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# The bacterial diversity in infected tissue pus of an East Asian finless porpoise

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**Two East Asian finless porpoises (*Neophocaena asiaeorientalis sunameri*) in Ningbo, East China Sea, China, were observed to be bacterially infected between the fat layer and muscle layer. The microbial communities in pus samples were identified by metagenome sequencing 16S in the PacBio platform and explored the relationship between emaciation and bacterial infection. The present paper was the first report on bacterial diversity in infected tissue pus of finless porpoise. In total, 101 bacterial species were identified, and the top nine species were *Rhodococcus qingshengii* (26.25%), *Rhodococcus jialingiae* (22.43%), *Ralstonia pickettii* (16.03%), *Moraxella osloensis* (9.97%), *Psychrobacter cibarius* (2.97%), *Flavobacterium johnsoniae* (2.11%), *Flavobacterium chungbukense* (1.80%), *Stenotrophomonas maltophilia* (1.73%) and *Serratia marcescens* (1.62%). These main bacteria could cause various diseases or metabolic disorder, such as endocarditis, meningitis, bacteraemia, septicaemia, and so on; they also involve microbiome metabolism of amino acids (12.47%), carbohydrates (11.58%), xenobiotic biodegradation (7.81%), lipids (6.01%) and energy (4.98%). Regardless of the type of disease metabolic disorder will firstly cause body weight loss. In conclusion, these bacteria could cause diseases or metabolic disorders, resulting in emaciation of East Asian finless porpoises. Admittedly, pathogenic assay is furthermore needed to determine the mechanisms regarding the pathological phenomena.**

**Key words:** Bacterial diversity, East Asian finless porpoises, Metagenome sequencing 16S.

## INTRODUCTION

Infectious diseases caused by bacteria were thought to be the primary cause of mortalities in aquatic mammals (Dunn et al., 2001). These bacteria contributed to different degrees of pathogenicity. For example, a *Helicobacter* sp. isolated from stomachs may play a role in the etiopathogenesis of gastritis and gastric ulcers in dolphins (Harper et al., 2000). Pathogenic vibrios have been found, which might lead to, secondary infections in debilitated (e.g., stranded) marine mammals was a distinct possibility (Buck and Stephen, 2010). *Brucella* sp. infections and associated lesions were described in a harbour porpoise (*Phocoena phocoena*) (Jauniaux et al.,

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2010). 20 bacterial pathogens were identified in captive and free-ranging killer whales or sympatric odontocetes species, and they were considered to affect fecundity or reproductive success (Gaydos et al., 2004).

Studies on infectious disease in marine mammals have seldom been conducted in China. Although nearly 45 marine mammal species harbor in Chinese waters (Zhou, 2004), the specimens of most animals were difficult to obtain except for finless porpoise. The East Asian finless porpoise (*Neophocaena sunameri*) was one of three finless porpoises species (Zhou et al., 2018), and most common marine mammal was found stranded or by caught. 170 porpoises were accidentally caught in Bohai and the Northern Yellow Sea from 2004 to 2008 (Liu, 2009). At present, bacterial infections in wild finless porpoises has rarely been reported due to scarce infected specimens and limitation of technology.

To identify these bacteria/microorganism species, 16S ribosomal RNA (rRNA) gene sequencing was commonly used as these genes comprise of highly conserved domains, interspersed with variable regions (You, 2005; Pootakham et al., 2017). To begin with, 16S rRNA gene profiling was performed using clone-based Sanger sequencing, which provides accurate, full-length or almost full-length sequences. Due to the high cost and low-output nature of the approach, the number of 16S rRNA sequencing used in Sanger-based bacterial profiling studies were often lower than 200 sequences per sample, which was insufficient to cover the complete diversity of the bacterial community (Sharp et al., 2012). Currently, various next generation sequencing platforms such as Roche 454 (Bayer et al., 2013), Illumina (Schmidt et al., 2013) and Ion Torrent PGM (Salipante et al., 2014) have gradually replaced the use of the Sanger sequencing method. Of these new sequencing platforms, the Pacific Biosciences (PacBio) single-molecule, real-time (SMRT) sequencing technology was recommended to be capable of analyzing the bacterial profiles based on the full length 16S rRNA gene (Amir et al., 2013; Pootakham et al., 2017). This technology favours metagenomic analysis and affords the researcher a relatively unbiased view of the microbial communities at different taxonomic levels of a sample (Charuvaka and Rangwala, 2011).

In Ningbo, East China Sea, China, two East Asian finless porpoises were observed to be bacterial infections between the fat and the muscle layers. The present paper focused on the bacterial diversity in the tissue pus sample, and the relationship between bacterial composition and infections symptoms. The recommended SMRT sequencing of the full-length 16S rRNA gene were applied.

## MATERIALS AND METHODS

### Sample collection

Within February and May 2009, 12 dead East Asian finless porpoises from fisheries by catch accidentally, in Ningbo, East China Sea were collected. The collection and research on specimens were authorized by Ningbo Ocean and Fisheries Bureau. Then pathological anatomy was conducted later on. During the necropsy, the green pus was found between the abdominal fat and muscle layers of two dead finless porpoises (Figure 1). Except of green pus, the fat and muscle tissues were normal, no lesions were found. The pus samples of one finless porpoise were collected in a sterile valve bags and stored at -20°C until its DNA was extracted.

Both infected porpoises were male with a body length of 114 and 118 cm. To explore the differences in nutritional status between infected porpoises and non-infected porpoises, six morphological characteristics, that is, dorsal blubber thickness, lateral blubber thickness, ventral blubber thickness, cervical blubber thickness, axilla girth and maximum girth were chosen. Meanwhile, to minimize the influence of aging and gender, data on five dead male finless porpoises with similar body lengths (113 to 119 cm) were collected in the same period, used for comparison (Table 1).

### 16S rRNA gene amplification and sequencing

Genomic DNA was extracted using the MoBio PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA quality was assessed on 0.8% agarose gel to ensure no degradation or rRNA contamination. The DNA samples were subsequently quantified using a spectrophotometer (Eppendorf, model RS-232C, Hamburg, Germany).

The diversity of bacterial communities was analyzed using single molecule real-time PacBio sequencing technology (Pacific Biosciences, Menlo Park, CA, USA). The full-length 16S ribosomal RNA gene was amplified from genomic DNA using the bacterial-specific primer. The primer sequences were 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'- ACCTTGTTACGACTT-3'.

To obtain barcoded 16S rRNA amplicons, the amplifications in two steps was performed. A first round of PCR was performed in a 25 µL PCR solution containing 5 µL NEB Q5 High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), 5 µL GC Enhancer (New England Biolabs, Ipswich, MA), 2 µL DNTP mixture (2.5 mmol/$L^{-1}$), 1 µL DNA template (20 ng/$\mu L^{-1}$), 1 µL of each primer (10 µmol/$L^{-1}$), and 10 µL sterilized distilled water. The thermal cycling conditions were as follows: 98°C for 2 min; 25 cycles of 98°C for 30 s; 50°C for 30 s; and a final extension time of 90 s at 72°C. The primer of the second round of PCR contained a set of 16-nucleotide barcodes for the barcoded SMRT sequencing of the full-length 16S rRNA gene. The primary PCR products were diluted to 2 ng/$\mu L^{-1}$, and the diluted products were used as templates for the secondary amplification. A second round of PCR was performed in a 25 µL PCR solution containing 5 µL NEB Q5 High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), 5 µL GC Enhancer (New England Biolabs, Ipswich, MA), 2 µL DNTP mixture (2.5 mmol/$L^{-1}$), 1 µL DNA template (2 ng/$\mu L^{-1}$), 1 µL of each primer (10µmol/$L^{-1}$), and 10 µL sterilized distilled water. The conditions used for amplification in the thermocycler were as follows: 98°C for 2 min; 15 cycles of 98°C for 30 s; 62°C for 30 s; and 72°C for 90 s; and a final extension time of 5 min at 72°C.

A PacBio library was constructed using the Template Prep Kit 1.0 (Pacific Biosciences, Menlo Park, CA, USA) containing a pool of barcoded amplicons from the bacterial sample. The full-length 16S rRNA gene was sequenced using P6-C4 chemistry on a PacBio RS II instrument (Pacific Biosciences, Menlo Park, CA, USA).

### Sequence data analysis and microbial population identification

Raw data were processed by the SMRT Portal, Version 2.7 (PacBio) to obtain effective sequences with a minimum of 3 full

**Figure 1.** A mass of green pus was found between the abdominal fat layer and muscle layer in a dead East Asian finless porpoise in Ningbo, East China Sea, China.

passes and a minimum predicted accuracy of 90 (Hou et al., 2015). The sequencing data were processed using the software package QIIME version 1.8.0 (Caporaso et al., 2010). Sequences shorter than 500 bp were removed prior to downstream analyses. Chimeric sequences were detected and removed using USEARCH version 5.2.236 (Edgar, 2010). The remaining sequences were clustered into OTUs (Blaxter et al., 2005) based on an "open-reference" OTU-selecting method at 97% identity using UCLUST (Edgar, 2010) (Figure 2). Taxonomy was assigned to the representative sequence of each OTU using the Greengenes database version 13.8 (Desantis et al., 2006), Ribosomal Database Project (RDP) database version 11.1 (Cole et al., 2009) and Silva database version 115 (Quast et al., 2013). The OTUs occurring only once or twice were discarded. After the sequencing data were rarefied, the following alpha diversity measures were calculated: Number of OTUs, Chao1 estimate of species richness (Chao, 1984), ACE estimate of species richness (Dawid, 1993), Shannon diversity (Shannon, 1948), and Simpson diversity (Simpson, 1949).

GraPhlAn is a new tool for compact and publication-quality representation of circular taxonomic and phylogenetic trees with potentially rich sets of associated metadata (Asnicar et al., 2015), and it was used to quickly detect the dominant microbe groups from the complex community data in this study. Then, KEGG PATHWAY Database (http://www.genome.jp/kegg/pathway.html) in PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013) was applied to predict the functional composition of the metagenome of the microbial community from its 16S profile.

## RESULTS

### Bacterial diversity

Following the removal of chimeras and circular consensus sequencing reads shorter than 500 bp, a total of 30768 reads of processed full-length 16S rRNA sequences were obtained for their samples. In total, 101 bacterial species were identified, and they belonged to six phyla: Actinobacteria (50.62%), Proteobacteria (42.24%), Bacteroidetes (5.06%), Firmicutes (1.82%), Cyanobacteria (0.22%) and Acidobacteria (0.05%). Four alpha diversity indices were calculated; the Chao1 estimator (307) was similar to the ACE estimator (307) while the Shannon diversity index and Simpson index were 4.2 and 0.86, respectively. Various colours on the GraPhlAn circular taxonomic and phylogenetic trees were used to distinguish among different taxa and their abundance were reflected through the node size (Figure 3).

Among the bacterial species, *Rhodococcus qingshengii* (26.25%), *Rhodococcus jialingiae* (22.43%), *Ralstonia pickettii* (16.03%) and *Moraxella osloensis* (9.97%) were the most prevalent (Table 2). Of these bacteria, two bacteria, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*, can produce green pigmentations consistent with the green pus in the two dead finless porpoises.

### Prediction of microbial metabolic function

The KEGG analysis indicated that 12 metabolic functions were associated with the microbial samples isolated from the infected finless porpoise. The major functions were amino acid metabolism (12.47%), carbohydrate metabolism (11.58%), xenobiotics biodegradation and

**Table 1.** The body measurements of infected finless porpoises and non-infected individuals and T-Test results.

| Sample ID | Infected porpoises | | | Non-infected porpoises | | | | | | *T* Test |
|---|---|---|---|---|---|---|---|---|---|---|
| | 200924 | 200935 | Average | 200931 | 2010055 | 2011005 | 2015039 | 2016011 | Average | P value |
| Sex | M | M | - | M | M | M | M | M | - | |
| Body length (cm) | 114 | 118 | 116 | 117.5 | 114.2 | 119 | 114 | 113 | 115.54 | 0.710 |
| Dorsal blubber thickness (cm) | 1.6 | 1 | 1.3 | 1.7 | 1.6 | 1.4 | 2.8 | 2.5 | 2 | **0.063** |
| Lateral blubber thickness (cm) | 1.4 | 1.6 | 1.5 | 1.6 | 1.7 | 1.9 | 3 | 2.5 | 2.14 | **0.074** |
| Ventral blubber thickness (cm) | 1.8 | 1.6 | 1.7 | 1.6 | 2 | 1.7 | 3.1 | 2 | 2.08 | 0.228 |
| Cervical blubber thickness (cm) | 1.4 | 1 | 1.2 | 1.9 | 1.5 | 2.2 | 2.2 | 2.5 | 2.06 | **0.007*** |
| Axilla girth(cm) | 64.5 | 68 | 66.3 | 70 | 62.1 | 69 | 67 | 72 | 68.02 | 0.353 |
| Maximum girth (cm) | 68 | 73 | 70.5 | 72 | 66.1 | 72 | 72 | 76 | 71.62 | 0.518 |

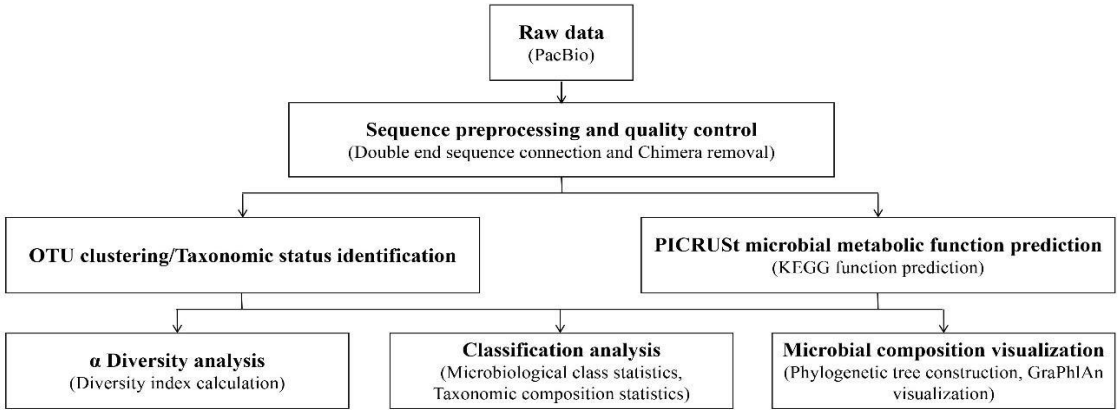


**Figure 2.** The framework of sequences data analysis of the bacterial diversity in an infected East Asian finless porpoise from the East China Sea.

metabolism (7.81%), lipid metabolism (6.01%) and energy metabolism (4.98%) (Figure 4).

## Nutritional status comparison

The mean value of the six bodies indices (dorsal blubber thickness, lateral blubber thickness, ventral blubber thickness, cervical blubber thickness, axilla girth and maximum girth) of the infected porpoises were less than in the non-infected porpoises (Figure 5). One sample t-test was performed using IBM SPSS Statistics version 21 (SPSS, Inc., Chicago, Ill., USA), and it showed significantly different cervical blubber thickness (p=0.007) and nearly significant lateral blubber thickness (p=0.074) and dorsal blubber thickness (p=0.063) (Table 1).

This indicated that the bacteria-infected individuals were more emaciated than the non-infected individuals.

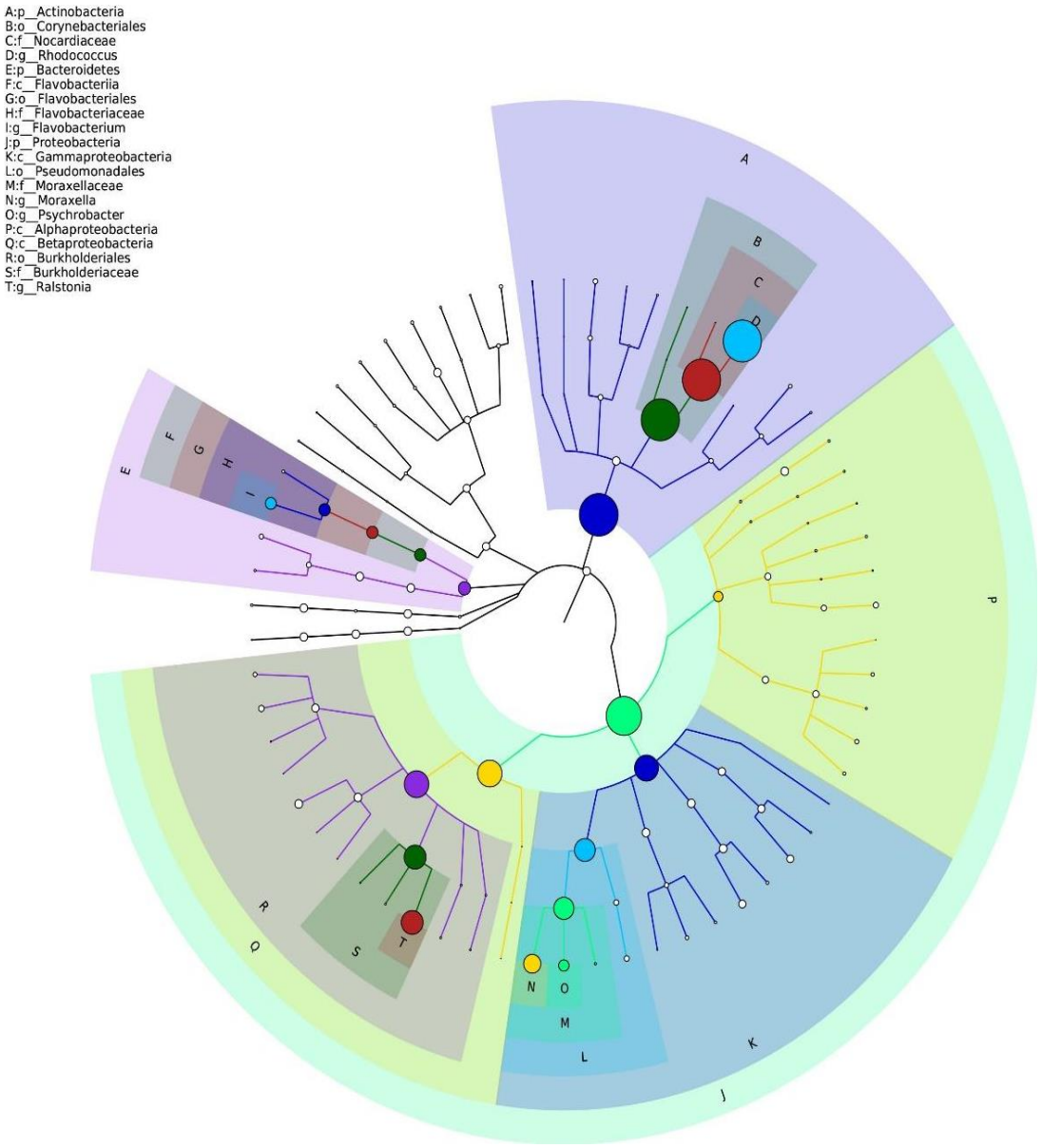


**Figure 3.** The hierarchical tree displays the relationships among all taxa, from phylum to species (from the inner circle to the outer circle arranged in turn). Average relative abundance is represented with node size, and the top 20 taxa are numbered with letters (A-T). p: Phylum, c: Class, o: Class, f: Family, g: Genus, s: Species.

## DISCUSSION

In this paper, single molecule real-time (SMRT) sequencing, developed by Pacific BioSciences (PacBio) were used to overcome the limitations of first- and second-generation sequencing (Rhoads and Au, 2015), which were hardly enough to capture the complete diversity of the bacteria (Pootakham et al., 2017). Therefore, this paper provided a more precise and reliable sequences than first- and second-generation technology.

The infected porpoises generally showed clear notable marasmus unlike the non-infected porpoises. This indicated that the porpoises might have been suffering from infections, which resulted in consumption of fats and body marasmus. Although these assumed diseases have not been determined, the analysis of the pathogenicity of the bacteria provides us with some insights.

A total of 101 bacteria were identified, among them, observations of *R. qingshengii* (26.25%) and *Rhodococcus jialingiae* (22.43%) have been reported in

**Table 2.** Bacterial species and pathogenicity from the sticky sample isolated from an infected finless porpoise from Ningbo, China, in 2009.

| Scientific name | Percentage | Possible source | Possible disease caused | Reference |
|---|---|---|---|---|
| *Rhodococcus qingshengii* | 26.25 | Carbendazim-contaminated soil | Pale liver, splenomegaly, melanosis in abdominal cavity, internal haemorrhaging | 1, 2 |
| *Rhodococcus jialingiae* | 22.43 | Carbendazim-contaminated water | | 1, 3 |
| *Ralstonia pickettii* | 16.03 | Industrial wastewater | Endocarditis, meningitis, bacteraemia, septicaemia, pneumonia, osteomyelitis, septic arthritis, spondylitis | 4, 5 |
| *Moraxella osloensis* | 9.97 | Unknown | Endocarditis, osteomyelitis, septic arthritis, bacteraemia, meningitis, pneumonia, endophthalmitis, respiratory failure | 6, 7, 8, 9 |
| *Psychrobacter cibarius* | 2.97 | Refrigeration | Unknown | 10 |
| *Flavobacterium johnsoniae* | 2.11 | Soil | Skin disease | 11, 12 |
| *Flavobacterium chungbukense* | 1.80 | Soil | Unknown | 13 |
| *Stenotrophomonas maltophilia* | 1.73 | Soil, water, air | Acral necrosis, endocarditis, meningitis, bacteraemia, osteochondritis, sinusitis, diffuse bronchitis, chronic enteritis, septic arthritis, otitis externa, spondylodiscitis, pyogenic liver abscess pyelonephritis, respiratory infection, pneumonia | 14, 15, 16, 17 |
| *Serratia marcescens* | 1.62 | Unknown | Respiratory tract infection, urinary tract infection, septicaemia, meningitis, endocarditis | 18 |
| *Aquabacterium commune* | 1.49 | Unknown | Unknown | |
| *Pelomonas saccharophila* | 1.08 | Unknown | Unknown | |
| *Pseudomonas aeruginosa* | 0.92 | Water, soil | Ulcerative keratitis, otitis extema, skin and soft tissue infections, pneumonia. | 18, 19, 20, 21 |

Reference Number: 1, Xu et al., 2007; 2, Avendaño-Herrera et al., 2011; 3, Wang et al., 2010; 4, Kulakov et al., 2002; 5, Ryan et al., 2006; 6, Shah et al., 2000; 7, Vuori-Holopainen and Pehola, 2001; 8, Berrocal et al., 2002; 9, Gargiulo et al., 2015; 10, Bowman, 2006; 11, Flemming et al., 2007; 12, Carson et al., 2010; 13, Lim et al., 2011; 14, Grimont and Grimont, 1984; 15, Fujita et al., 1996; 16, Hanes et al., 2002; 17, Falagas et al., 2009; 18, Kiska and Gilligan, 2003; 19, Paterson, 2006; 20, Driscoll et al. , 2007; 21, Avalos-Te´llez et al., 2010.

the sludge of a carbendazim wastewater treatment facility and carbendazim-contaminated soil (Wang et al., 2010). *Ralstonia pickettii* has been identified in biofilm formation in ultrapure water in industrial systems (Kulakov et al., 2002). The pathogenicity of these species has been reported in some aquatic species, e.g., genus *Rhodococcus* and *Flavobacterium johnsoniae* for freshwater and marine fish (Olsen et al., 2006; Flemming et al., 2007) and a West African dwarf crocodile that died from acute *Stenotrophomonas maltophilia* septicaemia (Harris and Rogers, 2001). The finless porpoise as a top predator in marine environments very likely to contacted these bacteria along the marine food cycle.

Meanwhile, *Moraxella osloensis* (9.97%), *Flavobacterium johnsoniae* (2.11%), *Stenotrophomonas maltophilia* (1.73%), *Serratia marcescens* (1.62%) and *Pseudomonas aeruginosa* (0.92%) were conditioned pathogens. Most of them are widely distributed in nature and exist in animals as normal flora. When this symbiotic relationship turns into parasitism caused by bacterial translocation, as in dysbacteriosis or host-lowered immunity, these normal floras might damage their hosts (Sun et al., 2011).

Additionally, species of the genus *Psychrobacter* have been isolated from various low-temperature habitats or sources, including sea ice, fish, chilled meat and blood products, krill stomach, Antarctic

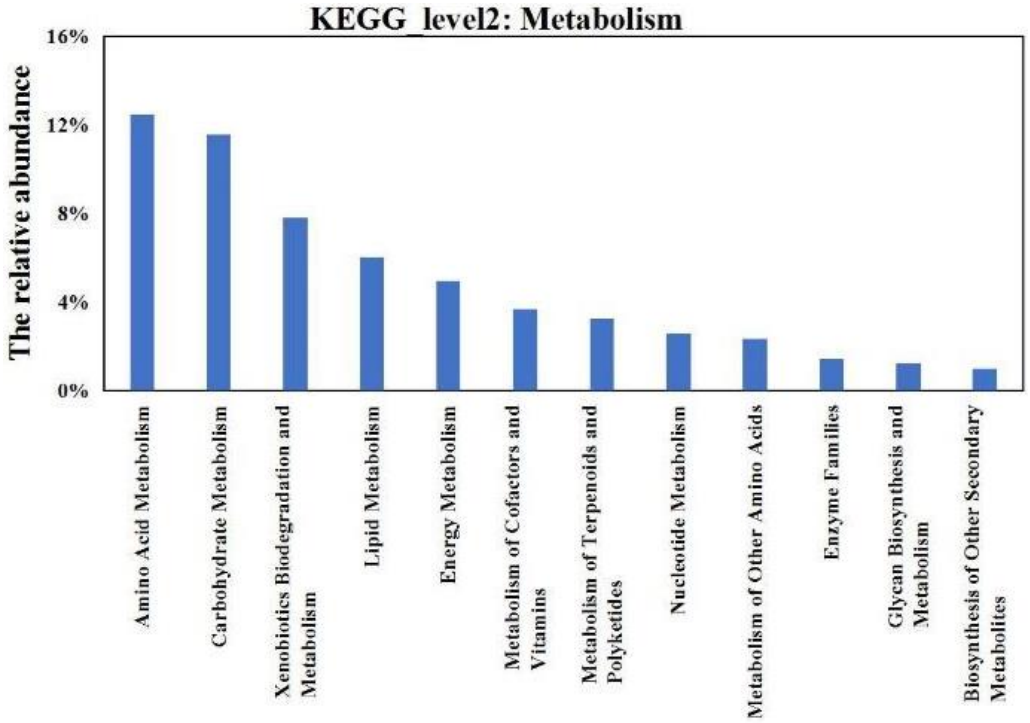


**Figure 4.** Histogram of prediction of microbial metabolic functions plotted with KEGG functional composition on the horizontal axis and the relative abundance on the vertical axis.

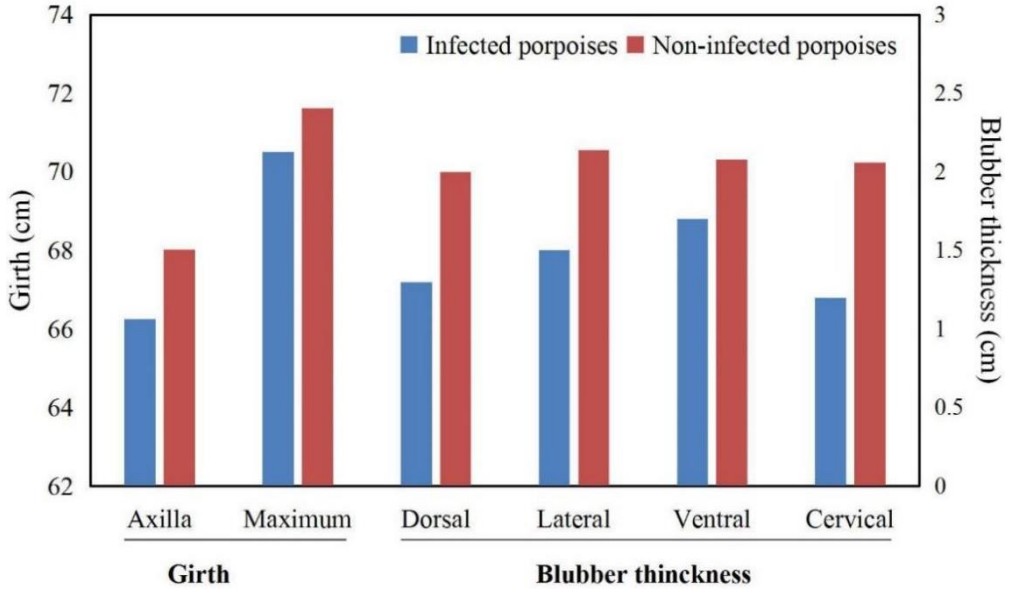


**Figure 5.** Comparison of the mean value of six body indices between infected porpoises and non-infected porpoises. Blubber thickness: Dorsal blubber thickness, lateral blubber thickness, ventral blubber thickness and cervical blubber thickness; Girth: Axilla girth and maximum girth.

ornithogenic soil and contaminants on lab media (Bowman, 2006), and *Psychrobacter cibarius* (2.97%) has never been detected as an animal pathogen. It can be presumed that the possible origin in the sample was refrigeration in the refrigerator.

Microorganisms and their hosts share the same

environment, and the activities of the microbiomes and their metabolic products influence a variety of aspects in metazoans (Lee and Hase, 2014). This was supported by the KEGG analysis in this study, that is, the microbial communities in finless porpoise involved in major metabolism functions which were the most fundamental requirements for survival, and metabolic disorders may lead to various health problems.

In fact, the main bacterial species have been proven to be related to various pathogenicities, skin diseases, respiratory diseases, diverse inflammations, hematologic diseases, and visceral lesions. For example, *Flavobacterium johnsoniae* and *Pseudomonas aeruginosa* were known as opportunistic skin pathogens (Driscoll et al., 2007; Carson et al., 2010), and they cause soft tissues infections in fish species (Carson et al., 2010). *Moraxella osloensis* was a pathogen that causes respiratory failure (Gargiulo et al., 2015). *Stenotrophomonas maltophilia* could cause endocarditis, meningitis, osteochondritis, sinusitis, diffuse bronchitis, chronic enteritis, septic arthritis, otitis externa, spondylodiscitis, pyogenic liver abscess pyelonephritis (Falagas et al., 2009) and pneumonia (Hanes et al., 2002). *Pseudomonas aeruginosa* has been reported to cause chronic bronchopneumonia in bottlenose dolphins (Avalos-Te´llez et al., 2010). *Ralstonia pickettii* has been associated with unusual clinical situations such as bacteraemia and septicaemia (Ryan et al., 2006). Infection from *Rhodococcus qingshengii* could cause severe visceral lesions, e.g., inducing the presence of the pseudo-membranes on the spleen, liver and heart. Histopathology revealed perivascular leucocytic infiltration, marked granulomas and increased fibroblastic reticular cells in spleen (Avendaño-Herrera et al., 2011).

Based on the analysis above, it was speculated that bacterial infections cause some diseases and metabolic disorders, which might impact the health of finless porpoises to some extent. Regardless of the type of diseases and metabolic disorders would first cause of body weight loss. Further study on pathogenicity experiment was needed in the future.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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