Current Research in Microbial Sciences 8 (2025) 100349

Contents lists available at ScienceDirect

Current Research in Microbial Sciences

journal homepage: www.sciencedirect.com/journal/current-research-in-microbial-sciences

Genetic diversity of single-celled microorganism *Blastocystis* sp. and its associated gut microbiome in free-ranging marine mammals from North-Western Mediterranean Sea

Marianna Marangi [a,*], Sonia Boughattas [b]

[a] Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Viale Luigi Pinto, 71122, Italy
[b] Biomedical Research Center, Qatar University, Doha, Qatar

ARTICLE INFO

Keywords:
Single-celled microorganisms
Marine mammals
Mediterranean Sea
Gut microbiome
Zoonosis
Antropic activities
One Health approach

ABSTRACT

Blastocystis sp. is frequently identified in humans and several animal hosts exhibiting a wide genetic diversity. Within One Health perspective, data on *Blastocystis* sp. distribution and its circulating subtypes (STs) from the terrestrial environment are available, while those from the marine environment remain still scare. A genetic and 16S rRNA gene sequencing analysis were conducted over the period 2022–2024 by screening fecal samples from four different species of free-ranging marine mammals (sperm, fin, long-finned pilot and Cuvier's beaked whales) circulating within North-Western Mediterranean Sea. 10 out of 43 fecal samples (23.2 %) were found positive to *Blastocystis* sp. using molecular tools. A predominance of zoonotic subtype ST3 among different species of marine mammals as well as the presence of ST1 allele 4 subtype and even untypable subtype within the fin whale specimen was reported. Moreover, Firmicutes, Bacteroidetes and Proteobacteria within the different *Blastocystis*-carrier marine mammal species as well the identification of Archaebacteria from Methanomethylophilaceae family within the fin whale isolate were detected by Illumina V3-V4 generated data. The present survey presents new insights regarding *Blastocystis* sp. prevalence and its circulating zoonotic ST1-ST3 subtypes from the marine environment, as well as its associated gut microbiome, providing hence baseline data for a better understanding of the associated risk and to prevent human and marine ecosystem exposure to these anthropogenic microorganisms.

1. Introduction

Blastocystis sp. is an enteric single-celled microorganism transmitted by oral-fecal route with a worldwide distribution (Guard, 2024). It is frequently identified in humans (Ning et al., 2020; Nemati et al., 2021; Rauff et al., 2021; Jimenez et al., 2023) and in a wide range of animal hosts, including non-human primates (NHPs) and other mammals (Deng et al., 2019; 2021) such as artiodactyls, perissodactyls, proboscideans, rodents, and marsupials, as well as birds, reptiles, amphibians, fish, annelids, and insects (Cian et al., 2017; Gantois et al., 2020; Hublin et al., 2021). In human, its prevalence reaches an average of 20 % in industrialized countries (Matovelle et al., 2022) and can largely exceed 50 % in developing countries where fecal peril represents a major risk associated with poor sanitary conditions, hygiene practices and low-quality drinking water and food (Alfellani et al., 2013; Safadi et al., 2014). Although its pathogenic potential and clinical significance remain controversial (Cao et al., 2024), several recent in vitro studies have clearly demonstrated the impact of this microorganism on the intestinal epithelium of the host, underlining various virulence factors and mechanisms potentially involved in its pathogenesis (Liao et al., 2023), likely due to subtypes variation and intestinal environments (Deng et al., 2022; 2023).

Such diversity of pathology was suggested to be linked to the protozoan genetic diversity (Matovelle et al., 2024). Indeed, based on the analysis of the polymorphisms within the small subunit ribosomal RNA-encoding SSU-rDNA gene, a large genetic diversity has been identified with at least 44 subtypes (STs) reported among different human and animal hosts (Villalobos et al., 2024), associated with different pathogenicity. Subtypes from ST1 to ST9 and ST12 have been mainly reported in human samples, with ST3 being the most frequent subtype followed by ST1, ST2 and ST4 which can be explained in large part by human-to-human fecal transmission (Jimenez et al., 2019;

* Corresponding author at: Department of Clinical and Experimental Medicine, University of Foggia, Viale Luigi Pinto, Foggia 71122, Italy.
E-mail address: marianna.marangi@unifg.it (M. Marangi).

https://doi.org/10.1016/j.crmicr.2025.100349

Available online 22 January 2025

Popruk et al., 2021; Marangi et al., 2023). The remaining STs, less frequently found in humans, colonize animal groups such as i.e. pigs with ST5, birds with both ST6 and ST7, with these hosts representing potential reservoirs of zoonotic transmission (Tantrawatpan et al., 2023) in addition to the 8 so-called non-mammalian and avian STs (NMASTs), identified within amphibians, reptiles and insect hosts (Gaintos et al., 2020). Moreover, although its impact on the microbiome is still being debated, *Blastocystis* sp. is frequently associated with imbalanced bacterial diversity and significant alterations within the gut microbiome (Aykur et al., 2024; Marangi et al., 2024). For instance, ST1 and ST4 are linked to improved gut bacterial diversity, whereas ST7 exacerbates colitis and decreases bacterial diversity (Deng et al., 2023b). ST4 also demonstrated effects on the HT-29 cell line when co-incubated with gut microbes (Deng and Tan, 2022).

Due to the risk of zoonotic transmission and its importance for the human health, within the One Health concept, the prevalence and STs distribution of *Blastocystis* sp. were investigated in numerous surveys focused on various animal groups mainly from the terrestrial environment (Naguib et al., 2023), but few data are available on animals living within the marine environment. In this context, marine mammals, and in particular cetaceans, due to their long lifespans, global distribution in both coastal and offshore waters, migratory patterns and their ecological role in the marine food web (Bossart 2011; Marangi et al., 2022), can serve as potential reservoir of enteric zoonotic microorganisms. In a study carried out on stranded marine mammals including cetaceans (common porpoise and sperm whales) and pinnipeds (common seal), *Blastocystis* sp. was reported to have a prevalence of 13.8 % (Gantois et al., 2020). Moreover, *Blastocystis* sp. has been microscopically and molecularly detected previously in free ranging individuals of two fin and one sperm whales circulating from Pelagos Sanctuary, North-Western Mediterranean Sea (Marangi et al. 2021).

Different aims of the current work were hence carried out. First, to add data on the distribution of *Blastocystis* sp., the genetic diversity and its subtypes within the marine ecosystem, taking into consideration four different species of free-ranging and alive marine mammals. In second, to investigate the potential zoonotic transmission by a detailed genetic and phylogenetic analysis with the *Blastocysts* subtype sequences available in literature. Third, to analyze the bacterial diversity related to *Blastocystis* sp. by marine mammals' gut microbiome community profile analysis and try to associate it with *Blastocystis* subtypes since some STs e.g. ST1 were even associated with beneficial effect on host health.

2. Material and methods

2.1. Study area

In the framework of a research project on the ecology of marine mammals spanning over the period 2022–2024, fecal samples of 24 individuals of free-ranging sperm (*Physeter macrocephalus)*, 12 individuals of fin (*Balaenoptera physalus)*, 6 individuals of long finned pilot

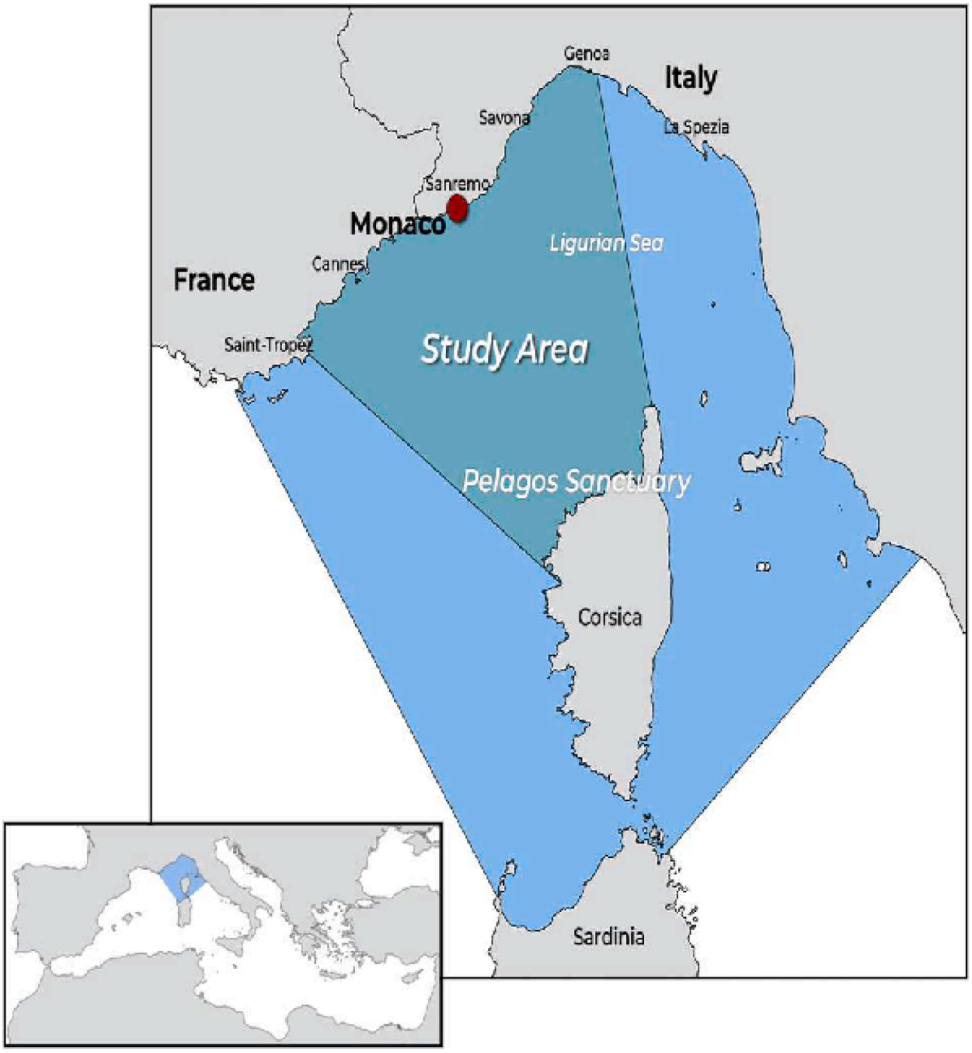


Fig. 1. Marine mammals species fecal sample collection study area in the context of Pelagos Sanctuary, Ligurian Sea, North-Western Mediterranean Sea.

(*Globicephala melas)* and 1 individual of Cuvier's Beaked (*Ziphius cavirostris*) whales were collected by Research Tethys Institute within the Pelagos Sanctuary, Ligurian Sea, North-Western Mediterranean Sea (Fig. 1 and Supplementary Fig. 1 (S1 a,b,c,d).

2.2. Sample collection

During summer boat surveys, photo identification data and floating feces were collected from individual whales using a fine nylon mesh net, avoiding direct contact with animals and any disturbance as reported and detailed in Marangi et al. (2021). The survey data collected, including photo identification, guaranteed that the sampled animals were unique and sampled only once during the monitoring. Fecal samples were immediately placed in sterile falcon, labelled for whale identification, refrigerated at +5 °C and transferred to the laboratory for future molecular and genetic investigations.

2.3. DNA extraction and single-celled microorganisms PCR amplification

The fecal samples (200 mg) were subjected to DNA extraction using MagCore® Nucleic Acid Extraction Kit (RBC Bioscience, Taiwan) and subsequently processed with the Allplex™ GI-Parasite Assay Real Time (Seegene Inc. Seoul, Korea) in order to detect and identify enteric protozoan microorganisms in accordance with the manufacturer's protocol. All the DNA samples were stored at -20 °C until further molecular investigations. Subsequently, all the DNA samples found positive to *Blastocystis* sp. by the Real Time assay were subjected to *Blastocystis* sp. conventional barcoding PCR and sequencing to identify the subtypes. Primers RD5 (5′- ATC TGG TTG ATC CTG CCA GT-3′) and BhRDr (5′-GAG CTT TTT AAC TGC AAC AACG-3′) were used to amplify approximately a fragment of 600 base pairs within the 1800 bp of *Blastocystis* SSU-rDNA gene (Scicluna et al., 2006) following the PCR and purification protocols previously described (Gazzonis et al., 2019).

2.4. Sequencing and phylogenetic analysis

Purified amplicons were directly sequenced in both directions using the ABI PRIMS Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) with the same primers as the respective PCR reaction, in accordance with the manufacturer's instructions. Obtained sequences were determined on the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA) and the chromatograms were inspected by eye using the FinchTV software v1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com).

Once the sequences had been cleaned up, primers trimmed, bad-quality regions removed, each sequence was compared with all the *Blastocystis* sp. homologous nucleotide sequences and isolates available in GenBank databases using the Blast tool (https://blast.ncbi.nlm.nih.gov). The collected sequences corresponding to *Blastocystis* SSU-rDNA gene portion were gathered in a "fasta" file and aligned with reference *Blastocystis* sp. subtypes sequences retrieved from http://entamoeba.lshtm.ac.uk/blastorefseqs.htm using MAFFT software (Kuraku et al., 2013). Maximum Likelihood Phylogenetic Analysis was achieved according to partitions and optimal substitution models identified by the BIC and AIC metrics with 1000 bootstrap replicates as implemented in MEGA X v10.0.5 (Kumar et al., 2018). Moreover, in order to assign the subtype alleles, each sequence has been submitted to a *Blastocystis* sp. public databases for molecular typing and microbial genome diversity (https://pubmlst.org/organisms/blastocystis-spp). The STs sequences generated within this study were submitted to GenBank under Accession Numbers PQ423749-PQ423758.

2.5. 16S rRNA gene sequencing library preparation

Library preparation was performed following the Illumina 16S rRNA gene Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA). The V3–V4 hypervariable regions of the 16S rRNA gene were PCR amplified in a 50 μL final volume containing 25 ng of microbial DNA, 2 × KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland) and 200 nmol/L of 341F (5′- CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) primers with Illumina adapter overhang sequences added (Klindworth et al., 2013). The PCR thermocycle consisted of 3 min at 95 °C, 25 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, and a final 5-min extension step at 72 °C (Turroni et al., 2016). PCR products were then purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA). Indexed libraries were prepared by limited-cycle PCR, using Nextera technology (Illumina), and cleaned-up as described above. Libraries were quantified using the Qubit 3.0 fluorimeter (Invitrogen, Waltham, MA, USA), normalized to 4 nM and pooled. The sample pool was denatured with 0.2 N NaOH and diluted to a final concentration of 4.5 pM with a 20 % PhiX control. Sequencing was performed on an Illumina MiSeq platform using a 2 × 250 bp paired-end protocol, according to the manufacturer's instructions.

2.6. 16S rRNA gene analysis

The raw sequences of *Blastocystis* sp. carrier samples were processed separately, according to the study of origin, using EasyMap pipeline incorporating modules from Quantitative Insights Into Microbial Ecology 2 (QIIME2), Linear Discriminant Analysis Effect Size (LefSe), and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) as described elsewhere (Hung et al., 2021). The Quality control was applied with the following parameters: maximum base number for each sequence "<1000″; maximum expected error "2″; truncated quality value "2″ and chimera filtering method "Consensus". For the taxonomic analysis and the phylogenetic tree generation, "q2-feature-classifier"; "q2-alignment" and "q2- phylogeny" modules were applied using SILVA Database. Alpha diversity was calculated as bacterial richness at the genus level using "observed OTUs", "Faith's Phylogenetic Diversity", and "Shannon index" metrics with Kruskal-Wallis test used to assess the difference distributions significance. Beta diversity was estimated by the "unweighted-unifrac", PcoA, PERMANOVA, pseudo-F ratio permutation metrics. *P*-values $\leq$ 0.05 were considered to be statistically significant.

3. Results

3.1. Single-celled microorganism PCR amplification

Overall, out of forty-three fecal samples subjected to molecular screening by qPCR, 10 (23.2 %) were found to be positive to *Blastocystis* sp. with 7 out of 24 sperm whales (29.2 %), 1 out of 12 fin whales (8.3 %) and 2 out 6 long finned pilot whales (33.3 %) infected. The only Cuvier's Beaked whale specimen was free from *Blastocystis* sp. presence. All the data were summarized in Table 1.

3.2. Sequencing and phylogenetic analysis

The 10 *Blastocystis* sp. positive samples were successfully amplified and unambiguously sequenced. Two subtypes (ST1 and ST3) were recorded frequently among the positive samples. Specifically, ST3 was identified in six sperm whale individuals (named SW1–5 and SW7) phylogenetic clustering strongly together (99 %) with the ST3 references and isolates sequences (Table 1 and Fig. 2). Such observations were supported by the phylogenetic analysis that included a dataset of 80 sequences with 56 covering the references ones, 13 from previously published marine mammals' isolates, 10 sequences generated within the current work and one sequence of *Proteromonas lacerate* used as outgroup (U37108.1) (Fig. 2).

ST1 was identified in three isolates: one sperm whale (named SW6)

Table 1
Blastocystis subtypes, GenBank sequences accession number and its BLAST similarity identified in the three different species of free-ranging *Blastocystis*-carrier marine mammals.

Number ID	Species	Subtype	GenBank Accession Number	BLAST similarity
SW1	Sperm whale isolate 1	ST3	PQ423749	MK770357.1 (100 %)
SW2	Sperm whale isolate 2	ST3	PQ423750	OR936084.1 (100 %)
SW3	Sperm whale isolate 3	ST3	PQ423751	OR936091.1 (100 %)
SW4	Sperm whale isolate 4	ST3	PQ423752	OR230226.1 (100 %)
SW5	Sperm whale isolate 5	ST3	PQ423753	OR936085.1 (100 %)
SW6	Sperm whale isolate 6	ST1	PQ423755	OR230220.1 (100 %)
SW7	Sperm whale isolate 7	ST3	PQ423754	MK770357.1 (100 %)
FW	Fin whale isolate	NMAST IV	PQ423756	OR987550.1 (94.22 %)
PW1	Long finned pilot whale isolate 1	ST1	PQ423757	OR230233.1 (100 %)
PW2	Long finned pilot whale isolate 2	ST1	PQ423758	OR230233.1 (100 %)

*SW: sperm whale; FW: fin whale; PW: long finned pilot whale.

and the two pilot whale's specimens (named PW1 and PW2). When using BLAST, exact match with Italian clinical specimens (Accession numbers: OR230233.1; OR230220.1; OR936083.1) was revealed and hence subtype them as ST1 allele 4.

Meanwhile, the only fin whale isolate (named FW) clusters separately (100 %) with other isolates identified previously within the same geographic region (Accession numbers: PQ436514, PQ436515, PQ436516) but far distant from any reference STs. Subsequently, the fin whale (Accession number: PQ423756) specimen and its closely similar sequences were re-run for a second phylogenetic analysis including the different NMAST subtypes and the previously reported untypable/unclassified *Blastocystis* sp. sequences. The Italian sequences cluster (53 %) with NMAST IV subtype and its related isolates from different tortoises and human hosts identified both within European countries (Fig. 3).

3.3. Microbiome analysis

The raw Illumina sequences of 16S rRNA gene were first demultiplexed, generating 150,664 sequence counts with the minimum count of 1447 observed within FW specimen and the maximum count of 25,202 recorded within the SW3. The sequences were then trimmed, filtered, denoised with dada2, chimeric removed, and finally merged with only those that successfully overlapped the forward and reverse strands resulting in 202 features with total frequency of 42,795. The taxonomy results revealed the presence of 15 phyla across our marine mammals' samples with the most abundant ones being Firmicutes, Bacteroidetes and Proteobacteria. Only the PW2 didn't include any Firmicutes bacteria. Meanwhile FW contained exclusively within its microbiome 5.18 % of Archaebacteria. Moreover, the phyla Patescibacteria, Planctomycetes, Acidobacteria were reported only within SW5, whereas Fusobacteria was reported solely within PW2 specimen. The microbial composition at phyla level was shown in Fig. 4.

At a lower taxonomic rank (Fig. 5), the Bacteroides included exclusively the Bacteroidia class whereas the Firmicute includes the Bacilli, Clostridia, Erysipelotrichia and Negativicutes classes followed by Gammaproteobacteria, Deltaproteobacteria, Alphaproteobacteria from the Alphaproteobacteria; Synergistia and both actinobacterium Coriobacteriia and Actinobacteria. Worth noting, the Cyanobacteria included Oxyphotobacteria and Melainabacteria classes and Tenericutes showed only Mollicutes class. However, at the species level, the majority of data was not available with a frequent uncultured bacterium result. At the genus level, uncultured Acidobacteria bacterium was the least abundant one observed only within SW5 with a relative frequency below 0.1 % whereas the Bacteriodetes Rikenellaceae RC9 gut group was the most abundant genus varing from 8.4 % within SW5 to 78.9 % within PW1 specimens with FW, PW2, SW4 totally free from it. Indeed, the microbiome of FW isolate is contituted mainly of 45.94 % of *Sarcina* spp. followed by 14.64 % of uncultred Victivallaceae, 9.234 % of Carnobacteriaceae, 9 % of Lachnospiraceae AC2044 group, 5.85 % of Oxyphotobacteria, 5.18 % of Methanomethylophilaceae, 4.27 % of *Psychrobacter*, 2.92 % of Ruminococcaceae UCG-0A4 and 2.92 % of *Enterococcus*. PW2 bacteriome is on the other hand constituted exclusively of Gram negative bacteria namely: 53.21 % of *Burkholderia-Caballeronia-Paraburkholderia*, 43.82 % of *Photobacterium*, 2.26 % of *Bacteroides* and 0.69 % of *Fusobacterium*. Meanwhile SW4 specimen exhibit a wide diversity within its microbiome profile constitueted of 40.69 % of uncultred bacterium from the Bacteroidetes F082, 18.29 % of Oxyphotobacteria, 17.65 % of uncultred bacterium from *Clostridium vadin* BB60 group, 4.10 % of *Clostridium* sensu stricto 1, 3.78 % of *Lachnoclostridium*, 3.78 % of *Ruminococcus* 2, 2.99 % of *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, 2.52 % of Clostridiales Vadin BB60 group, 1.57 % of *Enterococcus*, 0.94 % of *Sphingomonas*, 0.47 % of Erysipelotrichaceae UCG-004, and 0.47 % of Mollicutes RF39.

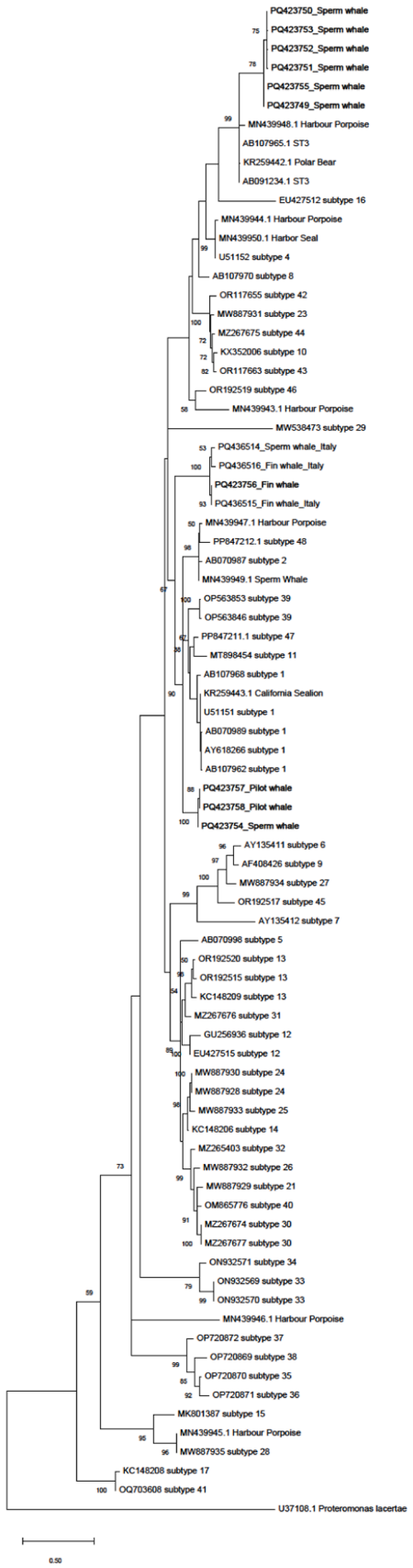
The richness of 16S rRNA gene reads of bacterial OTUs at species level within each sample was shown in Fig. 6. The alpha diversity analysis, based on the analysis on the parameters Species and Subtype was not retained significant as detailed in Table 2 and shown in Supplementary Fig. 7a, b (S7a,b). The beta diversity analysis was not considered statistically significant (data not shown).

4. Discussion

An ever-increasing number of studies on *Blastocystis* sp. prevalence, subtypes distribution and genetic diversity encompassing human and environmental ecosystems has been produced in the last ten years (Li et al., 2020; Sanggari et al., 2022; Attah et al., 2023). However, our knowledge of this single-celled microorganism within the marine environment is incomplete, and detailed genetic and phylogenetic studies are still lacking. In this work, *Blastocystis* sp. has been identified in three different species of free-ranging marine mammals with prevalence rate of 23.2 % and with a higher distribution in sperm whales (16.3 %, 7/43) compared to long finned pilot whales (4.6 %, 2/43) and fin whales (2.3 %, 1/43). The only Cuvier's Beaked whale was found negative to *Blastocystis* sp., but unfortunately, with a unique sample and due to the extremely difficult to collect samples from this species, any hypothesis can't be formulated.

The presence of *Blastocystis* sp. has been already reported in one sample of sperm whale collected from the Atlantic Northern Coast of France, although from a stranded and dead animal (Gaintos et al., 2020). The identification of this microorganism in fecal samples of our six different individuals of free-ranging sperm whales confirm what reported in a previous study (Marangi et al., 2021) and clearly shows a host-protozoan microorganism interaction advancing a potential colonization/infection of some marine mammal species by *Blastocystis* sp. This hypothesis is highly supported by the observation of this microorganism within other species of marine mammals, including the common porpoise and the common seal as reported in Gaintos et al., 2020, the fin whale (Marangi et al., 2021) and the long-finned pilot whales here detected. Therefore, to the best of our knowledge, the latter species represents a new host record and extends the known host range of this protozoan microorganism.

How *Blastocystis* sp. is transmitted to marine mammals is still unknown but most probably through the food chain which varies according to the marine mammal species from tiny zooplankton to other large



(caption on next column)

Fig. 2. The Maximum Likelihood (GTR + G + I substitution model) phylogenetic tree based on the analysis of the partial SSU-rDNA gene of *Blastocystis* isolates. Numbers next to the nodes represent posterior probabilities. Probabilities <50 % are not shown. Ten sequences from the present study (in bold) and 69 reference sequences representing *Blastocystis* subtypes (ST1-ST48) from literatures were included in the analysis for comparative purposes. *Proteromonas lacertae* was used as outgroup. Accession numbers of publicly available *Blastocystis* reference sequences are indicated.

fish (NOAA, 2023). For instance, the baleen whales as fin whales feed mainly on krill and small schooling fish (including herring, capelin, and sand lance), whereas, being toothed whales, sperm and pilot whales feed mainly on squid, as well as fish and octopus (NOAA, 2023). The consumption of such smaller fish and other aquatic animals, potentially carrying *Blastocystis* sp. may be indeed the source of contamination of the marine mammals, known to ingest massive quantities. Herring fish for example was previously reported contaminated by *Blastocystis* sp. when isolated from Eastern English Channel (Gantois et al., 2020). Other fish species collected from China (Wang et al., 2024), France (Gantois et al., 2020), Germany (Konig and Müller, 1997) and Iran (Asghari et al., 2024) were also reported infested with the protozoa. However, reports are limited so far to these few studies with unavailability of data when it comes to the prevalence of *Blastocystis* sp. among other whales' food chain hosts.

On the other hand, exposure of marine mammals, which may migrate along the coastline, to different protozoan microorganisms (Marangi et al., 2022) spread through contamination by sewage, agricultural and urban discharges (Bossart, 2011; Babuji et al., 2023) can be supposed. This could promote the infection with several protozoan microorganisms through fecal-oral transmission and their potential accumulation in these hosts. Indeed, different protozoa have been observed within sea waters specimens (Ben Ayed et al., 2009) alongside *Blastocystis* sp. identified within the Turkish Black Sea (Koloren et al., 2018) and North of France coastline (Ryckman et al., 2024). With the scare available data, a recent review (Ghafari-Cherati et al., 2024) reported that filtering marine animals were infected by *Blastocystis* sp., with the bivalves (mussels and oysters) showing the higher infection rate at 32 % (95 % CI: 13–59.7 %), exceeding the sponges with a prevalence of 10 % (95 % CI: 2.5–32.4 %). Such observations reinforce the suggestion of potential foodborne/waterborne transmission (Mahdavi et al., 2024).

Such observations confirm the *Blastocystis* sp. host range leading it to be one of the most prevalent microeukaryotes worldwide, opening up hence the question of protozoa-host specificity (Tan, 2004). Indeed, it is highly polymorphic with a complex life cycle not yet fully described (Stensvold et al., 2007) with the former name *B. hominis* replaced nowadays by *Blastocystis* sp. (Santin et al., 2011). Consequently, relying on the genetic polymorphism of the small subunit ribosomal RNA, *Blastocystis* nomenclature is presently dividing the protozoa into at least 48 subtypes: ST1-ST17, ST21, and ST23-ST48 (Maloney et al., 2021; Villalobos et al., 2024). The 'subtypes' ST18, ST19, ST20 and ST22 appear to be chimaeras, reported only on a single occasion by a single sequence suggesting it would be an artefact: hence they are recently recommended to be rejected as separate subtypes (Stensvold & Clark, 2020). Potential additional STs have also been proposed, observed mainly in amphibians, reptiles, insects and thus so-called NMASTs. In general, the different STs can be found within different hosts, as well as exclusively in humans. The most frequently isolated STs in humans are ST1 to ST4, with ST3 being the most common worldwide and the most pathogenic (Jiménez et al., 2019; Nemati et al., 2021) reflecting thus a large-scale human-to-human transmission of these STs.

Interestingly, in the present study, ST3 was the most prevalent ST among our marine mammal specimens. Its environmental occurrence is highly observed within wild mussels in France (Ryckman et al., 2024) and Cholga mussels in Chile (Suarez et al., 2023). Such widespread occurrence is likely linked to seawater contamination from human feces, which could help explain our findings. The investigated geographic area

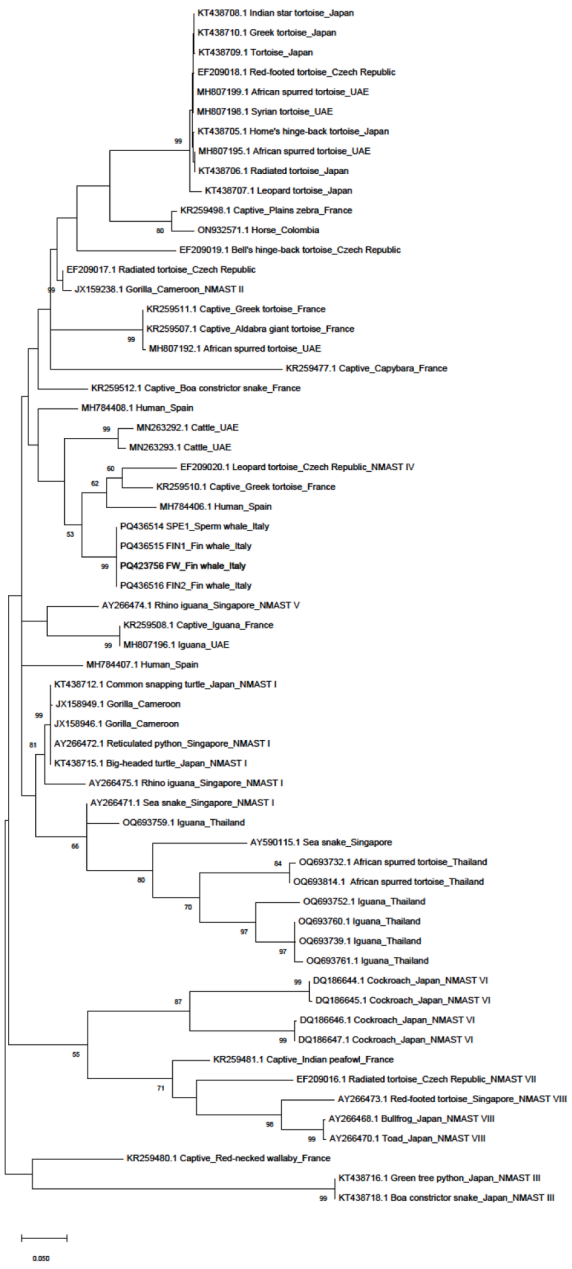


Fig. 3. Unrooted Maximum Likelihood (GTR + G + I substitution model) phylogenetic tree based on the analysis of the partial SSU-rDNA gene of the *Blastocystis* fin whale isolate (in bold) with the NMAST and untypeable subtypes. Numbers next to the nodes represent posterior probabilities. Probabilities <50 % are not shown. Accession numbers of publicly available *Blastocystis* reference sequences are indicated.

is surrounded by coastlines with water currents that may facilitate the spread of the microorganism. Additionally, our sampling took place during the summer, a period when *Blastocystis* sp. prevalence is known to increase compared to the winter months (Ryckman et al., 2024). The latter authors attribute the higher frequency of ST3 during this time to the increased population and recreational activities, which create greater anthropogenic pressure. This observation is consistent with findings in Malaysia, where the occurrence of *Blastocystis* sp. was notably higher during holidays, likely due to the surge in water-based activities by both locals and tourists (Ithoi et al., 2011).

The second most frequent *Blastocystis* subtype in the present study was ST1 as revealed by sequence and phylogenetic analysis. Beside it is frequence with human population especially within the same geographic study area either within diarrheic patients (Marangi et al., 2023) or COVID-19 patients (Marangi et al., 2024), ST1 was identified as the most widespread ST from various water sources (Guilavogui et al., 2022). It is commonly believed that is probably transmitted through water contaminated by feces from different hosts. In this respect, within Asiatic countries, one-fifth of water samples were detected to be positive for *Blastocystis* ST1 highlighting the probability of waterborne transmission as supported by the numerous waterborne outbreaks (Nemati et al., 2021). Moreover, ST1 was identified within cold-water fish samples as well as its surrounding water environment with authors speculating that *Blastocystis* sp. penetrated the intestinal tract of fish through feeding and water flow (Wang et al., 2024). It is worth noting that *Blastocystis* sp. not only remains alive in water with temperatures ranging from 4 to 25 °C but also seems resisting to conventional disinfectant treatment such chlorine (Ahmed and Karanis, 2018).

Interestingly, the fin whale isolate investigated in the current study harbored a poikilotherm-derived *Blastocystis* subtype. Indeed, NMASTs I to NMAST VIII represented amphibian and reptilian clusters. However, with the identification of a wild western lowland gorilla subtype within NMASTs, suggestion of probable accidental contamination of the primate with reptile feces in its natural environment were supposed (Cian et al., 2017). Our isolate sequence is clustering strongly with previously unclassified whale from Italy (Marangi et al., 2021) as well as, at a lesser extent, spur-thighed tortoise from France and the representative of the reptilian NMAST IV identified within Leopard tortoise from Czech Republic. The subtype seems to be widespread within the aquatic/marine animals, however the identification of similar subtype within human specimen from Spain, make the sense of transmission remains unclear. Indeed, fin whales, conversely to other whales, are not known to roam around the coastlines but dive more in depth even if *Blastocystis* sp. pollution was reported extended to offshore with the protozoa identification in seawater samples analyzed at depths of 2 or 3 m.

Although the most common STs found in humans have been identified within marine mammals, any direct interaction between humans and these animals was considered accidental and infrequent. As a result, the risk of zoonotic transmission is thought minimal, even though marine mammal feces can carry the protozoa and may contribute to the contamination of the marine environment (Gantois et al., 2020). However, historically, in regions where the climate is not conducive to intensive pastoral farming, communities such as the Chatham Island Moriori, Icelanders, Greenlanders, Faroe Islanders, Norwegians, and Eskimos have relied on seafood, particularly whale meat, as a vital source of protein. Whale meat is considered a nutritionally valuable delicacy by these cultures. In Japan, whale meat has been a part of the ethnic diet for at least 1000 years (Cawthorn, 1997). Recently, a case of suspected food poisoning from foodborne/waterborne protozoa linked to the consumption of raw common minke whale (*Balaenoptera acutorostrata*) meat was reported in Tokyo, Japan (Murata et al., 2024) which raise concerns about the marine mammal's involvement in zoonotic diseases.

Notwithstanding the fact that *Blastocystis* sp. is considered as a member of the gut microbiome, its impact on the bacterial composition and diversity is still being debated. Indeed, it is unclear whether *Blastocystis* sp. promotes a healthy gut and microbiome directly or whether it is more likely to colonize and persist in a healthy gut environment. Most of the studies has been carried out in the humans where recent reports suggest that *Blastocystis* sp. and its different subtypes are associated with differences in the composition and diversity of the bacterial gut microbiota (Deng et al., 2021b; Marangi et al., 2024b). Indeed, metagenomic studies have shown an association with increased abundances of the phylum Bacillota (syn. Firmicutes) and the class Clostridiales in the gut microbiomes of *Blastocystis*-colonized individuals, as well as a decreased abundance of Bacteroides (Mayneris-Perxachs et al., 2022; Aykur et al., 2024). On the other hand, it has also been reported that *Blastocystis* sp. can decrease the abundance of beneficial bacteria (Nourisson et al., 2014; Yasson et al., 2019).

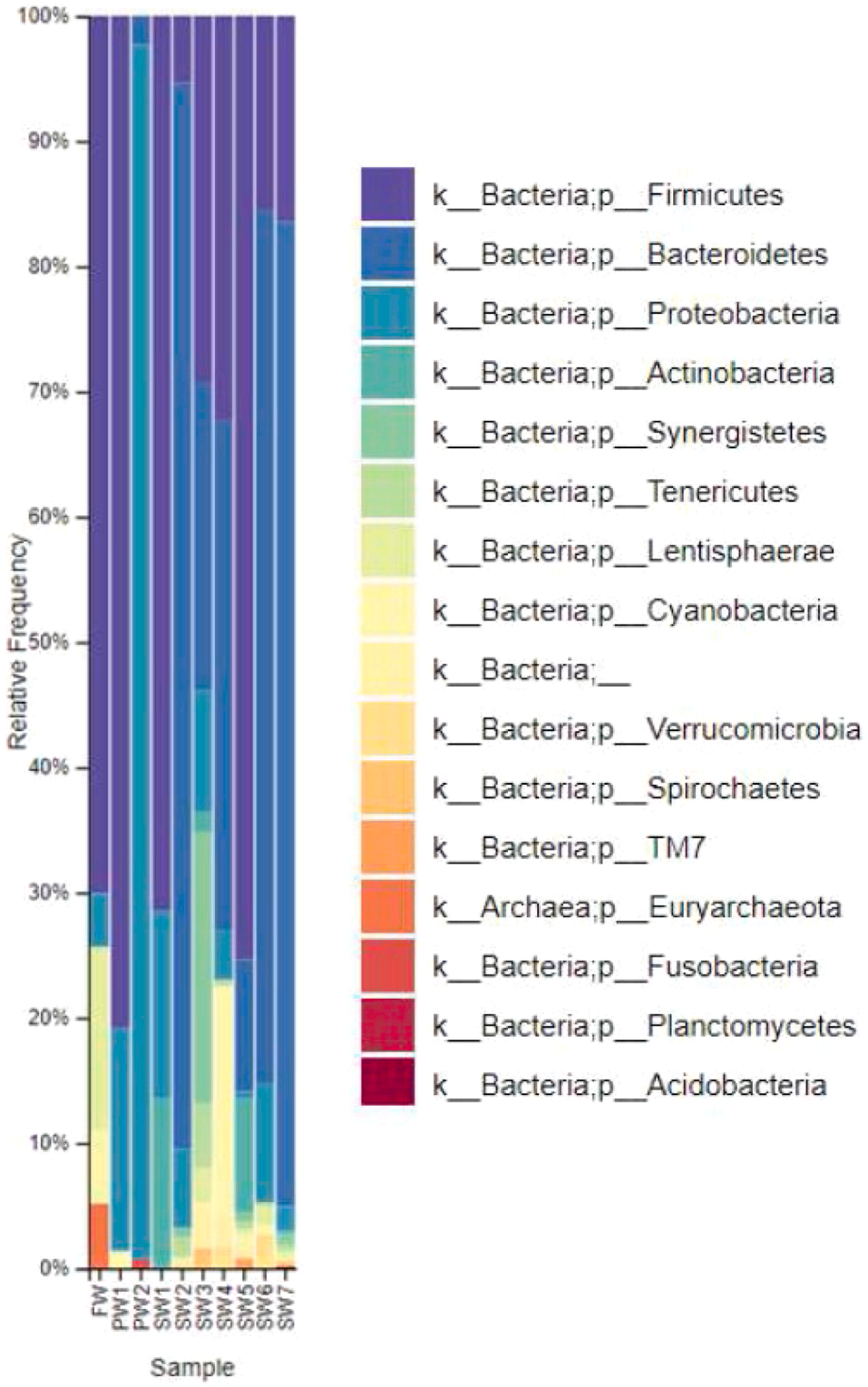


Fig. 4. Taxonomic microbial composition results at the phylum level identified within the ten *Blastocystis*-carrier marine mammals. SW: Sperm whale; FW: Fin whale; PW: Pilot whale.

In our current work, fifteen phyla with the most abundant ones being Firmicutes, Bacteroidetes and Proteobacteria were identified in all our *Blastocystis*-carrier free-ranging marine mammals supported by the recent gut microbiome profile among stranded cetaceans (Fan et al., 2024). PW2 isolate however didn't include any Firmicutes bacteria, while Fusobacteria was reported. These results are in accordance with what stated in a study by Bai et al. (2021) in which the gut microbiome of one stranded short-finned pilot whale was dominated by Firmicutes (mainly Clostridium) and Fusobacteria; whereas the pilot whale was composed of Gammaproteobacteria and Bacteroidetes (mainly Vibrio and Bacteroides, respectively), probably caused by intestinal disease. Firmicutes were frequently reported within marine mammals such as: the Risso's dolphin (*Grampus griseus*) (Wan et al., 2023), the Chinese white dolphin (*Sousa chinensis*) (Wan et al., 2021), Yangtze finless

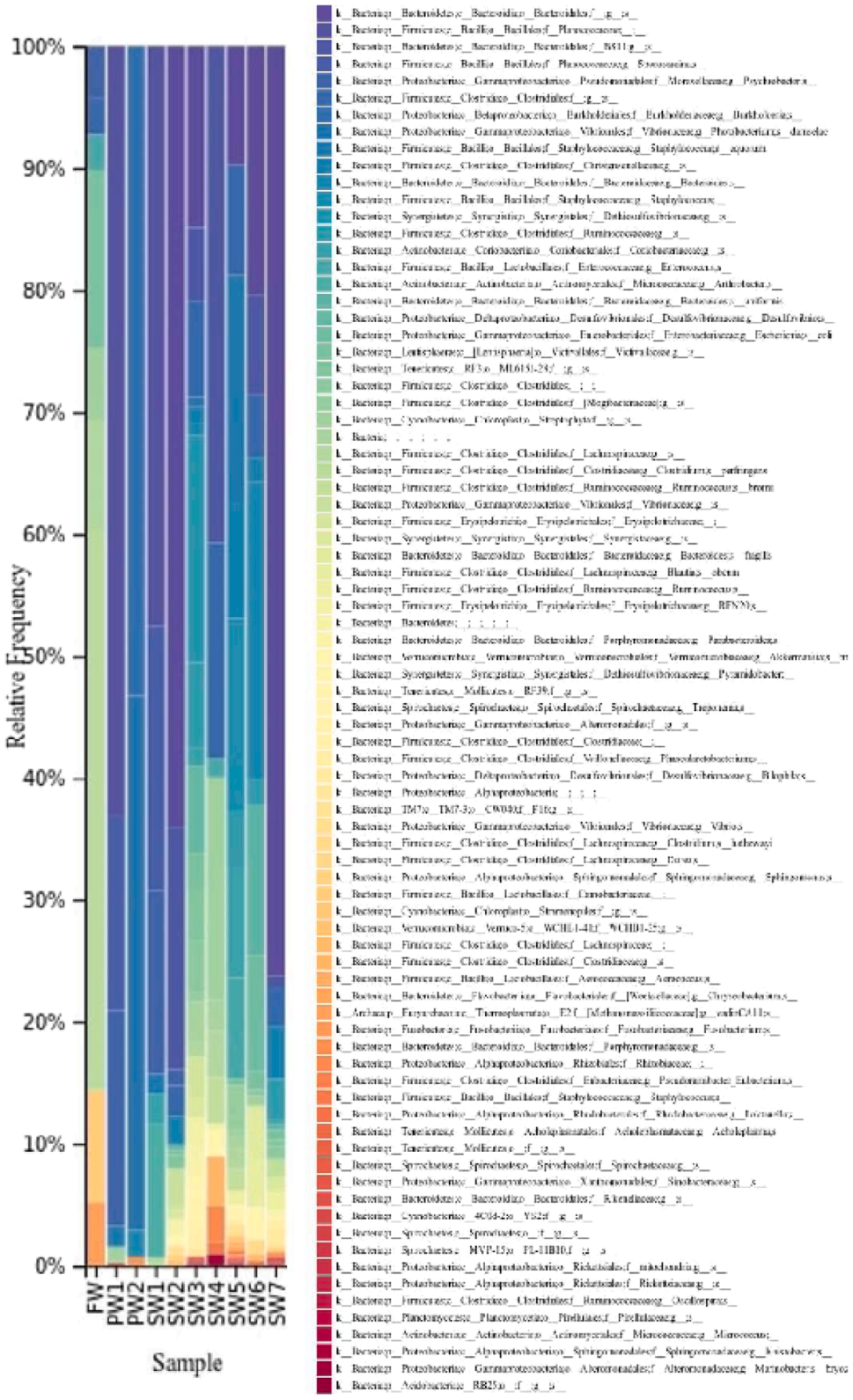


Fig. 5. Taxonomic microbial composition results at the species level identified within the ten *Blastocystis*-carrier marine mammals. SW: Sperm whale; FW: Fin whale; PW: Pilot whale.

porpoise (*Neophocena asiaeorientalis*) (Wan et al., 2016), bottlenose dolphin (*Tursiops truncatus*) (Tajima et al., 2022), large baleen (*Balaenoptera musculus, B. physalus, B. borealis*) and sperm whales (Glaeser et al., 2022), melon-headed whales (*Peponocephala electra*) (Bai et al., 2022), short-finned pilot whales (*Globicephala macrorhynchus*) (Bai et al., 2021), dwarf minke whale (*Balaenoptera acutorostrata*) (Tian et al., 2020), pygmy (*Kogia breviceps*), and dwarf (*Kogia sima*) sperm whales (Erwin et al., 2017). Other studies carried out on dead sperm whales reported an extensive microbial diversity, including *Enterococcus faecium, Fusobacterium nucleatum, Pseudomonas aeruginosa, Streptococcus*

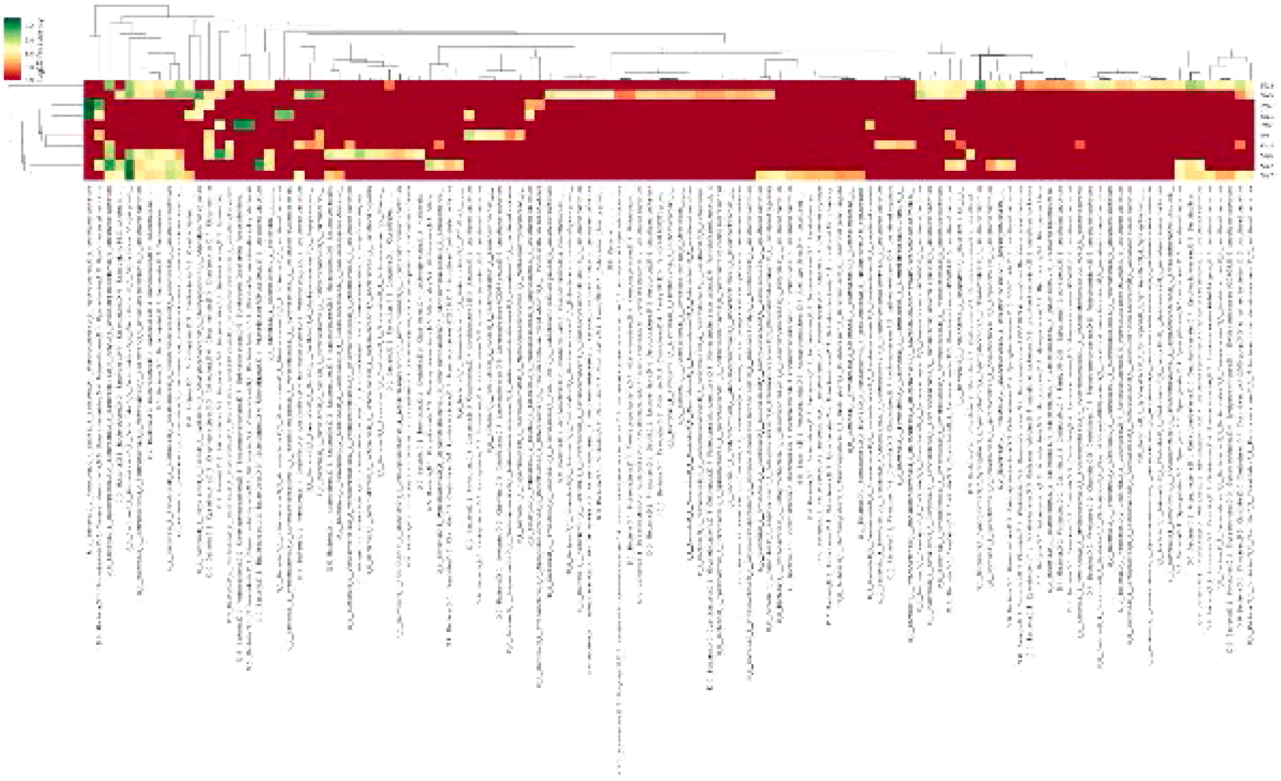


Fig. 6. Heat Map results at the species level identified within the ten *Blastocystis*-carrier marine mammals. The whale samples are indicated along the x-axis whereas OTUs are indicated along the y-axis. The abundance of each OTU is indicated by colors ranging from red (low abundance or absent) to dark green (high abundance) with double dendrograms based on average linkage hierarchical clustering.

Table 2
Alpha diversity results, calculated as bacterial richness at the genus level using "observed OTUs", "Faith's Phylogenetic Diversity", and "Shannon index" metrics with Kruskal-Wallis test (H) based on the comparison between marine mammals species and *Blastocystis* subtypes.

Kruskal-Wallis	Marine mammals' species			*Blastocystis* subtypes		
	Observed_OTUs	Faith_pd	Shannon	Observed_OTUs	Faith_pd	Shannon
H	5.926	5.142	4.644	3.713	3.763	1.781
p-values	0.051	0.076	0.098	0.156	0.152	0.410

*p-values $\leq$ 0.05 statistically significant

anginosus, Streptococcus pneumoniae, and *Streptococcus suis*, and five toxigenic *Clostridium* species usually associated with gastrointestinal infections (Li et al., 2019).

A frequent bacterium within our sperm whales (except SW4) is Rikenellaceae RC9 gut group belonging to a new type of Rikenellaceae family that exerts anaerobic metabolism in the digestive tract of different hosts and playing a major role in the digestion of crude fiber. Being part of the genus *Bacteroides* it is assumed to be involved in improving ruminant metabolism by metabolizing bile acid, protein, fat, and regulating carbohydrate metabolism in addition to metabolizing lipids (Jiang et al., 2022). Moreover, the majority of the sperm whale isolates investigated within the current study (except SW1) harbored Tenericutes bacteria with *Acholeplasma* within SW6; Izimaplasmatales within SW2, SW3, SW7 and RF39 within SW2, SW3, SW4 and SW5. The last one is one of the two main clades comprising uncultured Tenericutes. Such microorganisms are known to be frequent within different environmental matrices especially marine habitats infesting even several hosts as fish, sea star, oysters and mussel which may raise a foodborne concern (Wang et al., 2022).

The last result, but not less important, the gut microbiome of our only fin whale specimen is characterized by the abundance of *Sarcina* bacteria in addition to the Archaebacteria. *Sarcina* spp. are anaerobic, Gram-positive coccus, cellulose synthesizers and short-chain fatty acid (SCFA) producers (Khan et al., 2016). *Sarcina* spp. had been isolated from the gastrointestinal tracts of diverse mammalian hosts and are considered as well-recognized pathogens in infectious diseases literature. Indeed, their presence is often associated with host health complications, as is evident from many previously reports such as a presumptive cause of fatal acute gastric dilation and gastric emphysema in rhesus macaques (Lee et al., 2023) as well as a *Sarcina* bacterium linked to lethal disease in sanctuary chimpanzees in Sierra Leone (Owens et al., 2021). On the other hand, the archaeal community identified within our FW microbiome are from hydrogen-dependent and methylotrophic Methanomethylophilaceae family. Methanogenic Archaea occur in many natural and anthropogenic habitats, both aquatic such as ocean or lake sediments and wetlands, and terrestrial such as landfills (Weil et al., 2023) and/or the gastrointestinal tracts of numerous animals as reviewed recently by Volmer et al. (2024). Abundance of archaebacteria have been reported associated with *Blastocystis* sp. carriage within wild chimpanzees in Senegal (Renelies-Hamilton et al., 2019) that advance 12 % of untypables subtypes. Interestingly, our FW specimen harbored a poikilotherm-derived *Blastocystis* subtype. Although any hypothesis cannot be supposed, this result should be considered in the optical to have more samples in order

to support this data.

The microbial diversity identified in our *Blastocystis*-carrier marine mammals could lead to the hypothesis of a shift of microbiome composition driven by this protozoan. Unfortunately, due to the difficulty collecting samples from different species of free-ranging marine mammals, few studies on gut microbiome of whale species are available making a detailed comparison difficult (Erwin et al., 2017; Li et al., 2019; Bai et al., 2021). At the time of writing, there are no studies on the potential relationship between *Blastocystis* sp. and its associated microbiome in marine mammals' species.

5. Conclusion and study limitation

To the best of our knowledge, this is the first molecular and metagenomic study on the *Blastocystis* sp. prevalence, subtypes diversity and its associated microbial composition in four different species of free-ranging baleen and toothed whales carried out over the period 2022–2024. Although the main limitation of this work is related to the sampling, due to the extreme difficulty to collect fecal samples from marine mammals, for ecological and logistic reasons, these results shed lights on the circulation of this single-celled microorganism within an area of the Mediterranean Sea characterized by high marine biodiversity (Coll et al., 2010), as well as increasing anthropic pressure possibly due to the spread of microorganisms in coastal waters contaminated by sewage, agricultural and urban discharges, shared between humans and wildlife. These results give an overview of potential zoonotic microorganisms circulating in Mediterranean marine megafauna, indicating the need for more integrated research, within One Health approach, to understand the associated risk and to prevent human and marine ecosystem exposure to these anthropogenic microorganisms.

Consent for publication

Not applicable.

CRediT authorship contribution statement

Marianna Marangi: Conceptualization, Sampling, Methodology, Molecular analysis, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, **Sonia Boughattas:** Genetic analysis**,** Microbiome analysis, Writing – original draft, Writing – review & editing

Ethics approval and consent to participate

No approval from the institutional animal care and use or the ethics committee has been necessary in regard to the Italian law as no experiment involving animals was performed. To obtain faecal marine mammals samples, a collaboration agreement (2023–11–30) was signed between the University of Foggia and Tethys Research Institute (https://tethys.org/).

Declaration of competing interest

The authors declare that they have no conflict of interest and that they have no actual or potential competing financial interests.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or non-profit sectors. We are very grateful to all the researchers of Tethys Research Institute for their cooperation and collaboration in collecting samples and the skipper's boat Roberto Ranieri and Paolo Pinto.

Funding

The authors declare that no specific funding was used for this research.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2025.100349.

Data availability

Data will be made available on request.

References

Ahmed, S.A., Karanis, P., 2018. Comparison of current methods used to detect *Cryptosporidium* oocysts in stools. Int. J. Hyg. Environ. Health 221 (5), 743–763. https://doi.org/10.1016/j.ijheh.2018.04.006.

Alfellani, M.A., Jacob, A.S., Perea, N.O., Krecek, R.C., Taner-Mulla, D., Verweij, J.J., Levecke, B., Tannich, E., Clark, C.G., Stensvold, C.R., 2013. Diversity and distribution of *Blastocystis* sp. subtypes in non-human primates. Parasitology 140 (8), 966–971. https://doi.org/10.1017/S0031182013000255.

Asghari, A., Hanifeh, F., Mohammadi, M.R., 2024. Molecular epidemiology, subtype distribution, and zoonotic importance of *Blastocystis* sp. in camelids (camels and alpacas): a worldwide systematic review and meta-analysis. Foodborne Pathog. Dis. https://doi.org/10.1089/fpd.2024.0059.

Attah, A.O., Sanggari, A., Li, L.I., Nik Him, N.A.I.I., Ismail, A.H., Meor Termizi, F.H., 2023. *Blastocystis* sp. occurrence in water sources worldwide from 2005 to 2022: a review. Parasitol. Res. 122 (1), 1–10. https://doi.org/10.1007/s00436-022-07731-0.

Aykur, M., Malatyalı, E., Demirel, F., Cömert-Koçak, B., Gentekaki, E., Tsaousis, A.D., Dogruman-Al, F., 2024. *Blastocystis* sp.: a mysterious member of the gut microbiome. Microorganisms 12 (3), 461. https://doi.org/10.3390/microorganisms12030461.

Babuji, P., Thirumalaisamy, S., Duraisamy, K., Periyasamy, G., 2023. Human health risks due to exposure to water pollution: a review. Water (Basel) 15, 2532. https://doi.org/10.3390/w15142532.

Bai, S., Zhang, P., Lin, M., Lin, W., Yang, Z., Li, S., 2021. Microbial diversity and structure in the gastrointestinal tracts of two stranded short-finned pilot whales (*Globicephala macrorhynchus*) and a pygmy sperm whale (*Kogia breviceps*). Integr. Zool. 16 (3), 324–335. https://doi.org/10.1111/1749-4877.12502.

Bai, S., Zhang, P., Zhang, X., Yang, .Z, Li, S., 2022. Gut Microbial Characterization of Melon-Headed Whales (*Peponocephala electra*) Stranded in China. Microorganisms 10 (3), 572. https://doi.org/10.3390/microorganisms10030572.

Ben Ayed, L., Schijven, J., Alouini, Z., Jemli, M., Sabbahi, S., 2009. Presence of parasitic protozoa and helminth in sewage and efficiency of sewage treatment in Tunisia. Parasitol. Res. 105 (2), 393–406. https://doi.org/10.1007/s00436-009-1396-y.

Bossart, G.D., 2011. Marine mammals as sentinel species for oceans and human health. Vet. Pathol. 48 (3), 676–690. https://doi.org/10.1177/0300985810388525.

Cawthorn, M.W., 1997. Meat consumption from stranded whales and marine mammals in New Zealand: public health and other issues. Conservation Advisory Science Notes No. 164. Department of Conservation, Wellington.

Cian, A., El Safadi, D., Osman, M., Moriniere, R., Gantois, N., Benamrouz-Vanneste, S., Delgado-Viscogliosi, P., Guyot, K., Li, L.L., Monchy, S., Noël, C., Poirier, P., Nourrisson, C., Wawrzyniak, I., Delbac, F., Bosc, S., Chabé, M., Petit, T., Certad, G., Viscogliosi, E., 2017. Molecular epidemiology of *Blastocystis* sp. in various animal groups from two French zoos and evaluation of potential zoonotic risk. PLoS One 12 (1), e0169659. https://doi.org/10.1371/journal.pone.0169659.

Coll, M., Piroddi, C., Steenbeek, J., Kaschner, K., Ben Rais Lasram, F., Aguzzi, J., Ballesteros, E., Bianchi, C.N., Corbera, J., Dailianis, T., Danovaro, R., Estrada, M., Froglia, C., Galil, B.S., Gasol, J.M., Gertwagen, R., Gil, J., Guilhaumon, F., Kesner-Reyes, K., Kitsos, M.S., Koukouras, A., Lampadariou, N., Laxamana, E., López-Fé de la Cuadra, C.M., Lotze, H.K., Martin, D., Mouillot, D., Oro, D., Raicevich, S., Rius-Barile, J., Saiz-Salinas, J.I., San Vicente, C., Somot, S., Templado, J., Turon, X., Vafidis, D., Villanueva, R., Voultsiadou, E., 2010. The biodiversity of the Mediterranean Sea: estimates, patterns, and threats. PLoS One 5 (8), e11842. https://doi.org/10.1371/journal.pone.0011842.

Deng, L., Yao, J.X., Liu, H.F., Zhou, Z.Y., Chai, Y.J., Wang, W.Y., Zhong, Z.J., Deng, J.L., Ren, Z.H., Fu, H.L., Yan, X., Yue, C.J., Peng, G.N., 2019. First report of *Blastocystis* sp. in giant pandas, red pandas, and various bird species in Sichuan province, southwestern China. Int. J. Parasitol.: Parasites Wildlife 9, 298–304. https://doi.org/10.1016/j.ijppaw.2019.06.007.

Deng, L., Yao, J., Chen, S., He, T., Chai, Y., Zhou, Z., Shi, X., Liu, H., Zhong, Z., Fu, H., Peng, G., 2021. First identification and molecular subtyping of *Blastocystis* sp. in zoo animals in southwestern China. Parasit. Vectors. 14 (1), 11. https://doi.org/10.1186/s13071-020-04515-2.

Deng, L., Wojciech, L., Gascoigne, N.R.J., Peng, G., Tan, K.S.W., 2021b. New insights into the interactions between *Blastocystis* sp., the gut microbiota, and host immunity. PLoS. Pathog. 17 (2), e1009253. https://doi.org/10.1371/journal.ppat.1009253.

Deng, L., Wojciech, L., Png, C.W., Koh, E.Y., Aung, T.T., Kioh, D.Y.Q., Chan, E.C.Y., Malleret, B, Zhang, Y., Peng, G., Gascoigne, N.R.J., Tan, K.S.W., 2022. Experimental colonization with *Blastocystis* ST4 is associated with protective immune responses

and modulation of gut microbiome in a DSS-induced colitis mouse model. Cellular Mol. Life Sci. 79, 245. https://doi.org/10.1007/s00018-022-04271-9.
Deng, L., Tan, K.S.W., 2022. Interactions between *Blastocystis* subtype ST4 and gut microbiota in vitro. Parasite Vect. 15 (1), 80. https://doi.org/10.1186/s13071-022-05194-x, 8.
Deng, L., Wojciech, L., Png, CW., Kioh, DYQ., Gu, Y, Aung, TT., Malleret, B., Chan, ECY., Peng, G., Zhang, Y., Gascoigne, NRJ., Tan, KSW., 2023. Colonization with two different *Blastocystis* subtypes in DSS-induced colitis mice is associated with strikingly different microbiome and pathological features. Theranostics 13 (3), 1165–1179. https://doi.org/10.7150/thno.81583, 5.
Deng, L., Wojciech, L., Png, C.W., Kioh, Y.Q.D., Ng, G.C., Chan, E.C.Y., Zhang, Y., Gascoigne, N.R.J., Tan, K.S.W., 2023b. Colonization with ubiquitous protist *Blastocystis* ST1 ameliorates DSS-induced colitis and promotes beneficial microbiota and immune outcomes. npj Biofilms Microbiomes 9, 22. https://doi.org/10.1038/s41522-023-00389-1.
El Safadi, D., Gaayeb, L., Meloni, D., Cian, A., Poirier, P., Wawrzyniak, I., Delbac, F., Dabboussi, F., Delhaes, L., Seck, M., Hamze, M., Riveau, G., Viscogliosi, E., 2014. Children of Senegal River Basin show the highest prevalence of *Blastocystis* sp. ever observed worldwide. BMC Infect. Dis. 14, 164. https://doi.org/10.1186/1471-2334-14-164.
Erwin, P.M., Rhodes, R.G., Kiser, K.B., Keenan-Bateman, T.F., McLellan, W.A., Pabst, D. A., 2017. High diversity and unique composition of gut microbiomes in pygmy (*Kogia breviceps*) and dwarf (*K. sima*) sperm whales. Sci. Rep. 7 (1), 7205. https://doi.org/10.1038/s41598-017-07425-z.
Fan, J., Kang, H., Lv, M., Zhai, Y., Jia, Y., Yang, Z., Shi, C., Zhou, C., Diao, L., Li, J., Jin, X., Liu, S., Kristiansen, K., Zhang, P., Chen, J., Li, S., 2024. Taxonomic composition and functional potentials of gastrointestinal microbiota in 12 wild-stranded cetaceans. Front. Microbiol. 15, 1394745. https://doi.org/10.3389/fmicb.2024.1394745.
Gantois, N., Lamot, A., Seesao, Y., Creusy, C., Li, L.L., Monchy, S., Benamrouz-Vanneste, S., Karpouzopoulos, J., Bourgain, J.L., Rault, C., Demaret, F., Baydoun, M., Chabé, M., Fréalle, E., Aliouat-Denis, C.M., Gay, M., Certad, G., Viscogliosi, E., 2020. First report on the prevalence and subtype distribution of *Blastocystis* sp. in edible marine fish and marine mammals: a large scale-study conducted in Atlantic Northeast and on the coasts of Northern France. Microorganisms 8 (3), 460. https://doi.org/10.3390/microorganisms8030460.
Gazzonis, A.L., Marangi, M., Zanzani, S.A., Villa, L., Giangaspero, A., Manfredi, M.T., 2019. Molecular epidemiology of *Blastocystis* sp. in dogs housed in Italian rescue shelters. Parasitol. Res. 118 (10), 3011–3017. https://doi.org/10.1007/s00436-019-06424-5.
Ghafari-Cherati, M., Karampour, A., Nazem-Sadati, S.S., Asghari, A., 2024. Foodborne concerns of *Blastocystis* sp. in marine animals (fish, bivalves, and sponges): a systematic review and meta-analysis of global prevalence and subtypes distribution. Food Waterborne Parasitol. 36, e00242. https://doi.org/10.1016/j.fawpar.2024.e00242.
Glaeser, S.P., Silva, L.M.R., Prieto, R., Silva, M.A., Franco, A., Kämpfer, P., Hermosilla, C., Taubert, A., Eisenberg, T., 2022. A preliminary comparison on faecal microbiomes of free-ranging large baleen (*Balaenoptera musculus, B. physalus, B. borealis*) and toothed (*Physeter macrocephalus*) whales. Microb. Ecol. 83 (1), 18–33. https://doi.org/10.1007/s00248-021-01729-4.
Guard, G., 2024. *Blastocystis hominis*; friend or foe. Integrat. Med. (Encinitas) 23 (5), 28–33.
Guilavogui, T., Gantois, N., Even, G., Desramaut, J., Dautel, E., Denoyelle, C., Cissé, F.I., Touré, S.C., Kourouma, B.L., Sawant, M., Chabé, M., Certad, G., Viscogliosi, E., 2022. Detection, molecular identification and transmission of the intestinal protozoa *Blastocystis* sp. in Guinea from a large-scale epidemiological study conducted in the Conakry area. Microorganisms 10 (2), 446. https://doi.org/10.3390/microorganisms10020446.
Hublin, J.S.Y., Maloney, J.G., Santin, M., 2021. *Blastocystis* sp. in domesticated and wild mammals and birds. Res. Vet. Sci. 135, 260–282. https://doi.org/10.1016/j.rvsc.2020.09.031.
Hung, Y.M., Lu, T.P., Tsai, M.H., Lai, L.C., Chuang, E.Y., 2021. EasyMAP: A user-friendly online platform for analyzing 16S ribosomal DNA sequencing data. N Biotechnol. 63, 37–44. https://doi.org/10.1016/j.nbt.2021.03.001.
Ithoi, I., Jali, A., Mak, J.W., Wan Sulaiman, W.Y., Mahmud, R., 2011. Occurrence of *Blastocystis* sp. in water of two rivers from recreational areas in Malaysia. J. Parasitol. Res., 123916 https://doi.org/10.1155/2011/123916.
Jiang, Z., Su, W., Li, W., Wen, C., Du, S., He, H., Zhang, Y., Gong, T., Wang, X., Wang, Y., Jin, M., Lu, Z., 2022. *Bacillus amyloliquefaciens* 40 regulates piglet performance, antioxidant capacity, immune status, and gut microbiota. Ani. Nutr. 12, 116–127. https://doi.org/10.1016/j.aninu.2022.09.006.
Jimenez, P., Munoz, M., Ramirez, J.D., 2023. An update on the distribution of *Blastocystis* subtypes in the Americas. Heliyon. 8, e12592.
Jiménez, P.A., Jaimes, J.E., Ramírez, J.D., 2019. A summary of *Blastocystis* subtypes in North and South America. Parasit Vect. 12, 376. https://doi.org/10.1186/s13071-019-3641-2.
Khan, N., Vidyarthi, A., Nadeem, S., Negi, S., Nair, G., Agrewala, J.N., 2016. Alteration in the gut microbiota provokes susceptibility to tuberculosis. Front. Immunol. 7, 529. https://doi.org/10.3389/fimmu.2016.00529.
Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41 (1), e1. https://doi.org/10.1093/nar/gks808.
Koloren, Z., Gulabi, B.B., Karanis, P., 2018. Molecular identification of *Blastocystis* sp. subtypes in water samples collected from Black Sea, Turkey. Acta Trop. 180, 58–68. https://doi.org/10.1016/j.actatropica.2017.12.029.
König, G., Müller, H.E., 1997. *Blastocystis hominis* in animals: incidence of four serogroups. Zentralblatt für Bakteriologie 286 (3), 435–440. https://doi.org/10.1016/s0934-8840(97)80105-3.
Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35 (6), 1547–1549. https://doi.org/10.1093/molbev/msy096.
Kuraku, S., Zmasek, C.M., Nishimura, O., Katoh, K., 2013. aLeaves facilitates on-demand exploration of metazoan gene family trees on MAFFT sequence alignment server with enhanced interactivity. Nucleic Acids Res. 41, W22–W28. https://doi.org/10.1093/nar/gkt389. Web Server issue.
Lee, L.M., Owens, L.A., Krugner-Higby, L.A., Graham, M., Simmons, H.A., Climans, M., Brown, K., Bennett, A.J., Schaefer, J., Meyer, K., Goldberg, T.L., 2023. *Sarcina* spp. As a presumptive cause of fatal acute gastric dilation and gastric emphysema in rhesus macaques. J. Vet. Diag. Invest. 35 (6), 698–703. https://doi.org/10.1177/10406387231193965.
Li, C., Tan, X., Bai, J., Xu, Q., Liu, S., Guo, W., Yu, C., Fan, G., Lu, Y., Zhang, H., Yang, H., Chen, J., Liu, X., 2019. A survey of the sperm whale (*Physeter catodon*) commensal microbiome. PeerJ 7, e7257. https://doi.org/10.7717/peerj.7257.
Li, J., Wang, Z., Karim, M.R., Zhang, L., 2020. Detection of human intestinal protozoan parasites in vegetables and fruits: a review. Parasit. Vect. 13 (1), 380. https://doi.org/10.1186/s13071-020-04255-3.
Liao, C.C., Chen, C.H., Shin, J.W., Lin, W.C., Chen, C.C., Chu, C.T., 2023. Lipid accumulation in *Blastocystis* sp. increases cell damage in co-cultured cells. Microorganisms 11 (6), 1582. https://doi.org/10.3390/microorganisms11061582.
Mahdavi, F., Maleki, F., Mohammadi, M.R., Mehboodi, M., Hanifeh, F., Asghari, A., Mohammadi-Ghalehbin, B., 2024. A worldwide systematic review and meta-analysis of the prevalence and subtype distribution of *Blastocystis* sp. in water sources: a public health concern. Foodborne Pathog. Dis. https://doi.org/10.1089/fpd.2024.0107 [online] Available at.
Maloney, J.G., George, N.S., Molokin, A., Santin, M., 2021. An Illumina MiSeq-based amplicon sequencing method for the detection of mixed parasite infections using the *Blastocystis* SSU rRNA gene as an example. Methods Mol. Biol. 2369, 67–82. https://doi.org/10.1007/978-1-0716-1681-9_5.
Marangi, M., Airoldi, S., Beneduce, L., Zaccone, C., 2021. Wild whale faecal samples as a proxy of anthropogenic impact. Sci. Rep. 11 (1), 5822. https://doi.org/10.1038/s41598-021-84966-4.
Marangi, M., Carlucci, R., Carlino, P., Fanizza, C., Cirelli, G., Maglietta, R., Beneduce, L., 2022. Dolphins and sea turtles may host zoonotic parasites and pathogenic bacteria as indicators of anthropic pressure in the Gulf of Taranto (Northern Ionian Sea, Central-Eastern Mediterranean Sea). Vet. Res. Commun. 46 (4), 1157–1166. https://doi.org/10.1007/s11259-022-10011-y.
Marangi, M., Boughattas, S., De Nittis, R., Pisanelli, D., Delli Carri, V., Lipsi, M.R., La Bella, G., Serviddio, G., Niglio, M., Lo Caputo, S., Margaglione, M., Arena, F., 2023. Prevalence and genetic diversity of *Blastocystis* sp. among autochthonous and immigrant patients in Italy. Microb. Pathog. 185, 106377. https://doi.org/10.1016/j.micpath.2023.106377.
Marangi, M., Boughattas, S., Valzano, F., La Bella, G., De Nittis, R., Margaglione, M., Arena, F., 2024. Prevalence of *Blastocystis* sp. and other gastrointestinal pathogens among diarrheic COVID-19 patients in Italy. New Microbes. New Infect. 58, 101228. https://doi.org/10.1016/j.nmni.2024.101228.
Marangi, M., Boughattas, S., Benslimane, F., 2024b. Gut microbiome profile to the level species in diarrheic protozoan-carrier patients in Italy. Life Sci. 359, 123182. https://doi.org/10.1016/j.lfs.2024.123182.
Matovelle, C., Quílez, J., Tejedor, M.T., Beltrán, A., Chueca, P., Monteagudo, L.V., 2024. Subtype Distribution of *Blastocystis* spp. in Patients with Gastrointestinal Symptoms in Northern Spain. Microorganisms 12 (6), 1084. https://doi.org/10.3390/microorganisms12061084.
Matovelle, C., Tejedor, M.T., Monteagudo, L.V., Beltrán, A., Quílez, J., 2022. Prevalence and associated factors of *Blastocystis* sp. infection in patients with gastrointestinal symptoms in Spain: a case-control study. Trop. Med. Infect. Dis. 7 (9), 226. https://doi.org/10.3390/tropicalmed7090226.
Mayneris-Perxachs, J., Arnoriaga-Rodríguez, M., Garre-Olmo, J., et al., 2022. Presence of *Blastocystis* sp. in gut microbiota is associated with cognitive traits and decreased executive function. ISME J. 16, 2181–2197. https://doi.org/10.1038/s41396-022-01262-3.
Cao, M., Zhang, S., Nan, H., Huang, J., Zhang, C., Sun, Y., Liu, L., Wang, Y., Lu, X., Ma, L., 2024. Integrated omics reveal the pathogenic potential of B*lastocystis* sp. ST2. [online] Available at: https://doi.org/10.1155/2024/6025236.
Murata, R., Kodo, Y., Maeno, A., Suzuki, J., Mori, K., Sadamasu, K., Kawahara, F., Nagamune, K., 2024. Detection of *Toxoplasma gondii* and *Sarcocystis* sp. in the meat of common minke whale (*Balaenoptera acutorostrata*): a case of suspected food poisoning in Japan. Parasit. Vect. 99, 102832. https://doi.org/10.1016/j.parint.2023.102832.
Naguib, D., Gantois, N., Desramaut, J., Arafat, N., Mandour, M., Abdelmaogood, A.K.K., Mosa, A.F., Denoyelle, C., Even, G., Certad, G., Chabé, M., Viscogliosi, E., 2023. Molecular Epidemiology and Genetic Diversity of the Enteric Protozoan Parasite *Blastocystis* sp. in the Northern Egypt Population. Pathogens 12 (11), 1359. https://doi.org/10.3390/pathogens12111359.
Nemati, S., Reza Zali, M., Johnson, P., Mirjalali, H., Karanis, P., 2021. Molecular prevalence and subtype distribution of B*lastocystis* sp. in Asia and Australia. J. Water. Health 19, 687–694.
Ning, C.-Q., Hu, Z.-H., Chen, J.-H., Tian, L.-G., 2020. Epidemiology of *Blastocystis* sp. infection from 1990 to 2019 in China. Infect. Dis. Poverty. 9, 168. https://doi.org/10.1186/s41182-020-00308-w.
NOAA., 2023. https://www.fisheries.noaa.gov/whales#by-species.

Nourrisson, C., Scanzi, J., Pereira, B., NkoudMongo, C., Wawrzyniak, I., Cian, A., et al., 2014. *Blastocystis* sp. is associated with decrease of fecal microbiota protective bacteria: comparative analysis between patients with irritable bowel syndrome and control subjects. PLoS One 9, e111868.

Owens, L.A., Colitti, B., Hirji, I., Pizarro, A., Jaffe, J.E., Moittié, S., Bishop-Lilly, K.A., Estrella, L.A., Voegtly, L.J., Kuhn, J.H., Suen, G., Deblois, C.L., Dunn, C.D., Juan-Sallés, C., Goldberg, T.L., 2021. Publisher correction: A *Sarcina* bacterium linked to lethal disease in sanctuary chimpanzees in Sierra Leone. Nat. Commun. 12 (1), 2035. https://doi.org/10.1038/s41467-021-22494-5.

Popruk, S., Adao, D.E.V., Rivera, W.L., 2021. Epidemiology and subtype distribution of *Blastocystis* sp. in humans: a review. Infect. Genet. Evol. 95, 105085. https://doi.org/10.1016/j.meegid.2021.105085.

Rauff-Adetotun, A.A., Meor Termizi, F.H., Shaari, N., Lee, I.L., 2021. The coexistence of *Blastocystis* sp. in humans, animals, and environmental sources from 2010–2021 in Asia. Biology (Basel) 10, 990.

Renelies-Hamilton, J., Noguera-Julian, M., Parera, M., Paredes, R., Pacheco, L., Dacal, E., Saugar, J.M., Rubio, J.M., Poulsen, M., Köster, P.C., Carmena, D., 2019. Exploring interactions between *Blastocystis* sp., *Strongyloides* spp. and the gut microbiomes of wild chimpanzees in Senegal. Infect., Genet. Evol. 74, 104010. https://doi.org/10.1016/j.meegid.2019.104010.

Ryckman, M., Gantois, N., Dominguez, R.G., Desramaut, J., Li, L.L., Even, G., Audebert, C., Devos, D.P., Chabé, M., Certad, G., Monchy, S., Viscogliosi, E., 2024. Molecular identification and subtype analysis of *Blastocystis* sp. isolates from wild mussels (*Mytilus edulis*) in Northern France. Microorganisms 12 (4), 710. https://doi.org/10.3390/microorganisms12040710.

Sanggari, A., Komala, T., Rauff-Adedotun, A.A., Awosolu, O.B., Attah, O.A., Farah Haziqah, M.T., 2022. *Blastocystis* sp. in captivated and free-ranging wild animals worldwide: a review. Trop. Biomed. 39 (3), 338–372. https://doi.org/10.47665/tb.39.3.006.

Santín, M., Gómez-Muñoz, M.T., Solano-Aguilar, G., Fayer, R., 2011. Development of a new PCR protocol to detect and subtype *Blastocystis* sp. from humans and animals. Parasitol. Res. 109 (1), 205–212. https://doi.org/10.1007/s00436-010-2244-9.

Scicluna, S.M., Tawari, B., Clark, C.G., 2006. DNA barcoding of *Blastocystis*. Protist. 157 (1), 77–85. https://doi.org/10.1016/j.protis.2005.12.001.

Stensvold, C.R., Arendrup, M.C., Jespersgaard, C., Mølbak, K., Nielsen, H.V., 2007. Detecting *Blastocystis* sp. using parasitologic and DNA-based methods: a comparative study. Diagn. Microbiol. Infect. Dis. 59 (3), 303–307. https://doi.org/10.1016/j.diagmicrobio.2007.06.003.

Stensvold, C.R., Tan, K.S., Clark, C.G., 2020. *Blastocystis* sp. Trends. Parasitol. 36 (3), 315–316. https://doi.org/10.1016/j.pt.2019.12.008.

Suarez, P., Vallejos-Almirall, A., Fernández, I., Gonzalez-Chavarria, I., Alonso, J.L., Vidal, G., 2023. Identification of *Cryptosporidium parvum* and *Blastocystis hominis* subtype ST3 in Cholga mussel and treated sewage: Preliminary evidence of fecal contamination in harvesting area. Food Waterborne Parasitol. 34, e00214. https://doi.org/10.1016/j.fawpar.2023.e00214.

Tajima, Y., Sasaki, K., Kashiwagi, N., Yamada, T.K., 2022. A case of stranded Indo-Pacific bottlenose dolphin (Tursiops aduncus) with lobomycosis-like skin lesions in Kinko-wan, Kagoshima, Japan. J. Vet. Med. Sci. 77 (8), 989–992. https://doi.org/10.1292/jvms.14-0366.

Tan, K.S., 2004. *Blastocystis* sp. in humans and animals: new insights using modern methodologies. Vet. Parasitol. 126 (1-2), 121–144. https://doi.org/10.1016/j.vetpar.2004.09.017.

Tantrawatpan, C., Vaisusuk, K., Thanchomnang, T., Pilap, W., Sankamethawee, W., Suksavate, W., Chatan, W., Bunchom, N., Kaewkla, O., Stensvold, C.R., Saijuntha, W., 2023. Distribution of *Blastocystis* subtypes isolated from various animal hosts in Thailand. Res. Vet. Sci. 162, 104939. https://doi.org/10.1016/j.rvsc.2023.06.017.

Tian, J., Du, J., Lu, Z., Han, J., Wang, Z., Li, D., Guan, X., Wang, Z., 2020. Distribution of microbiota across different intestinal tract segments of a stranded dwarf minke whale. (Balaenoptera Acutorostrata). Microbiol. Open 9 (10), e1108. https://doi.org/10.1002/mbo3.1108.

Turroni, S., Fiori, J., Rampelli, S., Schnorr, S.L., Consolandi, C., Barone, M., Biagi, E., Fanelli, F., Mezzullo, M., Crittenden, A.N., Henry, A.G., Brigidi, P., Candela, M., 2016. Fecal metabolome of the Hadza hunter-gatherers: a host-microbiome integrative view. Sci. Rep. 6 (1), 1–9. https://doi.org/10.1038/srep32826.

Villalobos, G., Lopez-Escamilla, E., Olivo-Diaz, A., Romero-Valdovinos, M., Martinez, A., Maravilla, P., Martinez-Hernandez, F., 2024. Genetic variation among the partial gene sequences of the ribosomal protein large-two, the internal transcribed spacer, and the small ribosomal subunit of *Blastocystis* sp. from human fecal samples. Microorganisms 12 (6), 1152. https://doi.org/10.3390/microorganisms12061152.

Volmer, J.G., Evans, P.N., Soo, R.M, Hugenholtz, P., Tyson, G.W., Morrison, M., 2024. Isolation of a *Methanobrevibacter gottschalkii* strain from an Eastern Gray Kangaroo. Front. Microbiol. 15, 1483533. https://doi.org/10.3389/fmicb.2024.1483533.

Wan, X., Li, J., Ao, M., McLaughlin, R.W., Fan, F., Wang, D., Zheng, J., 2023. The intestinal microbiota of a Risso's dolphin (*Grampus griseus*): possible relationships with starvation raised by macro-plastic ingestion. Int. Microbiol. 26 (4), 1001–1007. https://doi.org/10.1007/s10123-023-00355-z.

Wan, X., Li, J., Cheng, Z., Ao, M., Tian, R., McLaughlin, R.W., Zheng, J., Wang, D., 2021. The intestinal microbiome of an Indo-Pacific humpback dolphin (*Sousa chinensis*) stranded near the Pearl River Estuary, China. Integr. Zool. 16 (3), 287–299. https://doi.org/10.1111/1749-4877.12477.

Wan, X., McLaughlin, R.W., Zhou, J., Hao, Y., Zheng, J., Wang, D., 2016. Isolation of culturable aerobic bacteria and evidence of *Kerstersia gyiorum* from the blowhole of captive Yangtze finless porpoises. Antonie Van Leeuwenhoek 109 (8), 1167–1175. https://doi.org/10.1007/s10482-016-0713-6.

Wang, H.J., Chen, D., Ma, Z.H., Liu, C.F., Li, W.D., Hao, Y., Yang, J.S., Lin, Q.B., Zhang, D.W., Li, Y., Yu, Y., Cong, W., Song, L.W., 2024. Molecular detection, subtyping of *Blastocystis* sp. in migratory birds from nature reserves in northeastern China. Acta Trop. 258, 107355. https://doi.org/10.1016/j.actatropica.2024.107355.

Wang, Y., Li, X., Zhao, X., Chen, J., Wang, Z., Chen, L., Zhang, S., Wang, K., 2022. Assessment of fish diversity in the Ma'an Archipelago Special Protected Area using environmental DNA. Biology. (Basel) 11 (12), 1832. https://doi.org/10.3390/biology11121832.

Weil, M., Wang, H., Zak, D., Urich, T., 2023. Spatial and temporal niche separation of *Methanomassiliicoccales* phylotypes in temperate fens. FEMS. Microbiol. Ecol. 99 (6), fiad049. https://doi.org/10.1093/femsec/fiad049.

Yason, J.A., Liang, Y.R., Png, C.W., Zhang, Y., Tan, K.S., 2019. Interactions between a pathogenic *Blastocystis* subtype and gut microbiota: In vitro and in vivo studies. Microbiome 7, 30.