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Short Note

Microbiological Assessment of Some Culturable Microbiota from Clinically Healthy Bottlenose Dolphins (*Tursiops truncatus*) Under Human Care

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The mammalian commensal microbiota constitutes over a thousand bacterial phylotypes (Suchodolski, 2014) and confers important functions, including a mucosal barrier function, a metabolic function, and an immune regulatory function, which contribute to the development and regulation of the gut immune system (Backhed et al., 2005). The composition of microbiota can be influenced by various factors, including diet, exposure to antibiotics, and the well-functioning mechanisms of immune tolerance (Koenig et al., 2011). Unfortunately, there are few reports describing gastrointestinal microflora in cetacean species, and the available information is related to free-ranging dolphins (Morris et al., 2011). The knowledge of normal gastrointestinal microflora from healthy dolphins under human care, in addition to allowing a wider understanding of the role of bacteria in animals with physiological disorders, could permit an evaluation of the general health status of wild marine mammals and the potential transmission risks to humans.

The aim of this study is to detect and enumerate some culturable microorganisms from the gastric juice and faeces of clinically healthy bottlenose dolphins (*Tursiops truncatus*) kept under human care to establish baseline data for this species for the first time. A total of 31 healthy bottlenose dolphins, 17 males and 14 females of 5 to 36 y of age (mean ± SE = 16 ± 1 y), weighing 149.9 to 266.7 kg (mean ± SE = 190.1 ± 4.3 kg) and hosted in three different facilities (A, B & C), were examined and sampled. The water in the first and the second facility (A & B) was an artificial salt water, disinfected by chlorine. The water in the third facility (C) was sea water, disinfected by ozone. Average water temperature in the different facilities was 23° C (73.4° F). Microbiological routine checks were carried out on the water of each facility before sampling to assess water quality.

The diet of all animals during the study consisted of capelin (*Mallotus villossus*), spratt (*Sprattus sprattus*), herring (*Clupea harengus*), mackerel (*Scomber scombrus*), horsemackerel (*Trachurus trachurus*), smelt (*Atherina boyeri*), squid (*Loligo opalescens*), and blue whiting (*Micromesistius poutassou*). Only the animals considered clinically healthy based upon physical exam, laboratory evaluation (i.e., complete blood count and serum chemistry, urinalysis, and faecal examination), cytology, and ultrasound evaluation were included in the study. Criteria for exclusion of individuals from the study included pregnancy and lack of good health or presence of ongoing disease.

Gastric samples were taken by inserting a 0.95-cm diameter sterile and flexible polyethylene tube (Pharmaplast, Milan, Italy) into the forestomach with mild pressure and rotation. Gastric juice samples were collected using a negative pressure siphon technique into a 120-ml polypropylene sterile container with screw cap (LP Italia, Milan, Italy). Faecal samples were collected by inserting a 0.40-cm diameter sterile and flexible polyethylene tube (Pharmaplast) into the anal orifice up to the intestinal tract. One mL of gastric juice of each bottlenose dolphin were put into sterile tubes together with 2 mL 0.9% sterile saline solution. The same procedure was carried out with 1 g of faeces. Each solution was brought to volume (10 mL)

with 0.9% sterile saline solution. Each sample (0.1 mL) was serially diluted via ten-fold dilutions (from 10-1 to 10-10). Starting from the lowest concentration, dilutions were plated and cultured on different media in triplicate using the spread plate method. Chromocult agar and Baird Parker agar were used, respectively, for the enumeration of *E. coli*/Coliforms and Staphylococci. All the plates were aerobically incubated at 37° C for 24 to 48 h. Reinforced Clostridial agar enriched with 5% sheep blood and 1 mg/mL K1 vitamin, and Rose Bengal Agar were used, respectively, for the enumeration of *Clostridium* spp. and yeasts. Anaerobic incubation was carried out in anaerobic jars (Oxoid) at 37° C for 48 to 72 h. The number of colonies was counted, and all the data were expressed as CFU × log/g.

The use of the good manual practice is of paramount importance to exclude potential external contamination by microorganisms or pathogens. For this reason, quality and assurance control methods were included during the analysis. Log transformation of the data was carried out before statistical analysis to meet the assumption of normal distribution criteria and reduce variability. For the determination of the reference intervals (RI) in healthy dolphins, the "Guidelines for the Determination of Reference Intervals in Veterinary Species" issued by Friedrichs et al. (2012) were followed. RI were calculated as arithmetic mean ± 2 SD for normal data (Bland, 2000). Outliers were examined using Horn's algorithm (Tukey's fences interquartile). Normality of data distribution of each variable and homoscedasticity among groups were assessed by a Shapiro-Wilk and Levene test, respectively. In cases for which variables failed tests for normality, the median was used as a measure of central tendency, and the RI was given as the central 95th percentile (Friedrichs et al., 2012).

To evaluate reliability and biological variations, analytical (CV_A) and interindividual (CV_G) variations were calculated, respectively, as the mean intra-assay coefficient of variation (CV) and the CV for values between individuals (Bland, 2000; Walton, 2012). Coefficient of quartile variation (CQV) was used for non-normal data (Bonett, 2006). In order to compare means between gastric juice and faeces, a paired sample *t* test or Wilcoxon signed-rank test was used. To describe the relative proportion of each examined microflora component, proportions of each component were presented wherein the total of the examined microorganisms was set at 100%. Comparisons between gender, age, weight, and location were performed using General Linear Model procedures. In the model, intercept was excluded while age was included as covariation where appropriate; $p < 0.05$ were considered to be statistically significant. All analyses were performed with *SPSS*, Version 20.0 (SPSS, Inc., Chicago, IL, USA), while an *Excel* spreadsheet was used for Tukey's fences interquartile and CV calculations.

Body weight was significantly higher in males than in females (193 ± 8 in males and 183 ± 9 kg in females; $p = 0.004$) and increased with age of the animal ($b = 10.3$, $p = 0.000$). A total of 31 and 30 samples of faeces and gastric juice, respectively, were analyzed. One dolphin was excluded from *E. coli* evaluation in gastric juice because the value exceeded the interquartile fence (1.65 to 4.83 Log CFU/gr). Mean or median, RIs, and CVs for the bacteria tested in the gastric juice and faecal samples are summarized in Table 1.

Dolphins were of different sexes, ages, and habitat conditions. However, no statistically significant differences were microbiologically found between male and female dolphins. In regard to faecal *E. coli*, values of seven dolphins were below or above the interquartile fence (4.14 to 5.21 Log CFU/gr) and were excluded. Due to non-normality of distribution, robust methods were used for Coliforms and yeast data, respectively, in gastric juice and faeces. *E. coli* is a ubiquitous organism and has been isolated from faeces obtained from free-ranging bottlenose dolphins in multiple geographic areas. The overall prevalence of *E. coli* was 48 to 52% (Greig et al., 2007). In our study, *E. coli* was detected in gastric juice and in faeces, respectively, with a mean of 3.25 and 4.70. The highest interindividual biological variations (CV or CQV > 18%) were recorded for faecal Staphylococci and for yeasts in both gastric juice and faeces. Microorganisms in gastric juice were not influenced by any variable considered (i.e., age, gender, and facility).

Faecal Staphylococci was affected by the facility ($F = 15.21$, $p = 0.000$), being higher in dolphins reared in B than in C (5.18 Log CFU/gr for B dolphins and 3.51 Log CFU/gr for C dolphins; $p = 0.000$). Dolphins reared in A showed intermediate values of Staphylococci (4.41 Log CFU/ gr). The Staphylococci count ($t = 5.31$, $df = 29$, $p < 0.001$) in faeces was increased compared to the count in gastric juice. These results can be attributed to the diet, gut morphology and physiology, and other environmental factors. These distributions also may be related to the quality of the waters in which the animals were located: bacteria would be expected to be higher in chlorinated artificial waters due to the higher sensibility to the ozone (Clark et al., 1993). A low rate of yeasts was found in both gastric juice and faecal samples. In our study, faecal yeasts tended to increase as the body weight of dolphins increased ($b = 0.011$, $F = 6.43$, $p = 0.017$), but they had the highest interindividual biological variations.

Table 1. Microflora composition (Log CFU/g) of gastric juice and faeces of healthy adult bottlenose dolphins (*Tursiops truncatus*)

		n	Mean	RI	95% CI for RI	CV_A (%)	CV_G (%)
Gastric juice	Coliforms#	30	5.13	3.36-5.91	—	1.4	11.9
	E. coli	29	3.25	2.10-4.39	[1.74, 2.46] [4.03, 4.75]	3.9	17.6
	Staphylococci	30	3.72	2.45-4.99	[2.05, 2.84] [4.60, 5.39]	2.6	17.1
	Yeasts	30	4.49	2.75-6.23	[2.21, 3.29] [5.70, 6.78]	2.6	19.4
	Clostridium spp.	30	6.07	4.82-7.32	[4.43, 5.21] [6.94, 7.71]	2.1	10.3
Faeces	Coliforms	31	5.11	3.70-6.52	[3.27, 4.13] [6.09, 6.95]	4.5	13.8
	E. coli	24	4.70	4.39-5.01	[4.28, 4.50] [4.91, 5,12]	3.3	3.3
	Staphylococci	31	4.62	2.62-6.63	[2.00, 3.23] [6.02, 7.24]	6.3	21.7
	Yeasts#	31	4.59	3.32-5.67	—	5.5	19.9
	Clostridium spp.	31	5.73	4.33-7.13	[3.91, 4.76] [6.71, 7.56]	4.5	12.2

Note: *n* = number of individuals sampled, CV_A = analytical variation, CV_G = interindividual variation, and # = robust method; median, central 95th percentile, and coefficient of quartile variation were reported instead of mean, RI by parametric method, and coefficients of variation, respectively.

This study provides preliminary but relevant data that could be very useful to clinicians. Cultivation based on analysis is still the simplest and most inexpensive system to quantify gut bacteria. While this technique does not permit detection of some species- or strain-level variations and does not require extensive bio-informatic analysis (Sekirov et al., 2010), it is functional to evaluate physiological parameters, and these can be carried out by the veterinarian as a routine examination via voluntary behaviour to assess the health of the animals.

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