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Research article

Faecal microbiota analysis and transplantation in four oceanaria-based short-finned pilot whales *Globicephala macrorhynchus*

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Abstract

The faecal microbiota of marine mammals has been studied to monitor enteric bacterial infections. Currently, there is no literature characterising the faecal microbiota of live pilot whales *Globicephala macrorhynchus*. Faecal microbiota is affected by genetics, environment, disease, and medications. Dysbiosis, disturbances in microbiota composition, involves pathobiont expansion, reduced diversity and/or loss of beneficial microbes. Faecal microbiota transplantation (FMT) is used in human and veterinary medicine to treat gastrointestinal diseases and to restore gut microbiota. The goals of this study were to characterise the faecal microbiota in four pilot whales in a managed care facility and assess the effects of FMT in one pilot whale to treat recurrent gastrointestinal disease. Baseline faecal microbiota analyses of the four whales via 16S rRNA and ITS next-generation sequencing identified bacteria within the phyla Bacteroidetes, Proteobacteria, Fusobacteria and Firmicutes, and the fungal phyla Ascomycota and Basidiomycota. Serial faecal samples were collected from one donor, the microbiota analysed, and samples processed for oral administration to the FMT recipient via capsules. The recipient's faecal microbiota, analysed on days 0, 5, 10, 16, 28 and 29 following FMT showed increased microbial diversity at the species level. Overall success of the FMT is unclear, as clinical signs of dysbiosis and microbial diversity initially resolved; however, the recipient later had recurring gastrointestinal upset, anorexia and lethargy, requiring aggressive antimicrobial administration for stabilisation.

Introduction

The microbiota consists of all genomes of the microbes in an environment including bacteria, viruses, fungi and archaea (Bojanova and Bordenstein 2016). Studies have shown a high diversity in bacterial composition depending on anatomical site and host species (Bik et al. 2016). Research studying the microbiota in marine mammals is growing, specifically with a focus on gastrointestinal microbiota. Nelson et al. (2015) review marine mammal gut microbiota studies, which have included multiple species: leopard seals *Hydrurga leptonyx*, southern elephant seals *Mirounga leonina*, grey seals *Halichoerus grypus*, hooded seals *Cystophora cristata*, harbour seals *Phoca vitulina*, Australian fur seals *Arctocephalus pusillus doriferus*, Australian sea lions *Neophoca cinerea*, Florida manatees *Trichechus manatus latirostris* and dugongs *Dugong dugong*. Across all these species the gut microbiota is composed largely of Firmicutes, Bacteroidetes and Proteobacteria (Merson et al. 2014; Nelson et al. 2013, 2015).

Bik et al. (2016) evaluated bacterial communities of multiple anatomical sites in 38 healthy bottlenose dolphins managed in human care at the US Navy Marine Mammal Program (MMP) in San Diego, California and 10 healthy wild bottlenose dolphins *Tursiops truncatus* during capture-release health assessments in Sarasota Bay, Florida. Firmicutes, Proteobacteria and Fusobacteria were the most abundant bacterial phyla noted in the rectal specimens of the MMP animals. Interestingly, there was no statistically significant difference between the rectal bacterial communities of the MMP and wild dolphins. Soverini et al. (2016) characterised the gut microbiota composition

of nine adult bottlenose dolphins managed under human care. Twenty-seven faecal samples were evaluated and revealed Firmicutes and Proteobacteria with the highest mean relative abundance, followed by Actinobacteria, Fusobacteria, Tenericutes and Bacteroidetes at the phylum level in the gut microbiota of adult dolphins. Clostridiaceae, Vibrionaceae, Staphylococcaceae, Lactobacillaceae, Peptostreptococcaceae, Ruminococcaceae, Fusobacteriaceae and Pasteurellaceae were the most represented families. These findings are consistent with those of Bik et al. (2016).

Suzuki et al. (2019) compared the gut microbiota of sixteen common bottlenose dolphins managed in human care at three different facilities in Japan and found that Fusobacteria, Firmicutes and Proteobacteria dominated overall. However, the highest mean relative abundance of each phylum varied within the three facilities. This was the first study to show differences in gut microbiota of dolphins reared in different facilities. A comparison of the faecal microbiota of wild and managed Indo-Pacific bottlenose dolphins *Tursiops aduncus* found statistically significant differences between wild and managed populations (Suzuki et al. 2021). Multiple pathogenic bacterial genera, including *Morganella* and *Mycoplasma*, were detected in the dolphins managed under human care, but not the wild dolphins. The wild dolphin faecal microbiota predominated with Proteobacteria (47.8%) and Fusobacteria (41.8%), followed by Firmicutes (5.9%) and Tenericutes (4.2%), whereas Firmicutes (60.2%) and Fusobacteria (39.2%) were identified in higher abundances in the managed dolphins.

Bai et al. (2021) reported findings from an investigation of microbial community composition from the gastrointestinal tract (stomach, midgut and hindgut) of two stranded short-finned pilot whales *Globicephala macrorhynchus*. The first animal was found deceased, whereas the second animal stranded alive and was treated with intravenous antibiotics. The rescued animal died three days following stranding. Samples were collected from both animals during necropsy. Results showed that the gut microbiota of the first stranded (deceased) short-finned pilot whale was dominated by Firmicutes (primarily *Clostridium* spp.) and Fusobacteria, whereas the second stranded (alive) pilot whale consisted of Gammaproteobacteria and Bacteroidetes (primarily *Vibrio* spp. and *Bacteroides* spp., respectively), potentially associated with intestinal disease and antibiotic treatment (Bai et al. 2021).

Bacterial and fungal agents associated with disease in marine mammals have been previously reviewed (Higgins 2000). While gastrointestinal bacterial infections are uncommon in cetaceans, the purported predominant bacterial phylum Firmicutes (*Clostridium* spp.) found consistently in dolphin microbiota has the propensity to cause significant dysbiosis. Buck et al. (1987) and Danil et al. (2014) describe cases of clostridial infection in captive and wild dolphins. Other reported microbial agents that reside in the cetacean gastrointestinal tract that can be pathogenic include *Bacteroides* spp., *Escherichia coli, Enterococcus faecalis, Vibrio* spp., *Morganella morganii* and *Actinobacillus* spp. (Greig et al. 2007).

The microbiota is affected by the host's phylogeny, genetics, gut anatomy, environment, diet, disease and medications (Bik et al. 2016; Ley et al. 2008; Muegge et al. 2011; Shreiner et al. 2015; Wang and Roy 2017). Homeostasis of the gut is preserved by commensal bacteria, functional organ barriers and a tolerant immune response (Wang and Roy 2017). Dysbiosis is an alteration in the microbiota composition and can be categorised as pathobiont expansion, reduced diversity or a loss of beneficial microbes (Petersen and Round 2014). Dysbiosis may contribute to a dysregulated immune response, gut barrier disruption and bowel dysfunction (Wang and Roy 2017).

Faecal microbiota transplantation (FMT) is the process of transferring faecal material from a clinically healthy donor to a recipient. It is becoming a mainstay treatment of dysbiosis and associated gastrointestinal diseases to restore commensal bacteria and optimise gut homeostasis. In human medicine, FMTs have been utilised to successfully treat recurrent *Clostridium difficile* infections and inflammatory bowel disease (Sha et al. 2014). Treatment with FMT was shown to reduce hospitalisation and recovery time in puppies with diarrhoea secondary to parvovirus infection (Pereira et al. 2018). Faecal microbiota transplantation has also shown improvement in faecal consistency in horses treated for antibiotic-induced or undifferentiated colitis (Mullen et al. 2018).

To the authors' knowledge, there are no published reports of an FMT performed in marine mammals. The goal of this study was to characterise the baseline faecal microbiota of four oceanaria-based pilot whales and to perform an FMT to restore homeostasis following a chronic period of dysbiosis in a single animal.

Materials and methods

Study subjects

Four short-finned pilot whales *Globicephala macrorhynchus* managed under human care participated in this study for baseline faecal microbiota analysis. The two males (PW-Rcpt and PW-Donor) and two females (PW1, PW2) shared a multi-pool natural seawater habitat (5.31×106 l) with seventeen bottlenose dolphins *Tursiops truncatus.* Their diets consisted of primarily 80–85% squid (mix of 10% *Loligo opalescens*, 75% *Illex illecebrosus*) and fish (15–20% *Clupea pallasii*) with Mazuri Vita-Zu Mammal Vitamins with lutein supplementation. PW-Rcpt (Pilot Whale-FMT Recipient) was an adult male that stranded in Japan as a juvenile. He was transported to SeaWorld San Diego, California, from Kamogawa Sea World, Japan in 2012 and was estimated to be 21 years of age at the time of this study. The remaining three pilot whales, PW-Donor (Pilot Whale-FMT Donor), PW1 and PW2, were estimated to be approximately 10 years of age, and were deemed non-releasable following a 2012 mass stranding in Fort Pierce, Florida. These animals were transported to SeaWorld Orlando, Florida for rehabilitation in 2012 and then transported to SeaWorld San Diego, California for further care in September 2019.

Sample collection, DNA extraction, library preparation and sequencing

Faecal samples were obtained with voluntary cooperation. The pilot whales presented in dorsal or lateral recumbency alongside the pool wall. An 18 French red rubber catheter was completely inserted into the rectum with sterile lubrication. Approximately 25 ml of sterile saline was infused into the rectum. The faecal sample was then gently aspirated with a 35 ml syringe, placed into a 50 ml conical vial, and transported on ice packs in a cool storage bin to the on-site laboratory. A sterile swab was inserted into the 50 ml conical vial containing the faecal sample, stirred throughout the entire sample, and the swab was placed in the provided MiDOG LLC collection tube for subsampling.

All subsamples used for microbiota analysis were immediately immersed and preserved in DNA/RNA ShieldTM (Zymo Research Corp. Cat. No. R1108, Irvine, California), and stored at room temperature until processing at the MiDOG LLC testing facility (Tustin, California). Genomic DNA was purified using the ZymoBIOMICS-96 DNA kit (Cat. No. D4304, Zymo Research Corp.). Sample library preparation and data analysis for bacterial and fungal profiling were performed by Zymo Research Corp. using the Quick-16S NGS Library Prep Kit (Cat. No. D6400, Zymo Research Corp.), with minor modifications. Primer sequences are proprietary to the MiDOG LLC service and targeted the 16S

rDNA V1-V3 region for bacteria and the ITS-2 region for fungal analysis. Libraries were sequenced using an Illumina HiSeq 1500 sequencer, and reads were filtered through Dada2 (R package version 3.4) (Callahan et al. 2016). Phylotypes were computed as percent proportions based on the total number of sequences in each sample. Relative abundances of bacteria compared to fungi were determined assuming an equivalency of one 16S rDNA copy to one fungal ITS copy, which is considered a rough estimate. Species-level resolution of the sequencing approach used here has been previously demonstrated by shot-gun sequencing (Tang et al. 2020).

Baseline faecal microbiota evaluation

In this study, baseline faecal samples for faecal microbiota evaluation were obtained from all four pilot whales in October 2019, following transport of the three animals (PW-Donor, PW1 and PW2) from SeaWorld Orlando, Florida. All animals were apparently healthy at the time of collection and not on any antibiotic or antifungal therapy. No clinically significant abnormalities were noted on physical examination, behavioural assessment and routine blood analysis (complete blood count, biochemistry panel, fibrinogen measurement, and erythrocyte sedimentation rate). Faecal samples were collected for faecal cytology with Gram stain in early October 2019. The cytological examinations showed variable distribution of Gram-negative rods, Gram-positive rods with spores, Gram-positive cocci, epithelial cells and digested fish spicules. No white blood cells or red blood cells were observed. Additional faecal samples were collected for microbiota analysis in late October 2019.

Serial donor faecal microbiota evaluation

The clinically normal PW-Donor was selected based on blood analysis and behavioural standards, and displayed a normal faecal microflora via faecal cytology with Gram stain and microbiome analysis. The donor had not received antimicrobials for at least seven months prior to donation collection. Faecal samples were collected from the donor over a 21-day period.

These serial collections were individually subsampled and analysed for evaluation of microbiota stability and pathogen screening for the FMT donation. Following subsampling for microbiota evaluation, if the sample appeared significantly diluted, the sample was centrifuged at 1,000 rpm for 5 minutes. Approximately 75% of the diluent was discarded and the pellet was then suspended, resulting in a more concentrated sample. The serial faecal donation samples were collated in 50 ml conical vials following each collection session and stored at -80°C for later evaluation and distribution; sample volumes varied in each collection session. Following the completion of donation sample collection, all samples were thawed on ice in preparation for the FMT and pooled for a single donation solution. Subsampling for microbiota analysis of the single faecal donation solution was performed, and the volume divided equally into two approximately 70 ml doses. Faeces were transferred into eight large gelatine capsules (Torpac, SIZE #07, 24 ml), carrying approximately 15–20 ml each. The first dose (approximately 70 ml) was administered orally immediately following processing. The second dose (approximately 70 ml) was stored in the gelatine capsules at -80°C for administration four days later.

Faecal microbiota transplantation procedure

An FMT was selected as a novel therapy for PW-Rcpt to treat chronic episodic gastrointestinal disease and dysbiosis that developed approximately two months after assimilation of other conspecifics (PW-Donor, PW1 and PW2) into the aquatic environment. PW-Rcpt's clinical signs were characterised by intermittent hyporexia, diarrhoea (loose, foamy stool), lethargy and abnormal behaviour (logging, disinterest in human or conspecific interactions). Cytological evaluation of PW-Rcpt's faecal samples showed recurrence of predominantly Gram-positive rod bacteria with sporulation, presumed to indicate clostridial overgrowth. Blood analyses performed during exhibition of clinical signs showed persistent evidence of inflammatory markers consisting of leukocytosis (elevated white blood cell counts) with a left-shift deviation, elevated fibrinogen and erythrocyte sedimentation rate, decreased alkaline phosphatase test, and low serum iron (Fe). Specific gastrointestinal markers such as cobalamin and folate were not evaluated during this study. These events of gastrointestinal dysbiosis occurred in roughly two-to-three month intervals, requiring medical intervention. Clinical signs resolved with antibiotic administration (including clindamycin, metronidazole and amoxicillin-clavulanic acid) and prophylactic antifungal (nystatin). After a year of episodic antibiotic and prophylactic antifungal treatment for dysbiosis (clostridial overgrowth) and other enteric pathogens (*Escherichia coli* and *Enterococcus* sp.), an FMT was sought to attempt to repopulate the gastrointestinal tract with normal microflora.

Three days prior to the FMT, current antimicrobial treatment (amoxicillin-clavulanic acid 8 mg/kg orally twice daily) was discontinued. A proton pump inhibitor (omeprazole 0.1 mg/kg orally once daily) was administered during the treatment period to reduce gastric acidity and minimise donor microbial degradation. One day prior to the FMT, the current prophylactic antifungal (nystatin 7000 IU/kg orally three times daily) was discontinued. An antacid was administered with minimal food approximately 2–4 hours prior to faecal microbiota transplantation. The first FMT dose (approximately 70 ml) was administered orally (PO) at 1200 on day 0. On day 3 following the FMT, a low-dose steroid (dexamethasone 0.05 mg/kg orally once daily) was administered to treat suspected post-transplantation discomfort and hyporexia. On day 4, the second dose (approximately 70 ml) of the FMT was administered orally (PO).

Prior to the FMT, PW-Rcpt blood analysis indicated analytes within normal range. Following the FMT, clinical markers of inflammation, notably leukocytosis with a left-shift deviation, elevated fibrinogen and erythrocyte sedimentation rate, and decreased total serum iron were observed (Table 1).

Results

Baseline faecal microbiota

Baseline faecal samples were collected for all four whales (PW-Rcpt, PW-Donor, PW1 and PW2) following the transfer of three animals (PW-Donor, PW1 and PW2) from SeaWorld Orlando, Florida to SeaWorld San Diego, California. These samples were analysed for their bacterial and fungal profile. In total, 72 different bacterial species were detected, of these 35 species represented at least 1% of the bacteria in one of the whales, and 31 species were present above 1% relative abundance on average across the four samples. These 31 species were very diverse between different samples. For readability, the species listed in Figure 1A are those that represent at least 2% abundance on average across the samples. On average across the four samples, the most abundant bacterial species were *Photobacterium damselae* (12.38%, 4 out of 4 whales), *Clostridiales* sp. (9.43%, 4/4), *Clostridium perfringens* (8.66%, 4/4), *Clostridium disporicum* (6.95%, 4/4) and *Terrisporobacter glycolicus* (5.20%, 4/4). On average, each whale had 44.5 different bacterial species at the baseline sample collection.

The fungal profile was less diverse than the bacterial profile. Only 18 different fungal species were detected, of which seven represented at least 1% of the mycobiome (Figure 1B). The most abundant fungi on average were *Candida albicans* (17.96%, 1/4),

Table 1. PW-Rcpt clinically relevant haematological and serum chemistry markers of inflammation and hepatic transaminase values associated with gastrointestinal dysbiosis and faecal microbiota transplantation (FMT) procedure. Blood values were monitored during FMT procedure, 3 days prior and up to 52 days after. Baseline values (mean of 2019 results) and mean for all PW-Rcpt blood values obtained since transfer to SeaWorld San Diego, CA (n=56) are provided for reference.

Blood values	Baseline	Day relative to FMT (Spring 2020)						Reference
	Mean 2019	-3	13	15	27	29	52	Mean n=56
Haemoglobin (g/dl)	14.2	13.9	15.4	14.8	14	11.7	11.1	13.2
Haematocrit (%)	42.1	41.6	45.5	43.8	42.8	34.9	31.9	38.5
White blood cell (103/µl)	6.10	6.49	24.04	22.36	18.24	14.87	7.61	8.35
Band neutrophil (%)	1	3	7	8	10	8	0	3
Erythrocyte sedimentation rate 60 min (mm/hr)	68	70	88	102	115	122	64	74.9
Total protein (g/dl)	5.9	5.5	6.9	6.4	6.6	5.3	5.2	5.81
Alkaline phosphatase (IU/L)	309	337	83	74	88	86	52	241.9
ALT (IU/L)	22	17	146	133	141	159	57	50.9
AST (IU/L)	227	111	356	351	556	547	315	270.5
GGT (IU/L)	27	24	104	90	206	171	112	58.4
CK (IU/L)	103	112	55	147	99	184	64	107.4
LD (IU/L)	446	316	742	810	1321	1504	688	633.9
Serum iron (µg/dl)	127	129	15	18	46	62	210	100.2
Fibrinogen (mg/dl)	426	483	>1000	>1000	>1000	966	770	580.1

Alternaria sp. (6.01%, 3/4), an unclassified fungal species (3.79%, 3/4), a species from the class Dothideomycetes (3.20%, 2/4) and *Candida parapsilosis* (2.68, 2/4). No single fungal species was found in all samples.

Serial donor faecal microbiota analysis

Serial faecal samples from the donor animal (PW-Donor) were collected at 14 time points over a duration of 21 days (23 November 2020 to 13 December 2020). In total, 105 different bacterial species were detected, and 43 different fungal species (Figure 2). Of these, five bacteria were present at all time points: *Photobacterium damselae* (48.54%, 14/14), *Actinobacillus* sp. (18.10%, 14/14), *Fusobacterium ulcerans* (5.55%, 14/14), *Clostridium perfringens* (3.63%, 14/14) and *Escherichia coli* (2.10%, 14/14). On average, nine bacterial species represented at least 1% abundance across all time points in addition to those mentioned above: *Fusobacterium mortiferum-necrogenes* (4.84%, 13/14), *Actinobacillus delphinicola* (1.95%, 13/14), *Fusobacterium* sp. (2.36%, 12/14) and *Paeniclostridium sordellii* (2.21%, 11/14). All other bacteria were not consistently present across the 14 samples. Interestingly, it appears that there may have been an antagonistic relationship between *Photobacterium damselae* and *Actinobacillus* sp. (Figure 2C).

No single fungal species was present at all 14 time points. The most frequent and abundant species were *Toxicocladosporium rubrigenum* (13.81%, 8/14), *Saccharomyces cerevisiae* (1.02%, 7/14), *Candida albicans* (14.85%, 6/7), *Stereum complicatum* (4.75%, 5/14) and *Malassezia restricta* (1.24%, 5/14).

Faecal microbiota transplantation

The FMT collated donation sample contained 33 different bacterial species in total, ten of which were present at above 1% relative abundance. The most abundant bacterial species were *Photobacterium damselae* (54.84%), *Clostridium perfringens* (7.70%), *Fusobacterium mortiferum-necrogenes* (6.23%), *Paeniclostridium sordellii* (6.03%), *Actinobacillus* sp. (5.66%), and *Actinobacillus delphinicola* (4.07%) (Figure 3). *Clostridium disporicum* and *C. baratii* were the only two species that were present in the donation sample but not in the recipient prior to the FMT and then were found on day 5 in the first post-FMT faecal sample.

The collated FMT donation sample contained nine different fungal species in total, eight of which were present at above 1% relative abundance. The most abundant fungal species were *Nakaseomyces glabrata* (46.70%), *Candida albicans* (29.12%), *Cladosporium* sp. (6.87%), *Kondoa aeria* (4.25%) and a species of the order Dothideales (3.73%). None of the fungal species in the donation samples were found in the first post-FMT faecal sample (day 5).

Clinical signs of decreased appetite and decreased behavioural cooperation were observed 5 days post-FMT and a low-dose appetite stimulant (dexamethasone 0.04 mg/kg orally) and anti-nausea agent (maropitant 1 mg/kg orally) was prescribed. Post-FMT nausea has been reported in the human medical literature, therefore symptomatic therapy was considered appropriate (Sha et al. 2014). On day 13, clinical signs including hyporexia and lethargy progressed; supportive care, including injectable antimicrobial therapy (ampicillin/sulbactam 30 mg/kg intramuscularly, amikacin 10 mg/kg intramuscularly, ceftazidime 20 mg/kg intramuscularly), oral antifungal therapy (voriconazole 0.5 mg/kg) and oral gastrointestinal supportive therapy (famotidine 0.5 mg/kg and simethicone 2.5 mg/kg) was implemented. Blood analysis revealed a marked leukocytosis of 24×103 cells/µl (χ=8.4×103 cells/µl), 7% band neutrophils (χ=3%), elevated hepatic transferases (AST 356 U/l, χ=278 IU/l; GGT 104 U/l, χ=60 IU/l; LDH 742 U/l, χ=646 IU/l), marked elevation in fibrinogen (>1000 mg/dl, χ=589 mg/dl) and elevated erythrocyte sedimentation rate (88 mm/hr, χ=77 mm/hr). Faecal samples were collected and analysed for

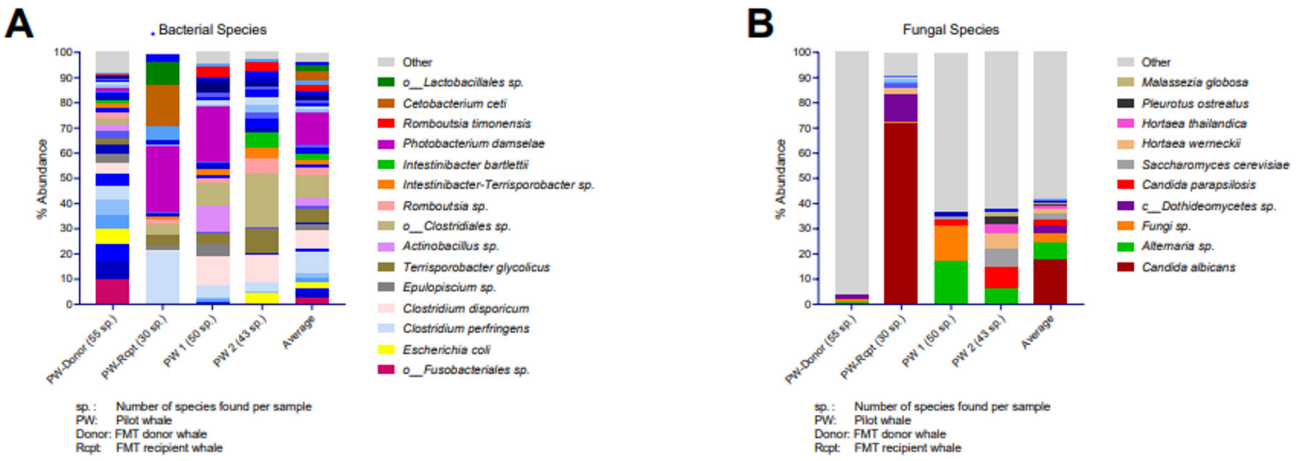

Figure 1. Microbial profile for each individual and the average across all four samples: (A) bacterial species profile and (B) fungal species profile. The number of detected species per individual are listed in parentheses following each whale's identifier. Listed bacteria species were present at a minimum of 2% average abundance, and listed fungi represent at least 2% of the mycobiome in any sample. Not all taxa could be identified to the species level. In those cases, taxonomic identity was established to the order (o_) or class (c__) level.

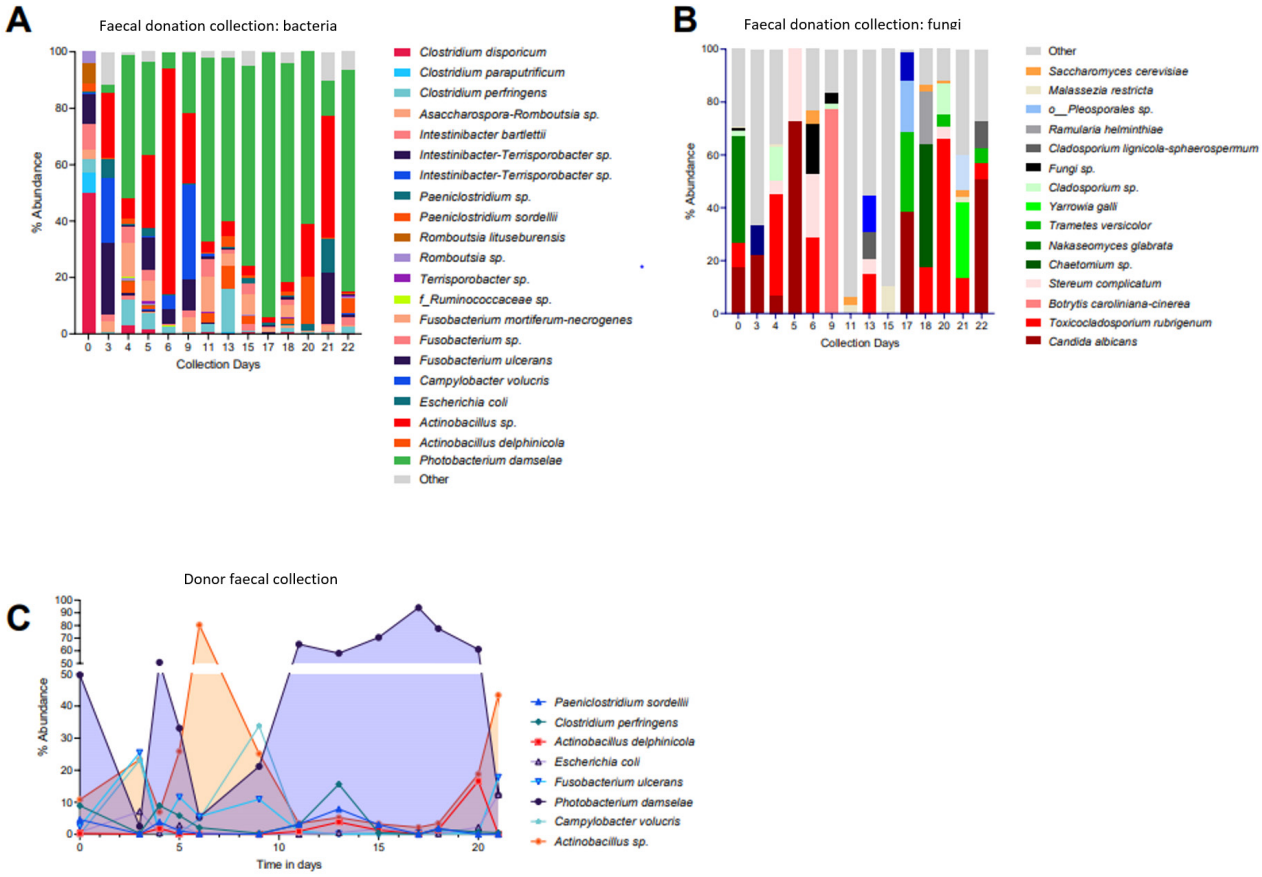

Figure 2. Microbial profile of donor faeces over time: A) bacterial composition, B) fungal composition and C) bacterial species over time for a subset of the species. For readability, in A) and B) only those species that represented at least 1% of the microbiota at a given time point and/or were present in at least half of the samples (n=7) are shown. In the fungal profile, any fungus representing more than 10% at a given time point is shown.

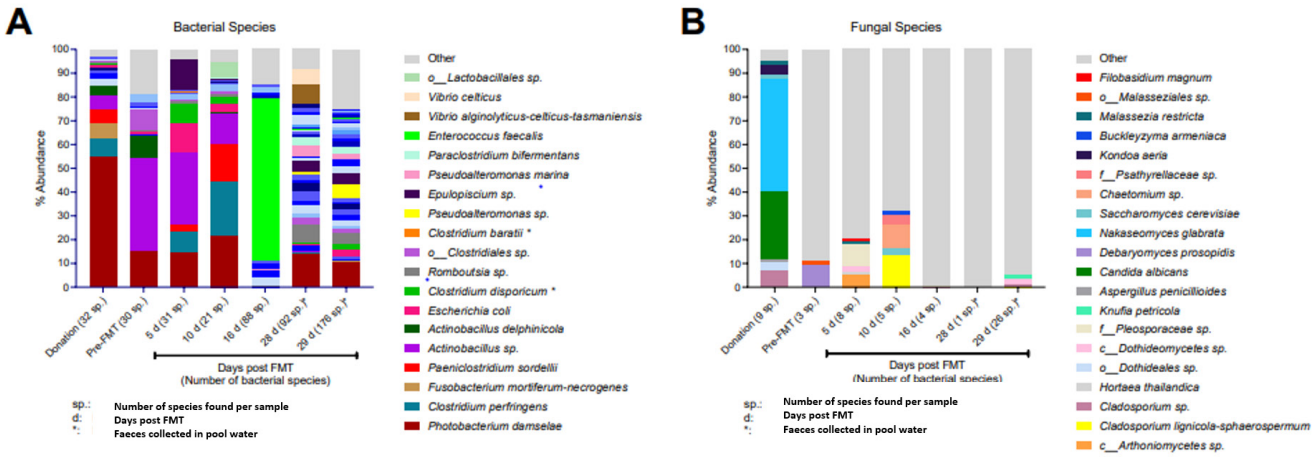

Figure 3. Microbial profile of faecal microbiota transplantation (FMT) donation sample and FMT-recipient over time: A) bacterial composition for species that represent at least 4% of the bacterial profile. (*) indicates those species that were not present in the recipient pre-FMT, but were in the donation sample and were then detected in the recipient post-FMT; B) fungal composition for species that represent at least 1% of the fungal profile. Number of bacterial and fungal species indicated by sp. Samples for days 28 and 29 were collected opportunistically by obtaining samples of pool water with faeces following defecation; microbiota samples may be contaminated or diluted. The number of detected species per sample are listed in parentheses following the day identifier.

microbiota on: days 0, 5, 10, 16, 28 and 29. Faecal samples from days 28 and 29 were collected opportunistically from pool water immediately following defecation. Faecal culture results revealed fairly antibiotic-resistant *Escherichia coli* and *Enterococcus faecalis* pathogens. This guided parenteral antimicrobial therapy (amikacin and ertapenem) with interventional supportive care consisting of oral fluids, oral antibiotics (metronidazole, amoxicillin-clavulanic acid and faropenem), prophylactic antifungal (voriconazole) and hepatic function support (silymarin, S-adenosylmethionine) for eight days to treat progressive ulcerative colitis that developed post-FMT. Approximately seven weeks post-FMT (day 52), PW-Rcpt was clinically stable on oral antibiotic treatment with haematological and chemistry analytes improving.

Discussion

This study assessed the faecal microbiota in four pilot whales managed under human care. These samples are considered baseline for this small population due to their normal clinical status and lack of antimicrobial administration at the time of collection. These baseline data were collected at a single time point following the transfer of PW-Donor, PW1 and PW2 from Florida to California. The most relatively abundant bacteria on average for the four individuals at the phylum (and species) levels were Proteobacteria (*Photobacterium damselae)* and Firmicutes (*Clostridium perfringens, Clostridium disporicum* and *Terrisporobacter glycolicus*).

Faecal samples from the clinically normal donor pilot whale were collected in the subsequent year, over the duration of three weeks. Five bacteria within the phyla Proteobacteria (*Photobacterium damselae, Actinobacillus* sp., and *Escherichia coli*), Firmicutes (*Clostridium perfringens*) and Fusobacteria (*Fusobacterium ulcerans*) were present at all time points. This may indicate the stability of these bacterial phyla within the donor pilot whale faecal microbiota. Previous literature from Bik et al. (2016) and Soverini et al. (2016) assessing the gut or faecal microbiota of dolphins further confirms that cetaceans may have a distinctive faecal microbiota population dominated by Firmicutes, Proteobacteria and Fusobacteria. Studies have also shown that differences in relative microbiota abundance can vary by anatomical site and be influenced by microbial communities present in the piscivorous diet and surrounding aquatic environment (Bik et al. 2016; Muegge et al. 2011; Robles-Malagamba et al. 2020; Suzuki et al. 2021). Robles-Malagamba et al. (2020) found consistent but distinct microbiota communities between anatomical sites in wild bottlenose dolphins that were unique to their surrounding aquatic environment and—similarly to Bik et al. (2016)—found no significant patterns between microbiota abundance and age, sex or environment.

Fungal and yeast infections have been reported in healthy and unhealthy cetaceans. To the authors' knowledge, there is limited published data assessing the comprehensive gut mycobiome in healthy marine mammals using next-generation sequencing technology. The microbiota analysis in the current study also describes the mycobiome found in the gastrointestinal tract of four pilot whales in a managed care facility. The baseline mycobiome of the four pilot whales assessed in this study was less diverse than the bacterial profile, with only 18 fungal species detected, 7 of which represented at least 1% of the mycobiome. The most relatively abundant fungi on average were *Candida albicans, Alternaria* sp., an unclassified fungal species, a species from the class Dothideomycetes and *Candida parapsilosis*. *Candida albicans* is considered a commensal fungal resident of mucous membranes and was identified in up to 54% of wild bottlenose dolphins examined in Sarasota Bay, Florida from 1990 to 1992 (Reidarson et al. 2001). The skin and mucosa are effective barriers against invasive *Candida* sp. in a healthy host; when these barriers are disrupted or the host is immunocompromised, local invasion and

infection may occur (Reidarson et al. 2001). *Candida parapsilosi*s was reported to be found on culture and cytology of a blowhole lesion of a pygmy sperm whale *Kogia breviceps* (Reidarson et al. 2001). *Alternaria* sp. has not been reported in marine mammals, but has been shown to cause infections in various organs of cats, horses, zebras and dogs (Seyedmousavi et al. 2013). No single fungal species was found in all samples, indicating variability within the group.

When assessing the mycobiome of the donor pilot whale's serial faecal samples, no single fungal species was present at all 14 time points, showing the variability of the mycobiome within an individual over a short duration of time. The most frequent and abundant fungi were *Toxicocladosporium rubrigenum*, *Saccharomyces cerevisiae, Candida albicans, Stereum complicatum* and *Malassezia restricta*. While *M. restricta* has not been reported as a fungal agent found in marine mammals, *Malassezia pachydermatis* has been identified via histopathology and culture from the skin of a California sea lion *Zalophus californianus* (Guillot et al. 1998; Reidarson et al. 2001). *Toxicocladosporium rubrigenum* has not been described as a pathogen, but only as an environmental fungus found on a *Eucalyptus camaldulensis* leaf growing in Madagascar (Crous et al. 2009). In a recent study, *Saccharomyces cerevisiae* was found in the gastrointestinal tracts and skin of marine fish and on the surface of mangrove trees in China, but was not found in surrounding water or sediments (Tian et al. 2021). *Stereum complicatum* is a fungus typically found on decomposing wood throughout eastern and central North America (Emberger 2008). The final fungus identified in the pilot whale mycobiome results, a species most closely related to the Dothideomycetes class, may be most similar to the marine Dothideomycetes and Sordariomycetes from mangroves and deep-sea sediments (Jones et al. 2020).

To the authors' knowledge, there are no published studies of FMT in marine mammals; a lack of literature provided scant species-specific guidance for procedural protocols. Methods were extrapolated from studies in humans and terrestrial species (Kao et al. 2017; Mullen et al. 2018; Stallmach et al. 2020). Routes of administration have varied over time from enemas, colonoscopy, nasoduodenal or nasogastric tube, and more recently orally via encapsulation in gastric-acid resistant capsules (Mullen et al. 2018; Stallmach et al. 2020). In a randomised clinical trial, FMT via oral capsules was as effective as via colonoscopy in treating recurrent *Clostridium difficile* infection in humans (Kao et al. 2017). Oral administration of FMT capsules was selected for this study as this method is the least invasive, least stressful and most logistically practical for the large size of the study patient. Donor screening protocols were also extrapolated from human and equine FMT protocols; donor selection criteria included positive health status without antimicrobial administration in the previous 3–6 months (Mullen et al. 2018; Stallmach et al. 2020). Mullen et al. (2018) recommend discontinuation of antimicrobials and treatment with proton pump inhibitors for FMT recipient preparation prior to FMT in horses; similar methods were followed in this study.

Determining the success of the FMT in this study is difficult. The microbial diversity of the faecal microbiota increased following the first dose of the FMT, from 30 species on day 0 to 88 species on day 16 post-FMT. However, the relative abundances of the post-FMT microbiota were not similar to the donation sample microbiota and gastrointestinal clinical signs recurred within two weeks following the initial dose of the FMT. The dominant growth of *Escherichia coli* and *Enterococcus faecalis* over commensal microflora suggests a complex pathogenic strategy to overwhelm luminal barrier defences with release of endotoxins and disabling proteins to infect the host via paracellular pathways (Steck et al. 2011). Without commensal microbiota established with the two doses of FMT, clinical disease ensued and medical intervention was required.

The microbiota results from the faecal sample 16 days post-FMT showed signs of marked microbiota alteration following antimicrobial administration, resulting in a dysbiosis predominated by *Enterococcus faecalis* (relative abundance of 68.27%). Costa et al. (2015) showed changes in intestinal microbiota in horses following the administration of systemic antimicrobials. Twenty-four healthy adult horses received either procaine penicillin or ceftiofur sodium intramuscularly, or trimethoprim sulfadiazine (TMS) orally for 5 consecutive days. Faecal samples were obtained for high throughput sequencing of the 16S rRNA prior to drug administration, on day 5 (following treatment), and on days 14 and 30. The most significant changes were observed immediately after treatment (day 5); though there was still a discernible difference on day 30, the microbiota was closer to the baseline results, indicating a recovery following antimicrobial administration. Microbiota analysis findings showed specific and different responses to each antimicrobial. TMS most significantly impacted the microbiota, with decreased bacterial species diversity and richness. Further studies may improve understanding of the effects on intestinal microbiota of the route of administration or spectrum of the drug (Costa et al. 2015).

This study contained multiple limitations. The small subject sample size of four pilot whales in a single managed care facility—the only pilot whales managed under human care at this time—is a significant limitation. The results of this study provide insight to the faecal microbiota of these four individuals rather than a representation of an entire wild population of this species. The difficulty of obtaining faecal samples from healthy wild cetaceans often leads to reliance on information gathered from stranded, ill or deceased animals for baseline comparisons. In this study faecal samples were collected with voluntary cooperation as part of routine husbandry behaviour. Maintaining these husbandry behaviours with positive animal-trainer interactions is crucial in minimising stress for animals managed under human care. The texture of cetacean faeces, along with limited opportunities for behavioural requests due to timing constraints and animal cooperation, led to a small quantity of faecal samples collected for donation. Mullen et al. (2018) recommend administration of 2–3 l of faecal solution for an adult horse averaging approximately 1,000 pounds (~454 kg) in weight. Therefore, the small faecal donation volume (two 70 ml doses) may have been ineffective for the goal of restoring a healthy gut microbiota. Further studies may include supplementary evaluation of this collection of animals on a temporal scale over several years, and during different seasons and life stages. Additional considerations may be provided for a potential use of probiotics or repeated FMT procedure with alterations to dose volume, sample processing, frequency, duration or route of administration.

Conclusion

The faecal microbiota transplantation treatment in this study did not achieve long-term resolution of clinical signs associated with dysbiosis and microbial diversity restoration in one individual as intended. However, this study contributes to investigations of marine mammal faecal microbiota, mycobiota and gastrointestinal disease pathogenesis, and supports discussion about the use of probiotics, the importance of judicious use of antibiotic therapy and the potential for promoting antibiotic resistance. This study may provide a framework for evaluating unconventional treatment of gastrointestinal disease in marine mammals with appropriate testing. The findings are comparable to other wild and managed cetacean faecal microbiota studies, including the mycobiome, and may be informative for future microbiota surveillance and health monitoring.

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References

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*). *Integrative Zoology* 16(3): 324–335.

Bik E.M., Costello E.K., Switzer A.D., Callahan B.J., Holmes S.P., Wells R.S., Carlin K.P., Jensen E.D., Venn-Watson S., Relman D.A. (2016) Marine mammals harbor unique microbiotas shaped by and yet distinct from the sea. *Nature Communications* 7: 10516.

Bojanova D.P., Bordenstein S.R. (2016) Fecal transplants: What is being transferred? *PLoS Biology* 14(7): e1002503.

Buck J.D., Shepard L.L., Spotte S. (1987) *Clostridium perfringens* as the cause of death of a captive Atlantic bottlenosed dolphin (*Tursiops truncatus*). *Journal of Wildlife Diseases* 23(3): 488–491. doi:10.7589/0090-3558-23.3.488

Callahan B.J., McMurdie P.J., Rosen M.J., Han A.W., Johnson A.J.A., Holmes S.P. (2016) DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods* 13: 581–583.

Costa M.C., Stämpfli H.R., Arroyo L.G., Allen-Vercoe E., Gomes R.G., Weese J.S. (2015) Changes in the equine fecal microbiota associated with the use of systemic antimicrobial drugs. *BMC Veterinary Research* 11: 19. doi:10.1186/s12917-015-0335-7

Crous P.W., Wingfield M.J., Groenewald J.Z. (2009) Niche sharing reflects a poorly understood biodiversity phenomenon. *Persoonia* 22: 83–94. doi:10.3767/003158509X439364

Danil K., St. Leger J.A., Dennison S., Bernaldo de Quirós Y., Scadeng M., Nilson E., Beaulieu N. (2014) *Clostridium perfringens* septicemia in a long-beaked common dolphin *Delphinus capensis*: An etiology of gas bubble accumulation in cetaceans. *Diseases of Aquatic Organisms* 111: 183–190. doi:10.3354/dao02783

Emberger G. (2008) *Stereum complicatum*. Retrieved from https://www.messiah.edu/Oakes/fungi_on_wood/crust%20and%20parchment/species%20pages/Stereum%20complicatum.htm

Greig T.W., Bemiss J.A., Lyon B.R., Bossart G.D., Fair P.A. (2007) Prevalence and diversity of antibiotic resistant *Escherichia coli* in bottlenose dolphins (*Tursiops truncatus*) from the Indian River Lagoon, Florida, and Charleston Harbor Area, South Carolina. *Aquatic Mammals* 33(2): 185–194. doi:10.1578/AM.33.2.2007.185

Guillot J., Petit T., Degorce-Rubiales F., Guého E., Chermette R. (1998) Dermatitis caused by *Malassezia pachydermatis* in a California sea lion (*Zalophus californianus*). *The Veterinary Record* 142(12): 311–312.

Higgins R. (2000) Bacteria and fungi of marine mammals: A review. *The Canadian Veterinary Journal* 41(2): 105–116.

Jones E., Devadatha B., Abdel-Wahab M., Dayarathne M., Zhang S., Hyde K., Liu J., Bahkali A., Sarma V., Tibell S., Tibell L., Wang M., Liu F., Cai L. (2020) Phylogeny of new marine Dothideomycetes and Sordariomycetes from mangroves and deep-sea sediments. *Botanica Marina* 63(2): 155-181.

Kao D., Roach B., Silva M., Beck P., Rioux K., Kaplan G.G., Chang H.J., Coward S., Goodman K.J., Xu H., Madsen K., Mason A., Wong G.K.S., Jovel J., Patterson J., Louie T. (2017) Effect of oral capsule- vs colonoscopy-delivered fecal microbiota transplantation on recurrent *Clostridium difficile* infection: A randomized clinical trial. *Journal of the American Medical Association* 318(20): 1985–1993. doi:10.1001/jama.2017.17077

Ley R.E., Hamady M., Lozupone C., Turnbaugh P.J., Ramey R.R., Bircher J.S., Schlegel M.L., Tucker T.A., Schrenzel M.D., Knight R., Gordon J.I. (2008) Evolution of mammals and their gut microbes. *Science* 320(5883): 1647–1651. doi:10.1126/science.1155725

Merson S.D., Ouwerkerk D., Gulino L.M., Klieve A., Bonde R.K., Burgess E.A., Lanyon J.M. (2014) Variation in the hindgut microbial communities of the Florida manatee, *Trichechus manatus latirostris* over winter in Crystal River, Florida. *FEMS Microbiology Ecology* 87(3): 601–615.

Muegge B.D., Kuczynski J., Knights D., Clemente J.C., González A., Fontana L., Henrissat B., Knight R., Gordon J.I. (2011) Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science* 332(6032): 970–974. doi:10.1126/science.1198719

Mullen K.R., Yasuda K., Divers T.J., Weese J.S. (2018) Equine faecal microbiota transplant: Current knowledge, proposed guidelines and future directions. *Equine Veterinary Education* 30(3): 151–160. doi:10.1111/eve.12559

Nelson T.M., Rogers T.L., Carlini A.R., Brown M.V. (2013) Diet and phylogeny shape the gut microbiota of Antarctic seals: A comparison of wild and captive animals. *Environmental Microbiology* 15(4): 1132–1145. doi:10.1111/1462-2920.12022

Nelson T.M., Apprill A., Mann J., Rogers T.L., Brown M.V. (2015) The marine mammal microbiome: Current knowledge and future directions. *Microbiology Australia* 36(1): 8–13. doi:10.1071/MA15004

Pereira G.Q., Gomes L.A., Santos I.S., Alfieri A.F., Weese J.S., Costa M.C. (2018) Fecal microbiota transplantation in puppies with canine parvovirus infection. *Journal of Veterinary Internal Medicine* 32(2): 707–711. doi:10.1111/jvim.15072

Petersen C., Round J.L. (2014) Defining dysbiosis and its influence on host immunity and disease. *Cellular Microbiology* 16(7): 1024–1033. doi:10.1111/cmi.12308

Reidarson T.H., McBain J.F., Dalton L.M., Rinaldi M.G. (2001) Mycotic disease. In: Dierauf L.A., Gulland F.M.D. (eds.). *CRC Handbook of Marine Mammal Medicine. 2nd edition*. Boca Raton, Florida: CRC Press, 337–355.

Robles-Malagamba M.J., Walsh M.T., Ahasan M.S., Thompson P., Wells R.S., Jobin C., Fodor A.A., Winglee K., Waltzek T.B. (2020) Characterization of the bacterial microbiome among free-ranging bottlenose dolphins (*Tursiops truncatus*). *Heliyon* 6(6): e03944. doi:10.1016/j.heliyon.2020.e03944

Seyedmousavi S., Guillot J., de Hoog G.S. (2013) Phaeohyphomycoses, emerging opportunistic diseases in animals. *Clinical Microbiology Reviews* 26(1): 19–35. doi:10.1128/CMR.00065-12

Sha S., Liang J., Chen M., Xu B., Liang C., Wei N., Wu K. (2014) Systematic review: Faecal microbiota transplantation therapy for digestive and nondigestive disorders in adults and children. *Alimentary Pharmacology and Therapeutics* 39(10): 1003–1032. doi:10.1111/apt.12699

Shreiner A.B., Kao J.Y., Young V.B. (2015) The gut microbiome in health and in disease. *Current Opinion in Gastroenterology* 31(1): 69–75. doi:10.1097/MOG.0000000000000139

Soverini M., Quercia S., Biancani B., Furlati S., Turroni S., Biagi E., Consolandi C., Peano C., Severgnini M., Rampelli S., Brigidi P., Candela M. (2016) The bottlenose dolphin (*Tursiops truncatus*) faecal microbiota. *FEMS Microbiology Ecology* 92(4): fiw055. doi:10.1093/femsec/fiw055

Stallmach A., Steube A., Grunert P., Hartmann M., Biehl L.M., Vehreschild M.J.G.T. (2020) Fecal microbiota transfer. *Deutsches Ärzteblatt International* 117(3): 31–38. doi:10.3238/arztebl.2020.0031

Steck N., Hoffmann M., Sava I.G., Kim S.C., Hahne H., Tonkonogy S.L., Mair K., Krueger D., Pruteanu M., Shanahan F., Vogelmann R., Schemann M., Kuster B., Sartor R.B., Haller D. (2011) *Enterococcus faecalis* metalloprotease compromises epithelial barrier and contributes to intestinal inflammation. *Gastroenterology* 141(3): 959–971. doi:10.1053/j.gastro.2011.05.035

Suzuki A., Segawa T., Sawa S., Nishitani C., Ueda K., Itou T., Asahina K., Suzuki M. (2019) Comparison of the gut microbiota of captive common bottlenose dolphins *Tursiops truncatus* in three aquaria. *Journal of Applied Microbiology* 126(1): 31–39. doi:10.1111/jam.14109

Suzuki A., Akuzawa K., Kogi K., Ueda K., Suzuki M. (2021) Captive environment influences the composition and diversity of fecal microbiota in Indo-Pacific bottlenose dolphins, *Tursiops aduncus. Marine Mammal Science* 37(1): 207–219. doi:10.1111/mms.12736

Tang S., Prem A., Tjokrosurjo J., Sary M., Van Bel M.A., Rodrigues-Hoffmann A., Kavanagh M., Wu G., Van Eden M.E., Krumbeck J.A. (2020) The canine skin and ear microbiome: A comprehensive survey of pathogens implicated in canine skin and ear infections using a novel next-generation-sequencing-based assay. *Veterinary Microbiology* 247: 108764. doi:10.1016/j.vetmic.2020.108764

Tian B.C., Liu G.L., Chi Z., Hu Z., Chi Z.M. (2021) Occurrence and distribution of strains of *Saccharomyces cerevisiae* in China Seas. *Journal of Marine Science and Engineering* 9(6): 590. doi:10.3390/jmse9060590

Wang F., Roy S. (2017) Gut homeostasis, microbial dysbiosis, and opioids. *Toxicologic Pathology* 45(1): 150–156. doi:10.1177/0192623316679898