25]. Sequences were grouped into operational
124 taxonomic units (OTUs) if they shared greater than 97% sequence identity. OTUs were
125 classified using the Ribosomal Database Project classifier with a minimum support
126 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.

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 of *C. perfringens* present in the gut of southern right whale calves (except for NetB, which, to our knowledge, has only been foundin 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

150 described [27]. All oligonucleotide primers used in this study [27] were purchased from
151 Integrated DNA Technologies (IDT). PCR reactions were performed using 1 µl of DNA
152 in a final volume of 50 µl.
153 The multiplex PCR program was run in a Peltier Thermal Cycler PTC-100™ (MJ
154 Research), with initial denaturation at 95°C for 15 min, followed by 35 cycles of 30 s at
155 94°C, 90 s at 55°C and 90 s at 72°C (denaturation, annealing and extension phases,
156 respectively), followed by a final extension cycle for 10 min at 72°C. Five microliters of
157 the PCR products were separated by electrophoresis on a 1% (w/v) agarose gel (Agarose
158 SFR™ Super Fine Resolution, AMRESCO®, code J234-25G), stained with 0.2 µg/ml of
159 ethidium bromide (AMRESCO®, code X328-10ML) for 20-30 min at 110V and
160 visualized by UV transillumination. The length of the amplification product of the
161 multiplex PCR could be easily discriminated in this gel because of a size difference of at
162 least 52 bp and compared to a molecular weight marker (Amplisize® Molecular Ruler,
163 50-2,000 bp Ladder, Cat. # 170-8200, Bio-Rad). DNA from two *C. perfringens* reference
164 strains (types B and E, respectively) both *cpb2* and *cpe* positive, were used as controls.
165 To determine the presence of enterotoxin (CPE) in the gut of stranded calves,
166 intestinal contents from all the animals were tested by a commercial ELISA, according to
167 the instructions of the manufacturer (Techlab, Blacksburg, VA).
168 **Histological analysis**
169 Samples collected from the small or the large intestine of dead calves were fixed
170 in 10% buffered (pH 7.2) formalin and processed using routine methods for histologic
171 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).

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

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 =

195 0.028), while the prevalence of *Cetobacterium* in the small intestine was lower (P =
196 0.032). There were no host clade-specific genera from either small or large intestinal
197 samples, suggesting that the two major phylogenetic clades of whales share similar
198 microbiotas. There were also no differences in the bacterial genera hosted by males
199 versus females.
200 We detected several possible geographic site-specific genera. *Allobaculum* was
201 more prevalent in the large intestine samples collected from Golfo San José (P = 0.004).
202 *Oscillospira* was specific to the small intestine of whales from Golfo Nuevo (P = 0.018),
203 and *Sarcina* was more prevalent in the small intestines of whales from Golfo San José (P
204 = 0.022).
205 Five genera decreased in prevalence over the duration of this study. *Dorea* and
206 *Prevotella* were more prevalent in 2005-2007, *Bifidobacterium* and *Oscillospira* in 2005-
207 2008, and *Erysipelothrix* in 2007. The genus *Sarcina* increased in prevalence in 2009-
208 2010. The genera *Bifidobacterium, Desulfovibrio, Dorea, Eggerthella, Erysipelothrix,*
209 *Oscillospira, Peptococcus, Prevotella, Proteus, Sutterella,* and *Treponema* were all more
210 prevalent in the large intestine of older calves (those ≥6 m) compared to younger calves
211 ($P < 0.05$ for all). Young calves did not exhibit higher prevalence of any microbes, and
212 there were no age-related differences in small intestine prevalences.
213 **Species richness**
214 There were no associations between species richness and state of post-mortem
215 decomposition, whale clade, sex, stranding location, or year. However, older calves
216 hosted more bacterial species. There were significant correlations between calf age
217 (length) and species richness in both the small intestine (Fig. 1A; $F_{1,16} = 5.89$, P = 0.027,

218 $R^2 = 0.27$) and large intestine (Fig. 1B; $F_{1,25} = 7.98$, $P = 0.009$, $R^2 = 0.25$). We
219 investigated decomposition state as a covariate, but it was not significant, so it was
220 removed from the final models.

221 **Pathogen identification and *Clostridium perfringens***

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

232 **Histopathology**

233 Samples were available for histologic review from 34 of the 44 whales. These
234 included: intestinal tissue (n=20; 9 small intestine; 7 large intestine; 9 intestine [not
235 further categorized due to autolysis]), and numerous other tissues (n=34; 24 skeletal
236 muscle; 23 lung, kidney; 20 skin; 10 heart; 15 liver; 13 spleen; 12 brain, 12 testis; 10
237 lymph node, stomach; 9 bone marrow; 8 connective tissue; 7 urinary bladder, pancreas,
238 artery, baleen; 6 tongue, esophagus; 5 thymus, ovary, penis, epididymis; 4 urethra; 3
239 cartilage; 2 trachea, adrenal gland, peripheral nerve, uterus, vagina, umbilicus; 1 gall
240 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 (4.1m 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 (4.96m 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 (5.05m 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.

5. 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.

263 The bacterial communities in stranded southern right whale calves were
264 represented by different genera that show similarities to the microbiomes described for
265 other cetaceans. Most bacterial sequences were identified as Firmicutes or Bacteroidetes,
266 which are the dominant phyla in other mammalian species [29-32], including cetaceans
267 [10,11,33-35]. For example, the clade 5 *Verrucomicrobia* and the genus *Treponema* were
268 found in the gut of both southern right and North Atlantic right whales. *Verrucomicrobia*
269 is more abundant in mammals whose diets contain fermentable animal polysaccharides
270 (such as chitinous zooplankton) [10].
271 Southern right whale calf microbiomes shared some similarities with the bacterial
272 taxonomic groups found in a bottlenose dolphin calf (*Tursiops truncatus*). Both species
273 shared several bacterial families, including *Clostridiaceae*, *Peptostreptococcaceae*,
274 *Ruminococcaceae*, *Enterococcaceae*, *Streptococcaceae*, *Prevotellaceae* and
275 *Sphingomonadaceae*. These bacterial families were also present in bottlenose dolphin
276 maternal milk suggesting milk influences the calf's microbiota [35]. In southern right
277 whale calves, maternal milk is most likely the only source of energy during the first three
278 months of life at their calving ground in Península Valdés [22] and probably is an
279 important source of bacteria. Accordingly, we found *Bifidobacterium* in 34% of the
280 examined calves. This genus is known to play a role in digesting milk oligosaccharides
281 [36], which are in especially high abundance in the milk of cetaceans [37-38].
282 Our inventories of bacterial genera also documented commensal or beneficial
283 microbes, such as *Oscillospira*, in the guts of whale calves. The functional capabilities of
284 *Oscillospira* are unknown, but it likely plays a role in fiber fermentation due to its
285 presence in numerous rumen systems and its greater abundance when hosts are fed fiber

286 [39]. The functional roles of these bacterial genera in southern right whales is currently
287 unknown. Also unknown is whether their presence or absence influences host fitness.
288 Our data suggest that there is no association between host phylogenetic clade,
289 location, sex and post-mortem decomposition, with the bacterial community structures of
290 southern right whale calves. Evidence to date indicates that post-mortem decomposition
291 is not an important structuring factor for mammal microbiomes, including studies on
292 mice [40] and two species of kogiid whales [11]. Bacterial community structure remains
293 largely unchanged, at least in the early stages of decomposition or before intestinal
294 rupture occurs and the gut microbiota is exposed to aerobic conditions [40]. Other studies
295 have also shown that sex has no significant effect on marine mammal microbiomes [8,11,
296 30,32] if the species do not display sexual size dimorphism [41].
297 In contrast, calf microbiome varied with year of stranding. Non-pathogenic
298 bacteria, such as *Dorea*, *Prevotella Bifidobacterium* and *Oscillospira* were more
299 prevalent in early study years; however, the genus *Sarcina* were more prevalent in later
300 years. *Bifidobacterium* perform important degradation of milk oligosaccharides [36].
301 Both *Oscillospira* and *Prevotella* are regularly found in ruminants [42,43], and are
302 thought to degrade complex carbohydrates. High abundances of *Oscillospira* are
303 associated with feeding on fresh forage [42], and so may play a role in fiber degradation.
304 *Prevotella* are non-cellulolytic, and instead degrade xylans [44]. While it is unclear what
305 carbohydrates these genera might be degrading in the guts of whale calves, previous
306 studies have demonstrated that whales tend to have some similarities to herbivores in
307 terms of bacterial community structure [10].

308 Calf age largely determined the bacterial community composition of right whale
309 calves. Microbiota composition changed with growth in a breast-fed bottlenose dolphin
310 calf from birth to 5-8 months of age and was probably due to nursing [35]. Southern right
311 whale calves in the Valdés population are on average 5.5 m long at birth and grow as
312 much as three meters during their first months of life at their calving ground [18,24].
313 Differences in the bacterial community composition between young (<6 m) and older
314 calves (≥6 m) might be due to nursing, which has been demonstrated for terrestrial
315 mammals [45]. In addition, infant humans and avian chicks exhibit increases in bacterial
316 diversity during ontogeny, which converge in adult-like communities [46,47]. This shift
317 is thought to be due to incidental exposure to environmental microbes that colonize the
318 gastrointestinal tract [46]. Our study only investigated the bacterial communities of
319 southern right whale calves; further studies are needed to characterize communities of
320 adults as well.
321 The genera *Bilophila, Peptococcus*, and *Treponema* were only found in the large
322 intestine of older calves (those ≥ 6m). Small calves did not harbor any unique microbes.
323 The abundance of *Bilophila* increases in response to dietary milk fats [48], and so may be
324 more abundant in older calves due to greater milk intake. *Peptococcus* is rare in human
325 children, but more abundant in adults [49]. Many other unidentified OTUs were present
326 only in older calves, suggesting that the microbiota obtains new members as whales
327 grow.
328 Several potential pathogens were detected in the intestine of stranded southern
329 right whale calves including the genera *Mycoplasma*, *Streptococcus*, *Erysipelothrix* and
330 *Clostridium. Mycoplasma spp*. have been associated with high mortality events in marine

331 mammals, especially pinnipeds. Primary clinical diseases include pneumonia and septic
332 polyarthritis [50-52]. In our study, *Mycoplasma* was present in the intestinal content of
333 three calves. Several tissues (testis, skin, skeletal muscle, penis, kidney, liver and spleen)
334 were available for histologic examination from only one and contained no lesions,
335 suggesting this to be an incidental finding. Other cases, particularly those with
336 pneumonia, should be analyzed to discard the role of this pathogen in right whale calf
337 deaths at Valdés.
338 *Streptococcus* spp. are known to produce pneumonia and septicemia in pinnipeds
339 [16], and two cases have been reported in odontocetes, the harbour porpoise (*Phocoena*
340 *phocoena*, [53]) and the pilot whale (*Globicephala melaena*, [54]). An apparent cause of
341 death related to infections by *Streptococcus* was not evident in the dead calves analyzed
342 in our study. Some species of *Erysipelothrix* can produce infections in odontocetes and
343 mysticetes. For instance, *Erysipelothrix rhusiopathiae* can cause lethal septicemia [16,55]
344 and has been found in skin lesions of southern right whales [56]. In this study,
345 *Erysipelothrix* was almost exclusively found in calves that died in 2007, a high mortality
346 year when most calves showed unusually severe skin lesions [57]. However, few samples
347 were available for histologic examination and the role of this potential pathogen remains
348 to be evaluated. Kelp gulls (*Larus dominicanus*) at Península Valdés feed on skin and
349 blubber of living right whales opening wounds on their backs of different size and
350 severity [58]. Fiorito et al. [56] reported *E. rhusiopathiae* in one living and one dead calf
351 with particular rhomboid shaped gull-inflicted lesions. Although the origin of the
352 bacterium is unknown, it could potentially be directly transmitted by gulls, constitute

353 normal skin microbiota, or be a direct or indirect (opportunistic infection in open
354 wounds) pathogen. Our findings show that right whale guts are another potential source.
355 *Clostridium perfringens* was the only OTU identified in all samples. This
356 bacterium is found at high prevalence in healthy individuals of multiple mammalian and
357 avian species [59], and its presence in carcasses, especially those in advanced stages of
358 decomposition, is relatively common. Detection in 100% of the stranded southern right
359 whale calves examined was not unexpected. The class Clostridia is well represented
360 among three species of baleen whales, the North Atlantic right whale, the humpback
361 whale (*Megaptera novaeangliae*) and the sei whale (*Balaenoptera borealis*) [10].
362 *Clostridium perfringens* has been also found in stranded pygmy (*Kogia breviceps*) and
363 dwarf (*K. sima*) sperm whales [11]) and has been detected in healthy North Atlantic right
364 whales [10]. The presence of *C. perfringens* in all carcasses analyzed for this study may
365 be also explained by changes in the gut environment under post-mortem conditions.
366 Some microbes opportunistically dominate the gut microbiome after death due to a
367 decreased intestinal blood flow and an increased digesta retention [60,61].
368 However, *C. perfringens* can also be a primary ante-mortem pathogen that causes
369 disease in a broad variety of avian and mammalian species. This microorganism can
370 produce a range of lethal toxins [62,63], and it has been associated with disease in several
371 aquatic mammals including dolphins [64], sea otters [65], Weddell seals [66] and hooded
372 seals [67]. Other *Clostridium spp*. can also affect marine mammals. These include *C.*
373 *septicum*, which was associated with the death of two adult sperm whales (*Physeter*
374 *macrocephalus*) in Denmark [20].

375 Pathogenicity of *C. perfringens* cannot be determined simply by its presence or
376 detection [59]. In several mammalian species, pathogenicity must be confirmed based on
377 gross and microscopic lesions, coupled with the presence of pre-formed toxins in the
378 intestinal content. In several mammals, intestinal lesions caused by *C. perfringens*
379 enterotoxin are characterized by necrosis and degeneration of the superficial epithelium,
380 edema and congestion [68,69]. In whales, however, no diagnostic criteria for *C.*
381 *perfringens* infections have been established. To date (2003-2017), enteritis or colitis has
382 been identified histologically in only two stranded right whale calves at Península Valdés
383 (n=39, [18]). Intestinal content from the calf with enteritis in the current study tested
384 positive for *C. perfringens cpe* toxin gene, but was negative for toxin production. In
385 addition, toxic changes (such as mucosal necrosis that can occur secondary to clostridial
386 toxins) were not observed in intestinal samples from this or other calves, and obvious
387 inflammation was not seen in other calves. However, histology may not be the most
388 sensitive indicator of toxin production, especially in animals in moderate to advanced
389 stages of autolysis since decomposition are known to occur rapidly in gastrointestinal
390 tissues of dead mammals [70,71] and can mask subtle lesions. Pre-formed toxins were
391 not detected in any dead calf. This finding may be because for CPE production, *C.*
392 *perfringens* must sporulate in the gastrointestinal tract of the host [72]. We did not detect
393 *C. perfringens* sporulation in dead southern right whale calves.
394 We further investigated the presence of toxin genes in the microbiota samples
395 from stranded calves. All cultured isolates of *C. perfringens* were identified as type A or
396 type F. *Clostridium perfringens* type A is generally considered a commensal, non-
397 pathogenic toxinotype in the intestine of most animals [73]. In contrast, *C. perfringens*

type F producing enterotoxin, causes foodborne 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.

6. 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

421 in this study are causative agents of calf deaths at Península Valdés. Our inventory also
422 provides insight into the bacterial ecology of baleen whale calves. Further research
423 related to the functions of various microbes within the calf gut is warranted.
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732 **Table 1.** Number of dead calves studied in each category/factor.

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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

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744 **Fig. 1.** Bacterial richness in small (A) and large (B) intestine as a function of calf length.

745 Longer calves exhibit higher bacterial richness in both intestinal sections.

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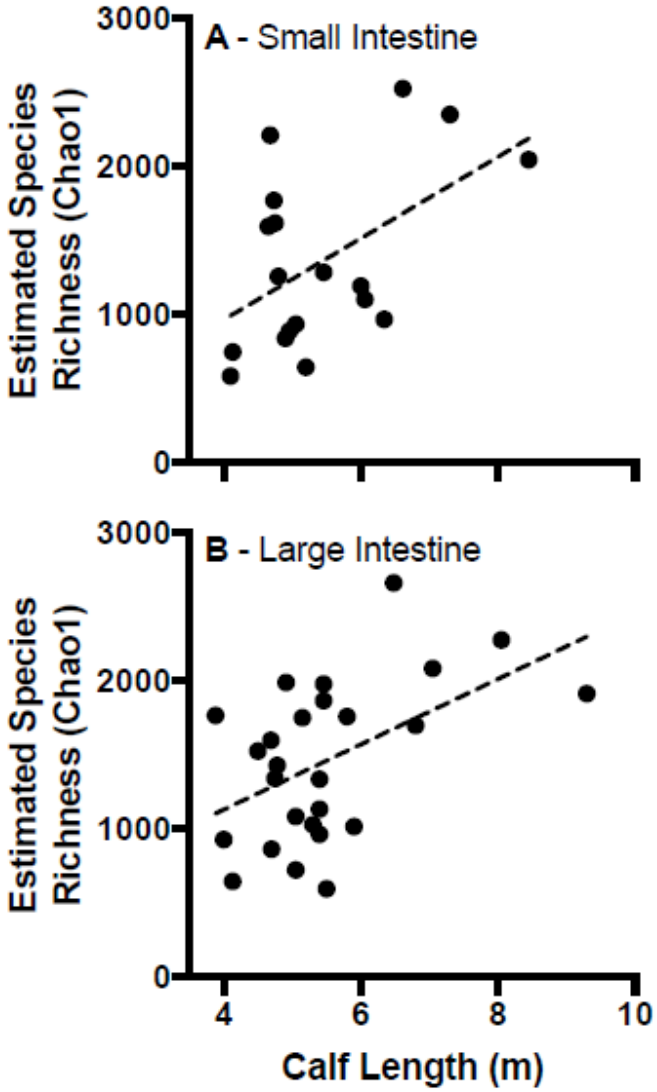

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