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Exploring the resident gut microbiota of stranded odontocetes: high similarities between two dolphin species *Tursiops truncatus* and *Stenella coeruleoalba*

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Original Article

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Abstract

The evaluation of symbiotic microbial communities occurring in the intestinal tract of animals has received great interest in recent years. However, little is known about gut microbial communities in cetaceans, despite their relevance in the ecology of marine communities. Here, we report an investigation using 16S rRNA gene amplicon sequencing of the resident gut microbiota of the two cetacean species *Stenella coeruleoalba* and *Tursiops truncatus* by sampling intestinal mucosa from specimens retrieved stranded along the Tyrrhenian coast of Tuscany (Italy). We found an abundance of members from *Clostridiaceae* and *Fusobacteriaceae*, which in total accounted for more than 50% of reads, in agreement with gut microbiota composition of other carnivorous mammals. Probably due to the limited number of samples available, sex, preservation status and also species, did not correlate with overall differences in the microbiota. Indeed, a high similarity of the taxonomic (family-level) composition between the gut microbiota of the two species was found. However, *Pedobacter* spp. was found abundant in amplicon sequencing libraries from *S. coeruleoalba*, while clostridia were more abundant from *T. truncatus* samples. Our results shed some light on the gut microbiota composition of two dolphin (*S. coeruleoalba* and *T. truncatus*) species, with specimens collected in the wild. Studies with a larger number of individuals are now needed to confirm these first results and evaluate the interspecific differences in relation to sex and age.

Introduction

The microbial communities present in the intestine of species ranging from invertebrates to humans have the attention of many investigators (Ley *et al.*, 2008*a*, 2008*b*; Round & Mazmanian, 2009; Zhu *et al.*, 2011; Huttenhower *et al.*, 2012; Keenan *et al.*, 2013; Kostic *et al.*, 2013; Mengoni *et al.*, 2013; Abdelrhman *et al.*, 2016; Bik *et al.*, 2016; Du Toit, 2016; Soverini *et al.*, 2016; Godoy-Vitorino *et al.*, 2017), in relation to their important role in their hosts' physiology and in adaptation (Kostic *et al.*, 2013). Enteric microorganisms have been revealed to contribute to the health of hosts (i.e. preventing opportunistic infections) besides contributing to other physiological functions such as mate selection, skeletal biology and lipid metabolism (Ley *et al.*, 2008*a*; Round & Mazmanian, 2009; Kostic *et al.*, 2013; Du Toit, 2016). Their involvement has been suggested in mammalian evolutionary radiation, allowing hosts to adapt to a wide range of dietary niches, due to extensive microbial metabolic capabilities (Ley *et al.*, 2008*a*; McFall-Ngai *et al.*, 2013; Nelson *et al.*, 2013); dietary regime has been indicated as the most decisive factor for the symbiotic gut microbiota composition (Ley *et al.*, 2008*a*, 2008*b*; Muegge *et al.*, 2011; Abdelrhman *et al.*, 2017*a*, 2017*b*). However, other parameters, such as environment, phylogeny and gut morphology, may in influence the intestinal microbiota (Langer, 2001; Ley *et al.*, 2008*a*, 2008*b*; Muegge *et al.*, 2011). For instance, in baleen whales, similarities with the gut microbiota of related terrestrial herbivores sharing a multichambered foregut have been revealed (Sanders *et al.*, 2015).

Investigation of the intestinal microbial content of threatened or vulnerable species may constitute an effective monitoring instrument that could reveal the presence of pathogenic microorganisms and indicate environmental health status (see Godoy-Vitorino *et al.*, 2017). Marine cetaceans are considered particularly sensitive to stressors present in their environment and several species have suffered drastic die-offs in the last decades due to human pressures. For example, both the striped dolphin *Stenella coeruleoalba* (Meyen, 1833) (http://www.iucnredlist.org/details/16674437/0) and the bottlenose dolphin *Tursiops truncatus* (Montagu, 1821) (http://www.iucnredlist.org/details/16674437/0) have been subjected to anthropogenic threats.

Studies on the gut microbiota communities of marine mammals have been performed (Nelson *et al.*, 2013) and research on whales, some dolphin and porpoise species is in the literature (see for instance, Wan *et al.*, 2018; Kim *et al.*, 2019; Miller *et al.*, 2020, Robles-Malagamba *et al.*, 2020). These studies showed a dominance of members from the

Table 1. Samples description and alpha diversity of gut microbiotas

	Species	Sex	State of preservation	Richness (no. of OTUs)	Simpson (1-D)	Shannon diversity	Evenness
RT104Sc	*S. coeruleoalba*	F	4	67	0.214	2.363	0.159
RT105Sc	*S. coeruleoalba*	F	2	78	0.117	2.778	0.206
RT102Sc	*S. coeruleoalba*	F	3	36	0.151	2.497	0.337
RT106Sc	*S. coeruleoalba*	F	2	102	0.099	3.020	0.201
RT107Sc	*S. coeruleoalba*	M	4	93	0.098	3.043	0.226
RT_Monterosso	*S. coeruleoalba*	F	4	57	0.181	2.440	0.201
Mean diversity *S. coerueoalba*				72 ± 24	0.14 ± 0.05	2.7 ± 0.3	0.22 ± 0.06
RT98Tt	*T. truncatus*	M	3	50	0.190	2.427	0.227
RT_tbd	*T. truncatus*	F	2	68	0.054	3.338	0.414
RT110Tt	*T. truncatus*	F	4	17	0.648	0.841	0.136
RT111Tt	*T. truncatus*	M	4	39	0.144	2.483	0.307
RT112Tt	*T. truncatus*	M	4	38	0.181	2.266	0.254
RT82Tt	*T. truncatus*	F	2	37	0.0917	2.822	0.454
Mean diversity *T. truncatus*				41 ± 17	0.22 ± 0.21	2.4 ± 0.8	0.30 ± 0.11

F, female; M, male. Preservation status of individuals on scale of 2–5 in accordance with standard guidelines for stranded cetaceans (Geraci & Lounsbury, 2005), see text for details. Shannon and Simpson diversity was computed using 'diversity' function of the vegan package whereas Evenness was computed using Pielou's formula J = H/log(S); where H is the Shannon index and S is the number of OTUs with abundance higher than 0. Mean values (±SD) for the two species are reported.

phylum *Firmicutes* and for marine carnivores a high representation of *Fusobacteria* (Nelson *et al.*, 2013). Within the phylum *Proteobacteria*, *Gammaproteobacteria* were particularly abundant, including members of the genus *Halomonas* which are thought to play a role in their hosts' digestive and immune systems (Wan *et al.*, 2018). For the dolphin species *T. truncatus* and *S. coeruleoalba* few reports are present, and mainly the enteric microbial composition of individuals maintained in captivity has been investigated (Soverini *et al.*, 2016; Suzuki *et al.*, 2019). Recently, analysis of swabs taken from different body parts of the same species has been reported (Robles-Malagamba *et al.*, 2020). In a few cases the gut microbiota from wild dolphins has been investigated (Bik *et al.*, 2016; Godoy-Vitorino *et al.*, 2017). These reports showed that the gut microbiota of animals from natural habitats is different from that of animals kept in captivity. However, analyses on wild animals are difficult and only very recently have data on wild animals been published (Robles-Malagamba *et al.*, 2020). Mainly stranded (in most case dead) animals are analysed, questioning the relevance of such results for inferring the gut microbiota in normal conditions (Godoy-Vitorino *et al.*, 2017).

The aim of this work was to characterize the associated gut microbiota from wild individuals of *S. coeruleoalba* and *T. truncatus* stranded along the coast of the Tyrrhenian Sea (Tuscany, Italy), to shed, with the precaution due to sampling stranded animals, some more light on the gut microbiota in wild conditions for such dolphin species.

Materials and methods

Sampling and sequence production

Sections of intestine (colon) of *S. coeruleoalba* and *T. truncatus* were removed from adult individuals found stranded along the Tyrrhenian coast in the Tuscany region (Italy) and collected in the years 2014–2017 in centres associated with the network of Tuscan Observatory for Biodiversity (OTC centres of the Regione Toscana). Colon sections were surgically taken in ARPAT (Agenzia Regionale per la Protezione Ambientale della Toscana, Livorno, Italy) premises, immediately after delivery of stranded animals, under sterile conditions. For each animal, three samples of ∼5 cm in length of intestine were taken and pooled in a composite sample, representing the single animal (thereafter defined as 'specimen'). A total of 12 specimens (six belonging to *S. coeruleoalba* and six to *T truncatus*) were sampled immediately after their retrieval (Table 1). For each individual the sex (M or F) and preservation status were registered in order to assess the eventual independence of the gut microbial composition of these parameters. The preservation status of individuals was evaluated in accordance with standard guidelines for stranded cetaceans (Geraci & Lounsbury, 2005) by assigning to each carcass a numeric value ranging from 2 to 5 (2: fresh carcass, <24 h after death, normal appearance, minimal external changes, no odour, minimum dehydration and wrinkling of skin, eyes, membranes and mucous membranes, normal eyes, no swelling carcass, not protruded tongue and penis; 3: carcass in moderate decomposition, intact carcass, evident bulge, protruded tongue and penis, desquamated skin, delicate odour, still wet membranes and mucous membranes, sunken eyes; 4: carcass in an advanced state of decomposition, the carcass can be intact, but more frequently collapsed; desquamated skin, smell strong and unpleasant, altered internal organs, sunken or missing eyes; 5: mummified carcass or skeleton remains, carcass often dried with dehydrated skin stretched over the bones, often missing organs).

All samples immediately after collection were stored at −20°C until the extraction of DNA (which was done within 1 month from the collection).

DNA was extracted from 500 mg of homogenized gut tissues using the FastDNA™ SPIN Kit for soil (MP Biomedicals, Italy). From the extracted DNA, the bacterial V4 region of 16S rRNA genes was amplified with specific primers (515F: 5′-GTG CCAGCMGCCGCGGTAA-3′, 806R: 5′-GACTACHVGGGTA TCTAATCC-3′, Klindworth *et al.*, 2013) in a 25 µl total volume with KAPA HiFi HotStart ReadyMix, 1 µM each primer with 25 cycles with the following temperature profile: 30 s 95°C, 30 s

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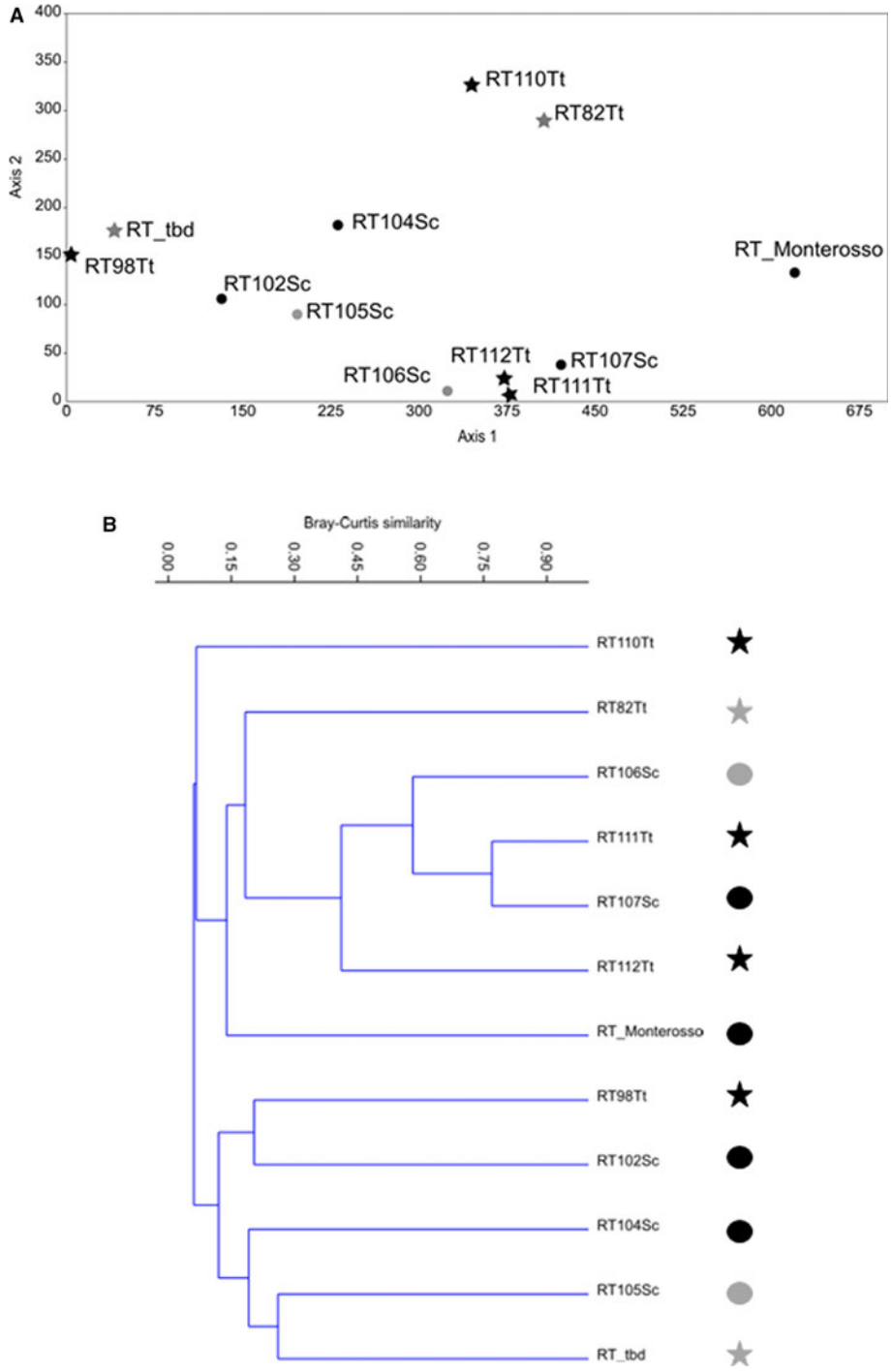

Fig. 1. Similarities among microbiota. (A) Detrended correspondence analysis (DCA). (B) UPGMA clustering based on Bray–Curtis dissimilarity matrices produced from OTU tables of partial 16S rRNA gene amplicon libraries from DNA extracted from gut sections of the dolphins *T. truncatus* (stars) and *S. coeruleoalba* (dots). Preservation status is displayed as black (3 and 4) and grey (2). No grouping according to either species or preservation status is present.

55°C, 30 s 72°C, as previously reported (Abdelrhman *et al.*, 2016). PCR products were sequenced in a single run using Illumina MiSeq technology with pair-end sequencing strategy and a MiSeq Reagent Kit v3 (Illumina, USA). Library preparation (Nextera XT, Illumina, USA) and demultiplexing were performed following Illumina's standard pipeline as previously reported (Abdelrhman *et al.*, 2017*a*).

Raw data processing and statistical analyses

Illumina sequences were clustered into Operational Taxonomic Units (OTUs) following the classical UPARSE pipeline (Edgar, 2013) as previously described in Abdelrhman *et al.* (2017*a*). Sequences were pre-processed with StreamingTrim (Bacci *et al.*, 2014) in order to remove low quality nucleotides which might interfere with downstream analysis. PANDAseq assembler (Masella *et al.*, 2012) was used for merging paired-reads into full-amplicon sequences. Singletons, namely sequences found only one time in all samples, were removed before the OTU clustering step that was performed using an identity threshold of 97% in UPARSE ('cluster_otus' command). Putative chimeric sequences were removed during the clustering step by UPARSE and no additional removal was conducted. A single representative sequence has been chosen from each cluster and taxonomically annotated using the SINA standalone classifier in combination with the 'Ref NR 99' database (Pruesse *et al.*, 2012). All steps were implemented with an in-house pipeline available at https://github.com/GiBacci/o2tab. Rarefaction analysis was carried out with

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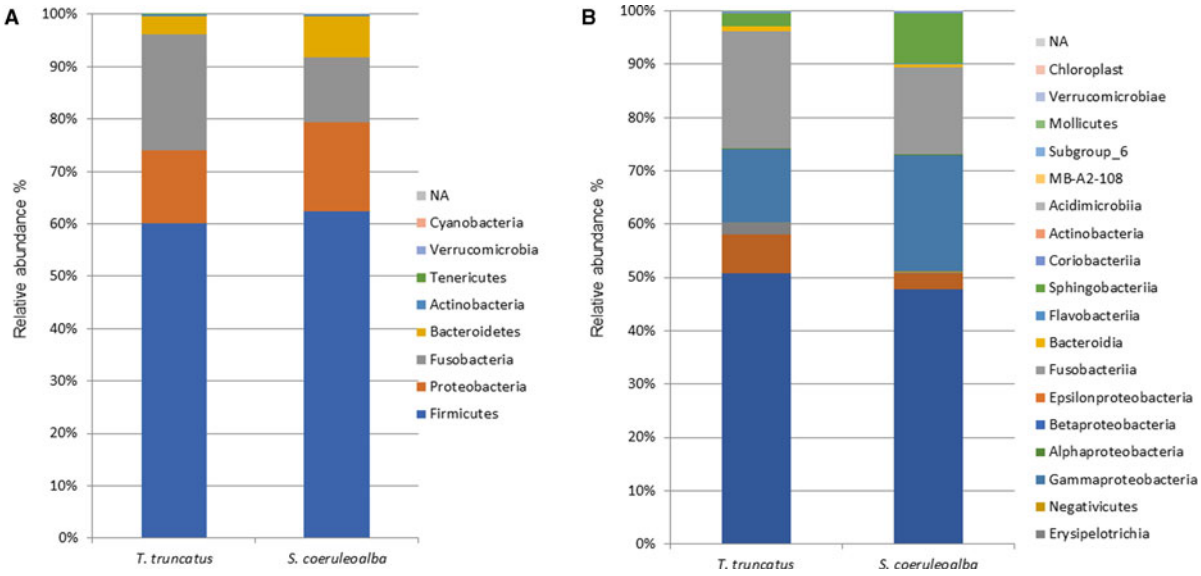

Fig. 2. Relative abundance of reads assigned at phylum (A) and class (B) levels. in partial 16S rRNA gene amplicon libraries from DNA extracted from gut sections of the dolphins *T. truncatus* and *S. coeruleoalba.* Colours in the legend indicate taxa order (from top to bottom in the plots). NA, not assigned. Firmicutes was the most abundant phylum, Clostridia the most abundant class.

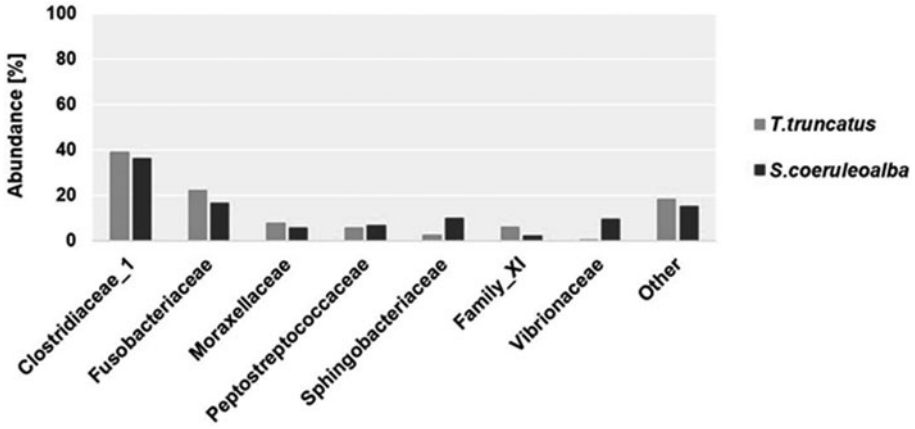

Fig. 3. Taxonomic composition at the family level of the gut microbiota of *S. coeruleoalba* (black bars) and *T. truncatus* (grey bars). The occurrence of discrete taxa is quoted as percentage abundance. Only families contributing to at least 5% of the total microbial communities in one of either species are reported.

SILVAngs (https://ngs.arb-silva.de/) (Supplementary Table S1). Good's estimator (Good, 1953) was used to calculate the percentage of coverage.

In order to inspect eventual differences in the OTUs distribution between *T. truncatus* and *S. coeruleoalba*, a Detrended Correspondence Analysis (DCA) was performed using the R package 'phyloseq' (McMurdie & Holmes, 2013). Moreover, further investigations were conducted to assess whether sex and preservation status affected the OTUs distribution by means of a PERMANOVA as implemented in R (Hoffman & Schadt, 2016) and LDA Effect Size (LEfSe) (Segata *et al.*, 2011). Specific differences in community composition were determined using similarity percentage (SIMPER) analysis as done in Past 3 software (Hammer *et al.*, 2001).

Links to deposited data

The sequences dataset was deposited in the SRA database under the BioProject PRJNA473403.

Results

The sequencing of 16S rRNA genes yielded a total of 7483–64,067 reads per sample (Supplementary Table S1). Rarefaction analyses (Supplementary Figure S1) showed that most samples were satisfactorily sampled (Good's coverage 99.99 ± 0.01). All samples were used for the following analyses.

After assigning reads to Operating Taxonomic Units (OTUs, 97% sequence similarity) and removing OTUs not assigned or assigned to Eukaryotes, a total number of 270 OTUs assigned to the bacterial taxonomy were detected (Supplementary Table S2). The OTU matrix was very sparse, with many OTUs represented in a few specimens only, indicating a high heterogeneity of OTUs abundance among specimens. The number of OTUs per specimen (Table 1) ranged from 17 to 102 and were significantly different between *S. coeruleoalba* and *T. truncatus* (Table 1) (*t*-test $P < 0.05$). The other diversity indices were not different between species. No significant differences with respect to sex or preservation status were found.

DCA and UPGMA clustering revealed a general lack of separation of OTUs representation between *T. truncatus* and *S. coeruleoalba* (Figure 1). Indeed, either a variance partition or a PERMANOVA analysis did not find significant differences in relation to species and on the whole dataset in relation to preservation status, but a slightly significant effect of preservation status on *S. coeruleoalba* was found (Supplementary Table S3). An LDA Effect Size (LEfSe) (Segata *et al.*, 2011) confirmed a lack of significant separation between species and among preservation statuses

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Table 2. SIMPER analysis on genera representation. The differences with respect to conservation status on gut microbiotas were inspected separately for *S. coeruleoalba* (a) and *T. truncatus* (b). In (c) the differences between *S. coeruleoalba* and *T. truncatus* considering samples in good conservation status only are reported

Taxon	Average dissimilarity	Contribution (%)	Cumulative (%)	Mean 3 + 4	Mean 2
(a) *S. coeruleoalba*					
Pedobacter	12.86	15.41	15.41	0	5.62×10^3
Photobacterium	9.15	10.96	26.36	117	4.01×10^3
Paeniclostridium	5.86	7.025	33.39	278	2.63×10^3
Clostridium_sensu_stricto_1	5.24	6.28	39.66	572	2.77×10^3
Cetobacterium	5.02	6.01	45.68	1.39×10^3	2.34×10^3
Clostridium_sensu_stricto_7	4.28	5.13	50.81	1.90×10^3	478
Clostridium_sensu_stricto_15	4.21	5.05	55.86	2.18×10^3	0
Clostridium_sensu_stricto_1	3.18	3.73	59.59	637	1.38×10^3
Clostridium_sensu_stricto_11	2.09	2.50	62.09	994	16
Photobacterium	1.81	2.17	64.27	11.3	774
Taxon	Average dissimilarity	Contribution (%)	Cumulative (%)	Mean 2	Mean 3 + 4
(b) *T. truncatus*					
Clostridium_sensu_stricto_1	11.63	12.71	12.71	150	3.29×10^3
Cetobacterium	7.44	8.13	20.83	691	1.94×10^3
Fusobacterium	4.54	4.96	25.79	987	1.25
Genus not assigned, Family XI Clostridiales	4.29	4.68	30.47	1.19×10^3	2.75
Peptostreptococcus	3.57	3.90	34.37	880	3.5
Psychrobacter	3.22	3.52	37.89	117	615
Clostridium_sensu_stricto_1	3.21	3.50	41.40	0	908
Fusobacterium	3.16	3.45	44.84	703	30.5
Clostridium_sensu_stricto_11	2.39	2.60	47.45	0	481
Cetobacterium	2.34	2.56	50.01	105	600
Taxon	Average dissimilarity	Contribution (%)	Cumulative (%)	Mean *T. truncatus*	Mean *S. coeruleoalba*
(c) *T. truncatus vs S. coeruleoalba*					
Clostridium_sensu_stricto_1	10.79	11.99	11.99	3.16×10^3	390
Pedobacter	6.71	7.45	19.44	0	2.81×10^3
Clostridium_sensu_stricto_7	6.57	7.31	26.75	0	1.95×10^3
Photobacterium	5.43	6.03	32.78	15	2.11×10^3
Paeniclostridium	3.85	4.28	37.06	311	1.50×10^3
Cetobacterium	3.62	4.02	41.08	409	1.18×10^3

(*Continued*)

Table 2. (*Continued.*)

Taxon	Average dissimilarity	Contribution (%)	Cumulative (%)	Mean 3 + 4	Mean 2
Clostridium_sensu_stricto_1	3.46	3.84	44.92	56.3	1.41×10^3
Psychrobacter	3.44	3.82	48.75	673	389
Clostridium_sensu_stricto_11	2.80	3.11	51.86	111	749
Clostridium_sensu_stricto_11	2.35	2.61	54.47	481	22

Top 10 bacterial genera are reported (Supplementary Table S2). The average dissimilarity (Bray–Curtis) and the percentage of contribution to variance is reported for the contrasts between individuals with good preservation status (status = 2) and bad preservation status (status 3 and 4) (a and b). See Table 1 for specifications on preservation status. In (c) only individual with preservation status = 2 are considered.

(data not shown). This could be due to the lack of sharing of the highly abundant OTUs (Supplementary Figure S2).

On the overall dataset, taxonomic compositions of the two species are very similar to each other. At the level of phylum, Firmicutes (mean relative abundance 60–62%), Proteobacteria (14–17%) and Fusobacteria (12–22%) dominated the gut microbiota ecosystem of both *T. truncatus* and *S. coeruleoalba* (Figure 2). Clostridia accounted for nearly 50% at the class level. The dominant families (Figure 3) were *Clostridiaceae 1* (36.0% in *S. coeruleoalba* and 38.7% in *T. truncatus*) and *Fusobacteriaceae* (16.2% in *S. coeruleoalba* and 21.9% in *T. truncatus*) contributing in total to the 52.2% and the 60.6% of the gut microbial ecosystem of *S. coeruleoalba* and *T. truncatus* respectively. Other mainly representative families are *Sphingobacteriaceae* (9.5%) and *Vibrionaceae* (9.4%) in *S. coeruleoalba* and *Moraxellaceae* (7.6%) and *Family XI* (5.6%) in *T. truncatus*. When considering *T. truncatus* and *S. coeruleoalba* datasets separately, a differential abundance of taxa (meaning relative abundance in amplicon sequencing libraries) was found in relation to a comparison between good and bad preservation statuses (2 *vs* 3 + 4). Indeed, under SIMPER analysis samples of *S. coeruleoalba* with good preservation status were more abundant in members of *Pedobacter*, *Photobacterium* and *Paeniclostridium*, while poor preservation status samples had higher numbers of clostridia (Table 2). Similar differential abundance of clostridia associated with samples with poor preservation status was found for *T. truncatus* also (Table 2). The same approach was used to inspect possible differential occurrence of taxa between *T. truncatus* and *S. coeruleoalba* individuals in good preservation status (Table 2). Most of the differences were due to clostridia (with two groups being more abundant in *T. truncatus* and *S. coeruleoalba*, respectively), *Pedobacter*, *Photobacterium* and *Paeniclostridium*. However, due to the extremely limited number of samples, these results should be treated with great caution.

Discussion

Results obtained in our work pointed out high similarities in the gut microbial composition between the cetacean species *T. truncatus* and *S. coeruleoalba*. The slight (not statistically significant) differences between the two species could be due to the diversity in their prey consumption, putatively reflecting different metabolic necessities. Indeed, it has been shown that although both cetaceans exhibit a piscivorous dietary regime, they feed on prey partially different in relation to their discrete sea habitats (see Scuderi *et al.*, 2011). However, the gut microbiota taxonomic composition revealed in our *T. truncatus* specimens partially differs from that identified in previous works (Bik *et al.*, 2016; Soverini *et al.*, 2016). Besides bacterial taxa typically occurring in the gut of carnivorous species (i.e. *Clostridiaceae*, *Fusobacteriaceae* and *Peptostreptococcaceae*) (Nelson *et al.*, 2013) that were found in our investigations, previous works on animals in captivity detected a higher presence of members of *Staphylococcaceae* and *Lactobacillaceae* (Soverini *et al.*, 2016) (here 13% *vs* <5%, see Supplementary Table S2). Conversely, in a recent analysis on the gut microbiota of captive *T. truncatus* in aquaria in Japan (Suzuki *et al.*, 2019), abundance of *Fusobacteriaceae*, *Peptostreptococcaceae* and *Vibrionaceae* was found as in our analysis, reflecting the typical taxonomic composition of carnivorous species in the wild. However, the discordance between studies from animals in aquaria suggests that local dietary supplements/condition may bias the estimates on normal gut microbiota composition (as for instance in individuals analysed in Bik *et al.*, 2016; Soverini *et al.*, 2016). Recently, in agreement with our report, analyses of faecal samples from free *T. truncatus* (Robles-Malagamba *et al.*, 2020) found a high abundance of Firmicutes and Fusobacteria, suggesting that our samples may

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give a realistic representation of the gut microbiota of free-ranging animals. Of course, we cannot a priori exclude that the preservation status of samples may have biased our results, in particular in relation to the number of clostridia. Biases among studies can in theory be due to the use of different procedures in DNA extraction and bioinformatic analyses, possibly limiting the comparison. However, the taxonomic level of analysis we have chosen (family) strongly limits any bias in terms of single species/genus representation.

Unfortunately, regarding our findings on *S. coeruleoalba*, no comparison can be made with previous works since the sole study focused on the characterization of the gut microbiome of this species considered only one specimen (Godoy-Vitorino *et al.*, 2017). In that work colon microbiota were rich in Firmicutes, Fusobacteria and Proteobacteria, which is in agreement with our results. However, at genera level few clostridia were found by those authors on their single animal. However, we may expect that the same impact on the gut microbiota of captivity conditions would be present in this species also. Contrarily to *T. truncatus*, in *S. coeruleoalba* some differences in relation to preservation status were found, mainly in a possibly higher abundance in clostridia for samples with poor preservation status (as would be expected due to anaerobic digestion of carcasses) and in Bacteroidetes (*Pedobacter* spp.). Moreover, *Pedobacter* spp. was found exclusively in *S. coeruleoalba*. The presence of members of this latter genus may deserve further attention in relation to a reservoir of antibiotic resistant strains in *S. coeruleoalba* gut. In fact, *Pedobacter* has been claimed as a superbugs genus since species in this group are intrinsically resistant to several classes of antibiotics, including colistin (Viana *et al.*, 2018).

Overall, our results could not confirm the supposition advanced by Sanders *et al.* (2015) on the importance of phylogeny on the microbial gut communities of mammalians. Indeed, our findings may suggest that the dolphin gut microbiota could be similar to that of other carnivorous (phylogenetically unrelated) mammals (Bik *et al.*, 2016), which could lead to an hypothesis on the influence of diet (carnivorous) on the taxonomic shaping of the gut microbiota. However, since there is a limited number of samples this point deserves more attention in future sampling and a careful evaluation of biases inherent to the preservation status (such as the massive presence of clostridia).

In conclusion, our work shed light on the gut microbiota composition of wild animals of *T. truncatus* and *S. coeruleoalba*, by analysing 12 stranded individuals, with different preservation status. We emphasize here the importance of a careful recording of preservation status of stranded animals, as well as the availability of specimens from a relatively high number of animals to provide reliable estimates of the gut microbiota composition of marine mammals in the wild.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0025315420000983.

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