Identification of *Lactobacillus* strains with probiotic features from the bottlenose dolphin (*Tursiops truncatus*)

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Abstract

**Aims:** In order to develop complementary health management strategies for marine mammals, we used culture-based and culture-independent approaches to identify gastrointestinal lactobacilli of the common bottlenose dolphin, *Tursiops truncatus*.

**Methods and Results:** We screened 307 bacterial isolates from oral and rectal swabs, milk and gastric fluid, collected from 38 dolphins in the U.S. Navy Marine Mammal Program, for potentially beneficial features. We focused our search on lactobacilli and evaluated their ability to modulate TNF secretion by host cells and inhibit growth of pathogens. We recovered *Lactobacillus salivarius* strains which secreted factors that stimulated TNF production by human monocytoid cells. These *Lact. salivarius* isolates inhibited growth of selected marine mammal and human bacterial pathogens. In addition, we identified a novel *Lactobacillus* species by culture and direct sequencing with 96.3% 16S rDNA sequence similarity to *Lactobacillus ceti*.

**Conclusions:** Dolphin-derived *Lact. salivarius* isolates possess features making them candidate probiotics for clinical studies in marine mammals.

**Significance and Impact of the Study:** This is the first study to isolate lactobacilli from dolphins, including a novel *Lactobacillus* species and a new strain of *Lact. salivarius*, with potential for veterinary probiotic applications. The isolation and identification of novel *Lactobacillus* spp. and other indigenous microbes from bottlenose dolphins will enable the study of the biology of symbiotic members of the dolphin microbiota and facilitate the understanding of the microbiomes of these unique animals.

Introduction

Little is known about the composition and functions of the symbiotic gastrointestinal microbiota of marine mammals, or the exact contribution of various microbes to disease in these animals (Venn-Watson et al. 2008). Gastroenteritis occurs in bottlenose dolphins (*Tursiops truncatus*), and diagnosis can be based upon abnormal faecal or gastric content appearance, changes in gut motility and appetite, or overgrowth of *Candida* spp. or *Clostridium perfringens* in faeces. While the aetiology of gastroenteritis in dolphins is not commonly identified, *Campylobacter* spp., *Cryptosporidium* spp., *Edwardsiella tarda*, enteropathogenic *Escherichia coli* (EHEC), *Giardia* spp., *Listeria* spp, *Salmonella* spp. and *Vibrio* spp. are common terrestrial mammalian gastrointestinal pathogens that are found in marine...
mammals (Minette 1986; Higgins 2000; Venn-Watson et al. 2012).

Dolphins appear to be particularly susceptible to gastric ulcers due to a lack of glands in the forestomach to protect itself against digestive fluids and hydrochloric acid (Gaskin 1978). Further, Helicobacter infections may affect the health status of dolphins by contributing to the pathogenesis of gastric ulcers. In effect, novel helicobacters have been found in wild and captive marine mammals (Goldman et al. 2011). Helicobacter cetorum has been isolated from the gastric mucosa of dolphins (Harper et al. 2000, 2002), although its relation to gastric disease remains unclear. Given the variety of potential causes of gastroenteritis in dolphins, and the difficulty in acquiring a definitive diagnosis, there is a need for broad-spectrum protection against disease.

Probiotics, as defined by the Food and Agricultural Organization of the United Nations, are ‘live microorganisms which when administered in adequate amounts confer a health benefit on the host’ (FAO/WHO 2001). Previous studies, extensively reviewed elsewhere (Rastall et al. 2005; Guarner et al. 2012), have enumerated the necessary traits and potential benefits of probiotic use for prevention and treatment of disease in animals and humans. Some of the beneficial features include inhibition of pathogen growth and prevention of colonization, suppression of virulence factor expression, modulation of host microbiota, modification of energy utilization and of pain perception, enhancement of epithelial cell function, protection from physiological stress and modulation of host immune responses, including alteration of cytokine and antibody production by host cells and regulation of T-lymphocyte function (Ryan et al. 2009; Thomas and Versalovic 2010). Beneficial microbes and probiotics have been delivered to various animals of agricultural importance such as cattle (Nader-Macias et al. 2008), swine (Mori et al. 2011), poultry (Pascual et al. 1999; Brisbin et al. 2011) and fish (Balcazar et al. 2007; Gatesoupe 2008), in applications such as animal feed, for growth promotion, modulation of the gut microbiota and prevention of infectious diseases (Bernardeau et al. 2006; Czarnecki-Maulden 2008; Gaggi et al. 2010). Lactic acid bacteria, which include the genera Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus and others, have received particular interest as probiotics, because many of them are classified as generally regarded as safe (GRAS) organisms (Bernardeau et al. 2006; Heczko et al. 2006). Lactobacillus spp. produce antimicrobial factors and bacteriocins which make them attractive candidates for prevention and treatment of a variety of infectious diseases (Aiba et al. 1998; Pascual et al. 1999; Corr et al. 2007). In effect, lactobacilli have been used to reduce Salmonella loads and eradicate various pathogens from chickens, pigs and other animals (Pascual et al. 1999; Walsh et al. 2008; Chen et al. 2012), making them a sensible choice to evaluate for marine mammal health promotion.

With the aim of exploring complementary health management strategies for marine mammals, we embarked on an effort to isolate and identify candidate probiotic lactobacilli from the indigenous microbiota of bottlenose dolphins (T. truncatus).

Materials and methods

Sample acquisition

Samples were collected from 38 bottlenose dolphins at the Navy Marine Mammal Program (MMP), a programme that has been active for more than 50 years. Navy dolphins are housed in netted enclosures in San Diego Bay, California, and routinely work in the open ocean. They are fed restaurant-quality frozen–thawed fish (mackerel, herring, capelin and squid), receive routine anthelmintics, are observed daily, have routine physical examinations by highly experienced veterinarians and are part of a vigilant preventive medicine programme. Voluntary, trained behaviours are used to aid in routine sample collection, including blood, gastric fluid and faecal samples, and blood panel reference ranges amongst healthy animals have been published for this group of dolphins (Venn-Watson et al. 2007, 2011b). While the average age of dolphins in the wild is approximately 24 years, an increasing number of Navy dolphins are living 40–50 years (Venn-Watson et al. 2011a).

The Navy Marine Mammal Program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and adheres to the national standards of the United States Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the Animal Welfare Act. As required by the Department of Defense, the MMP’s animal care and use programme is routinely reviewed by an Institutional Animal Care and Use Committee (IACUC) and the Department of Defense Bureau of Medicine.

Samples included in this study were oral swabs (from the gingival sulcus), rectal swabs and approximately 5 ml of gastric fluid from each dolphin, all taken from each animal on the same date. Oral and rectal swabs were collected using BD BBL™ CultureSwab™ Plus Amies Medium (Sparks, MD, USA) and subsequently stored at −80°C. Gastric fluid samples were collected and frozen in Brucella broth supplemented with 20% glycerol. Thirty-one dolphins were considered healthy at the time of sampling. Of the remaining seven dolphins in the study, gastric endoscopic evaluation confirmed that two
dolphins had erosive or ulcerative esophagitis and five animals had erosive or ulcerated gastritis. Dolphins were sampled at multiple time points from November 2007 to December 2008, creating a total of 119 oral swabs, 119 rectal swabs and 119 gastric fluid specimens. In addition, milk samples were collected from three lactating female dolphins as lactobacilli have been isolated from human breast milk and that of other mammals (Diaz-Ropero et al. 2007; Lara-Villoslada et al. 2007), and oral swabs were collected from their nursing calves as part of routine health assessments. For milk collection, the skin surrounding the teat was wiped with 70% isopropyl alcohol and then rinsed and wiped with sterile water prior to application of the modified manual breast pump for sample acquisition. Milk samples were stored in Brucella broth with 20% glycerol. All samples were immediately stored at −80°C and shipped frozen to our (M.A.D., E.P.G., J.V.) laboratory on dry ice.

**Isolation, culture and identification of candidate probiotic strains**

Samples were either plated directly on de Man, Rogosa and Sharpe (MRS), Brucella, sheep blood or chocolate agar, or enriched for 24–48 h in MRS or Brucella broth and then plated on these agar media. Blood and chocolate agar were obtained from Remel (Lenexa, KS, USA); MRS and Brucella media were obtained from BD (Franklin Lakes, NJ, USA). Blood, Brucella and chocolate media were used to obtain greater isolate diversity, while MRS media was chosen for enrichment and selection of lactic acid bacteria. A greater emphasis was given to screening rectal swabs and gastric fluid, as these specimens were likely to contain lactobacilli, according to studies carried out in other animals (Neville and O’Toole 2010) and to preliminary data obtained by 16S rDNA clone library sequencing from these dolphin specimens (E.M. Bik et al., personal communication). All samples were incubated under anaerobic or microaerobic culture conditions at 37°C to simulate the host environment, for as long as 7 days, until colony growth was apparent. Isolates displaying different colony morphologies (about 2–3 per plate) were subcultured for further study. Pure cultures made from isolated colonies were stored at −80°C in Brucella broth supplemented with 20% glycerol.

Cultured bacterial isolates were identified by analysis of the 16S ribosomal RNA gene (rDNA). Partial 16S rDNA sequencing was performed to screen for lactobacilli; only isolates identified as *Lactobacillus* spp. were fully sequenced. PCR was performed using the FastStart PCR Master Mix (Roche, Indianapolis, IN, USA) and universal bacterial primers 27f (5′-GAG TTT GAT CCT GGC TCA G-3′) and 1525r (5′-AGA AAG GAG GTG ATC CAG CC-3′) (Rainey et al. 1996). Other primers used include universal bacterial primers B-V3 (5′-ACG ACA GCC ATG CAG CAC CT-3′) and BR5-V1 (5′-GAA GAG TTT GAT CAT GGC TCA G-3′) (Luna et al. 2007), and 18S rRNA gene primers ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS4 (5′-TCC TGG GTC TAT GTA TAT GC-3′) to detect yeasts (Zachow et al. 2009). Template DNA was obtained by colony lysis, or purified from pure bacterial cultures using MO BIO UltraClean DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) or MagNA Pure Nucleic Acid Isolation kit (Roche). A variety of DNA extraction and PCR sequencing conditions were employed as these can vary considerably for different organisms. Purified amplicons (QIAquick kits; Qiagen, Valencia, CA, USA) were sequenced at SeqWright. Sequence analysis was performed using VectorNTI (Invitrogen, Carlsbad, CA, USA), SeqMatch, available through the Ribosomal Database Project II (RDPII database, http://rdp.cme.msu.edu/index.jsp) (Maidak et al. 2001), and BLAST (Altschul et al. 1997) (National Center for Biotechnology Information, available through http://www.ncbi.nlm.nih.gov/BLAST/). Isolates identified as *Lactobacillus* spp. by 16S rDNA sequencing were further characterized biochemically using API 50 CHL (bioMérieux, Marcy l’Etoile, France) and by DiverSiLab repPCR (bioMérieux) genomic fingerprinting. The repPCR fingerprinting was performed as instructed by the manufacturer (Woods et al. 1993; Healy et al. 2005), using genomic DNA extracted from pure bacterial cultures with the MO BIO UltraClean DNA isolation kit.

Ribosomal RNA sequences of isolates identified as *Lactobacillus* spp. were aligned using the Greengenes NAST aligner (DeSantis et al. 2006) and phylogenetically analysed using the Greengenes version of the ARB software package (Ludwig et al. 2004). A neighbour-joining tree was generated using a 1323-bp column filter, a Jukes-Cantor correction and a bootstrapped version building 1000 trees.

**Pathogen growth inhibition assays**

Pathogen growth inhibition assays were performed to assess the probiotic properties of candidate probiotic strains, as previously described (Schillinger and Lucke 1989; Jacobsen et al. 1999; Tzortzis et al. 2004), with modifications. These assays involved growth of spot inocula of probiotic candidate strains (‘effectors’) on agar to allow secretion and diffusion of growth inhibitory factors. This step was followed by overlaying the plate with a pathogen culture (‘indicator’) and ultimately measuring the pathogen growth inhibition zone. Pathogens used as ‘indicator’ organisms included a marine mammal-derived isolate of *Salmonella enterica* serotype Enteritidis (strain...
MMP-3466467), a human enterohaemorrhagic *E. coli* (EHEC; strain EHEC-JV.112) and a human-derived enterotoxigenic *E. coli* (ETEC; strain ETEC-JV.3A5). Strains EHEC-JV.112 and ETEC-JV.3A5 were clinical isolates obtained from the Microbiology Laboratories of the Department of Pathology and Microbiology at Texas Children’s Hospital, Houston, TX. The *Salmonella* MMP-3466467 strain had been isolated from a sea lion faecal sample collected in June 2006 at the MMP location in San Diego. The animal was diagnosed with gastroenteritis and mixed viral (PCR positive for Calicivirus) and bacterial infections (heavy growth of *E. coli* and *Salmonella*). Clinical signs included anorexia, lethargy and an erosive oral lesion.

Candidate probiotic ‘effector’ strains were grown 18–24 h anaerobically at 37°C in MRS broth. Two microlitre (2 μl)-droplets of standardized culture broth containing approximately 10^5 cells ml⁻¹ (as estimated by OD_600 absorbance readings) were spotted onto MRS agar and incubated anaerobically for 24 h at 37°C. The three pathogen ‘indicator’ strains were grown aerobically in brain–heart infusion (BHI) broth for 18–24 h at 37°C and used to inoculate 7 ml soft BHI agar (0-7% agar; molten and tempered to 45°C) to obtain standardized bacterial suspensions of 10^7 cells per plate. Plates containing effector strain growth spots (3 mm radius each) were carefully overlaid with indicator organism cell suspensions and incubated aerobically for 18–24 h at 37°C to obtain bacterial lawns. The radius of each pathogen growth inhibition zone was measured in millimetres. Assays were performed twice, both times in triplicate, and results are presented as the means and standard deviations. Statistical analyses *(ANOVA, P < 0.05)* were performed using Stata (StataCorp, College Station, TX, USA). An inhibition radius <4 mm was considered noninhibitory; a radius between 4 and 7 mm was designed as intermediary inhibitor, and a radius exceeding 7 mm was considered strongly inhibitory. *Lactobacillus salivarius* ATCC 11741, originally isolated from the human oral cavity (Rogosa *et al.* 1953), and recently sequenced as a reference genome by the NIH Human Microbiome Consortium (http://genome.jgi.doe.gov/HumanMicr/HumanMicr.info.html), was used as a reference strain and positive control in these assays.

**TNF secretion assays**

A high-throughput quantitative immunoassay was developed and used to screen for candidate bacteria with the ability to affect TNF secretion by host cells, based on previously described methods (Lin *et al.* 2008; Jones and Versalovic 2009), with the following modifications. Wells from 96-well plates containing MRS broth were inoculated using a fresh colony of the probiotic candidate and incubated anaerobically at 37°C for 24 h. Cultures were subcultured using fresh 96-well plates with MRS broth, incubating anaerobically at 37°C for 24 h. OD_600 readings were taken to ensure bacterial growth to late log phase and to make adjustments when necessary. Conditioned media containing secreted bacterial factors were prepared by filtering the culture material (0.22 μm pore size) to remove bacterial cells, then vacuum drying the filtrates and resuspending them in equal volumes of RPMI 1640 media (Sigma-Aldrich, St Louis, MO, USA). Plates with conditioned media were kept at 4°C for 24 h and then stored for up to 1 week at 4°C or at −20°C until use.

THP-1 human mononcytoid cells (ATCC TIB-202) were cultured in RPMI 1640 media supplemented with 10% foetal bovine serum. To challenge THP-1 cells, conditioned media (1% v/v; 200 μl final volume) were added to cell cultures containing approximately 50 000 THP-1 cells and incubated at 37°C with 5% CO_2 for 3 h 30 min. MRS broth-matched controls (no bacteria; also vacuum-dried and resuspended in RPMI) were used to detect the effects of culture media on THP-1 cells. Supernatants of the THP-1 cells were tested for TNF production, which was measured by quantitative ELISA (R&D Systems, Minneapolis, MN, USA) as previously described (Jones and Versalovic 2009). These assays were performed in duplicates, and results are presented as the means and standard deviations of TNF quantities secreted by THP-1 cells exposed to bacterial factors. Statistical analyses *(ANOVA, P < 0.05)* were performed using Stata. As references, we tested a collection of type strains of *Lactobacillus*, which included *Lact. acidophilus* ATCC 4796, Lact. *brevis* subsp *gravesensis* ATCC 27305, Lact. *buchneri* ATCC 11577, Lact. *casei* ATCC 334, Lact. *delbrueckii* subsp *bulgaricus* ATCC 11842, Lact. *fermentum* ATCC 14931, Lact. *gasseri* ATCC 33323, Lact. *johnsonii* ATCC 33200, Lact. *paracasei* ATCC 25302, Lact. *plantarum* ATCC 14917, Lact. *reuteri* ATCC 53609, Lact. *reuteri* ATCC 55148, Lact. *reuteri* ATCC 55730, Lact. *reuteri* ATCC PTA 6475, Lact. *rhamnosus* ATCC LSM2-1, Lact. *ruminus* ATCC 25644 and Lact. *salivarius* ATCC 11741.

**Results**

**Isolation and identification of candidate probiotics**

A total of 307 isolates were cultured from 21 rectal swabs, 25 gastric fluid specimens, 2 oral swabs and 3 milk specimens obtained from 38 dolphins, using MRS and Brucella media. Each isolate corresponded to a distinct morphotype obtained per plate with the sample and culture conditions employed.
The majority of isolates were obtained using MRS (150 isolates, 48.9%) and Brucella (118 isolates, 38.4%) media; some isolates were cultured using sheep blood (27 isolates, 8.8%) and chocolate (12 isolates, 3.9%) agar. One hundred and fifty-three (49.8%) isolates were recovered from rectal swabs, 97 isolates (31.6%) were obtained from gastric fluid, 53 isolates (17.3%) were derived from milk, and four isolates (1.3%) were cultured from nursing calf oral swabs.

Two hundred and fifty isolates (81.4%) were identified by partial 16S rDNA sequencing; 58 (18.9%) isolates remained unidentifiable. The most prevalent genera cultured from dolphin samples under the conditions employed were Staphylococcus (77 isolates, 25.1%) and Escherichia/Shigella (61 isolates, 19.9%), not surprisingly as both are able to grow in MRS media. Staphylococci were isolated from all specimen types and were predominant in gastric fluid and milk, whereas Escherichia/Shigella were mainly isolated from rectal swabs. Other genera recovered included Actinobacterium, Actinobacillus, Alcanivorax, Bacillus, Citrobacter, Clostridium, Corynebacterium, Desulfovibrio, Edwardsiella, Enterococcus, Eubacterium, Lactobacillus, Macrococcus, Ochromobacterium, Peptostreptococcus, Photobacterium, Pseudomonas, Rhodococcus, Stenotrophomonas and Streptococcus. Although 52 isolates were recovered from dolphin milk samples, no lactobacilli were recovered from these samples; the isolates were predominantly staphylococci. A detailed list of isolates recovered and their specimen of origin is provided in Table 1.

Of the 307 bacterial strains isolated from dolphin samples, seven were Lactobacillus spp. Four Lact. salivarius isolates were recovered from rectal swab samples, all from dolphin ‘C’. The isolates had 16S rDNA sequences that were more than 99.8% identical to each other. White, circular, smooth colonies formed on MRS and Brucella agar after 24 h of anaerobic incubation at 37°C, and an alpha-haemolytic phenotype was evident when they were cultured on sheep blood agar. These isolates were nonmotile and did not form spores. They were Gram-positive, catalase-negative, facultative anaerobic bacilli that appeared as single cells and as pairs of cells by microscopy.

In addition, we obtained three novel Lactobacillus spp. isolates from the gastric fluid of dolphin ‘Z’, with 16S rDNA sequences 99.8% identical to each other and with 96.3% 16S rDNA sequence identity to Lactobacillus cetti, a species previously isolated from beaked whales (Vela et al. 2008). The isolates formed small, grey, nonhaemolytic round colonies after 3–7 days of incubation on sheep blood agar at 37°C, under anaerobic conditions. They were Gram-positive, anaerobic, catalase-negative bacilli present as single cells and as pairs of cells. The 16S rDNA sequences obtained from these isolates were 99.8% identical to sequences detected directly in gastric fluid and rectal swab samples from several dolphins studied by whole-community, broad-range 16S rDNA PCR and clone library sequencing (E.M. Bik et al., personal communication). The novel Lactobacillus sp. and the Lact. salivarius isolated in this study had 92.71% 16S rDNA sequence identity to each other. The results of a 16S rDNA-based phylogenetic analysis of lactobacilli found in both studies are provided in Fig. 1. These sequence data have been submitted to the GenBank database under accession numbers JX142127 through JX142133.

RepPCR genomic fingerprinting of lactobacilli isolated in this study revealed that, as with ribosomal DNA sequence analysis, the four Lact. salivarius isolates, MMP005, MMP006, MMP007 and MMP007, obtained from dolphin ‘C’, grouped together to form one clade, as did the novel Lactobacillus isolates MMP239, MMP241 and MMP242, obtained from dolphin ‘Z’ (data not shown). Further comparative genomic analyses using microarray or whole-genome sequencing technologies will allow us to refine the phylogenetic relation and genome variability amongst the Lact. salivarius and the novel Lactobacillus sp. isolates, respectively, and determine whether these isolates represent distinct strains of each species (Rafits et al. 2010).

Lactobacillus salivarius isolates were characterized using API 50 CHL strips. These biochemical test kits consist of 50 carbohydrate utilization/fermentation profiles differed slightly from reference strain Lact. salivarius ATCC 11741, which displayed a typical carbohydrate fermentation profile of Lact. salivarius (Jacobsen et al. 1999; Neville and O’Toole 2010), and did not utilize D-ribose or D-adenitol, but was positive for l-rhamnose. The three novel Lactobacillus isolates (MMP239, MMP241 and MMP242) were fastidious and failed to yield sufficient growth for API 50 CHL assays.

Pathogen growth inhibition properties

Lactobacillus salivarius candidate probiotic isolates MMP005, MMP006, MMP007 and MMP007, obtained from dolphin ‘C’ rectal swabs, strongly inhibited growth of the marine mammal-derived Salm. enterica serotype Enteritidis (strain MMP-3466467) and human pathogens.
EHEC-JV.112 and ETEC-JV.3A5 (Fig. 2). Similarly, Lact. reuteri ATCC 55730 and Lact. salivarius ATCC 11741, references established as probiotic strains (Rogosa et al. 1953; Valeur et al. 2004), also inhibited these enteric pathogens. Other marine mammal-derived isolates tested included Staphylococcus sp. MMP123 and strains of Bacillus thuringiensis, Edwardsiella ictaluri, Enterococcus casseliflavus, Escherichia/Shigella, Photobacterium damselae and Staphylococcus spp., all of which yielded inhibition radii smaller than 4 mm and were noninhibitory (not

| Organism*          | Rectal swab | Gastric fluid | Oral swab | Milk | Total (%) |
|--------------------|-------------|---------------|-----------|------|-----------|
| Achromobacter denitrificans | 1           |               |           | 1    | (0.3%)    |
| Actinobacillus scottiae    | 1           | 5             |           | 6    | (2.0%)    |
| Alcanivorax dieselolei     | 2           |               |           | 2    | (0.7%)    |
| Bacillus thuringiensis     | 1           |               |           | 1    | (0.3%)    |
| Citrobacter braakii        | 1           |               |           | 1    | (0.3%)    |
| Citrobacter freundii       | 4           |               |           | 4    | (1.3%)    |
| Clostridium ghonii         | 1           |               |           | 1    | (0.3%)    |
| Clostridium perfringens    | 3           |               |           | 3    | (1.0%)    |
| Clostridium sordellii      | 2           |               |           | 2    | (0.7%)    |
| Corynebacterium tuberculostearicum | 1         |               |           | 1    | (0.3%)    |
| Desulfovibrio spp.         | 1           |               |           | 1    | (0.3%)    |
| Edwardsiella ictaluri      | 1           |               |           | 1    | (0.3%)    |
| Edwardsiella tarda         | 13          |               | 13 (4.2%) |      |           |
| Enterococcus casseliflavus | 3           |               |           | 3    | (1.0%)    |
| Enterococcus silesiacus    | 2           |               |           | 2    | (0.7%)    |
| Enterococcus termitis      | 10          | 3             |           | 13   | (4.2%)    |
| Eubacterium tenue          | 1           |               |           | 1    | (0.3%)    |
| Escherichia/Shigella       | 57          | 4             | 61 (19.9%)|      |           |
| Lactobacillus salivarius   | 4           |               |           | 4    | (1.3%)    |
| Lactobacillus spp.         | 3           |               |           | 3    | (1.0%)    |
| Macrococcus caseolyticus   | 1           |               |           | 1    | (0.3%)    |
| Ochrobactrum tritici       | 1           |               |           | 1    | (0.3%)    |
| Peptostreptococcus stomatis| 2           |               |           | 2    | (0.7%)    |
| Photobacterium damselae    | 5           | 2             |           | 7    | (2.3%)    |
| Pigmentiphaga kulae        | 1           |               |           | 1    | (0.3%)    |
| Propionibacterium avidum   | 2           |               |           | 2    | (0.7%)    |
| Pseudomonas otitidis       | 1           |               |           | 1    | (0.3%)    |
| Rhodococcus corynebacteroides| 1          |               |           | 1    | (0.3%)    |
| Staphylococcus caprae      | 1           | 1             | 1         | 9    | (3.9%)    |
| Staphylococcus cohnii      | 1           |               | 1         | 1    | (0.3%)    |
| Staphylococcus delphini    | 1           | 8             |           | 9    | (2.9%)    |
| Staphylococcus epidermidis | 4           | 6             | 2         | 10   | (7.2%)    |
| Staphylococcus hominis     | 2           |               | 8         | 10   | (3.3%)    |
| Staphylococcus pasteuri    | 2           | 4             | 1         | 3    | (3.3%)    |
| Staphylococcus warneri     | 3           | 6             | 4         | 13   | (4.2%)    |
| Stenotrophomonas maltophilia| 1           |               |           | 1    | (0.3%)    |
| Streptococcus australis    | 2           |               |           | 2    | (0.7%)    |
| Streptococcus mitis       | 1           |               |           | 1    | (0.3%)    |
| Streptococcus parasanguinis| 1           |               | 1         | 2    | (0.7%)    |
| Possible yeast (18S rDNA positive) | 1            | 25            |           | 26   | (8.5%)    |
| Not sequenced or identified| 23          | 25            | 10        | 58   | (18.9%)   |
| Total                   | 154 (50.2%) | 97 (31.5%)    | 4 (1.3%)  | 52 (16.9%) | 307 |

* A total of 119 sample sets including an oral swab, a rectal swab and gastric fluid were collected from 38 dolphins. Milk samples were obtained from three lactating dolphins; oral swabs were collected from their nursing calves. Samples were collected from November 2007 to December 2008, by personnel at the U.S. Navy Marine Mammal Program in San Diego, CA. de Man, Rogosa and Sharpe (MRS), Brucella, chocolate and blood agar were used for isolation and culture of micro-organisms. Isolate identification was based on 16S rDNA sequence analysis; the listed species correspond to the closest RDP hit, using SeqMatch.
shown). As mentioned earlier, the novel Lactobacillus sp. isolates were not studied further as they were fastidious and yielded insufficient growth. The growth medium alone had no growth inhibitory effect on any of the bacterial pathogens tested (not shown).

Secreted factors from Lact. salivarius isolates MMP005, MMP006, MMP007 and MMP007 were assayed for their ability to modulate TNF production by THP-1 human macrophages. The growth medium alone had no growth inhibitory effect on any of the bacterial pathogens tested (not shown).

Table 2 Summary of characteristics of lactobacilli analysed in this study

| Isolate | Species* | Specimen | Dolphin | Enrichment media | Isolation media | Pathogen growth inhibition | TNF modulation | API-CH profile† |
|---------|----------|----------|---------|------------------|----------------|---------------------------|----------------|-----------------|
| MMP 005 | Lactobacillus salivarius | Rectal swab | C | Brucella | MRS | +++↑ | RIB, ADO, GAL, GLU, FRU, MNE, MAN, SOR, NAG, MAL, LAC, MEL, SAC, TRE, RAF |
| MMP 006 | Lact. salivarius | Rectal swab | C | Brucella | MRS | +++↑ | RIB, ADO, GAL, GLU, FRU, MNE, MAN, SOR, NAG, MAL, LAC, MEL, SAC, TRE, RAF |
| MMP 007 | Lact. salivarius | Rectal swab | C | Brucella | MRS | +++↑ | RIB, ADO, GAL, GLU, FRU, MNE, MAN, SOR, NAG, MAL, LAC, MEL, SAC, TRE, RAF |
| MMP 077 | Lact. salivarius | Rectal swab | C | None | MRS | +++↑ | RIB, ADO, GAL, GLU, FRU, MNE, MAN, SOR, NAG, MAL, LAC, MEL, SAC, TRE, RAF |
| MMP 239 | Novel Lactobacillus sp. | Gastric fluid | Z | Brucella | Blood | ND | ND |
| MMP 241 | Novel Lactobacillus sp. | Gastric fluid | Z | Brucella | Blood | ND | ND |
| MMP 242 | Novel Lactobacillus sp. | Gastric fluid | Z | Brucella | Blood | ND | ND |
| ATCC 11741† | Lact. salivarius | – | – | – | – | +++↑ | GAL, GLU, FRU, MNE, RHA, MAN, SOR, NAG, MAL, LAC, MEL, SAC, TRE, RAF |

MRS, de Man, Rogosa and Sharpe; +++↑, strongly inhibitory.

*According to phylogenetic analysis shown in Fig. 1.
†Lactobacilli obtained from Marine Mammal Program (MMP) dolphin samples were characterized biochemically using API 50 CHL (bioMérieux). D-Ribose (RIB), D-adonitol (ADO), D-galactose (GAL), D-glucose (GLU), D-fructose (FRU), D-mannose (MNE), D-mannitol (MANN), D-sorbitol (SOR), N-acetylglucosamine (NAG), D-maltose (MAL), D-lactose, (LAC), D-melibiose (MEL), D-saccharose (SAC), D-trehalose (TRE) and D-raffinose (RAF) and L-rhamnose (RHA).
‡Lact. salivarius ATCC 11741 was used as a reference strain.
monocytoïd cells. Bottlenose dolphin cell lines (Beineke et al. 2010) were not available for this study, so use of a human cell line was a valuable model for screening purposes. We found that conditioned media from *Lact. salivarius* isolates stimulated TNF production significantly more than the MRS medium control (Fig. 3). Secreted factors from the bottlenose dolphin-derived *Lact. salivarius* strains exhibited TNF-stimulatory features similar to the effects of human-derived reference probiotic strains *Lact. salivarius* ATCC 11741 and *Lact. reuteri* ATCC 55730. The latter is a known TNF-stimulatory strain previously studied in our laboratory and elsewhere (Valeur et al. 2004; Lin et al. 2008; Jones and Versalovic 2009). Other reference lactobacilli tested yielded various degrees of TNF inhibition.

**Discussion**

The main objectives of this study were to isolate gastrointestinal bacteria from bottlenose dolphins and to evaluate selected isolates for beneficial properties. Following the laboratory screening of 307 bacterial isolates from oral, gastric and rectal specimens collected from 38 dolphins in the U.S. Navy Marine Mammal Program, gastrointestinal lactobacilli and other Gram-positive bacteria were evaluated for defined beneficial features. Novel *Lactobacillus* spp. phylogenetically related to *Lact. ceti* were identified by direct sequencing and bacteriological culture. Gastrointestinal *Lact. salivarius* isolates obtained by bacteriological culture from *T. truncatus* demonstrated the ability to suppress the proliferation of enteric pathogens and stimulated TNF production in mammalian myeloid cells. These new isolates represent an initial foray into the characterization of lactobacilli with potentially beneficial health-promoting features in marine mammals.

Microbiome studies suggest that microbial communities have co-evolved with their hosts (Ley et al. 2008; Spor et al. 2011) and highlight the roles that microorganisms may play in their co-habitation with them (Muegge et al. 2011; Clemente et al. 2012). The mammalian gastrointestinal microbiota includes a diverse group of micro-organisms that exert functions with potential probiotic activities (Ley et al. 2008; Gerritsen et al. 2011). Probiotics may confer beneficial effects as modulators of the host microbiota, enhancing resilience and resistance to pathogens and other perturbations by promoting a healthier microbial ecology of the gut (Gerritsen et al. 2011; Wallace et al. 2011). These studies bring to light the value of culture-based methods to complement the predominantly molecular-based approaches used to study...
the microbiome at bacterial membership and functional levels (16S rDNA surveys, metagenomics, metabolomics, etc.). Efforts to culture symbiotic microbes enable a better understanding of the biology of the host microbiota, particularly given the historical priority given to studying organisms of traditional medical and veterinary relevance, namely pathogens (Spor et al. 2011).

*Lactobacillus salivarius* is part of the indigenous microbiota of the mammalian gastrointestinal tract and was first isolated from the human oral cavity (Rogosa et al. 1953). The species has also been isolated from human breast milk (Martin et al. 2006), a plausible route for gastrointestinal colonization (Martin et al. 2006; Jara et al. 2011), and from food and environmental sources (Neville and O’Toole 2010). *Lactobacillus salivarius* displays a high level of genomic diversity (Raftis et al. 2010). The 2-13-Mb genome of strain UCC118 has been annotated (Claesson et al. 2006), revealing a circular genomic megaplasmid, which is a novel feature amongst lactobacilli, and may be implicated in its adaptation to a wide range of environments and probiosis (Raftis et al. 2010). Comparative genomics studies will provide further understanding of strain diversity within the species as genomic characterization of various *Lact. salivarius* strains isolated from different habitats is completed by scientists at the NIH Human Microbiome Consortium and other laboratories (Raftis et al. 2010; Kergourlay et al. 2012; Langa et al. 2012).

To our knowledge, *Lactobacillus* spp. have not yet been reported in dolphins. Given the growing scientific evidence supporting successful probiotic applications of lactobacilli in humans and other animals, these organisms were the focus of this study. We successfully recovered *Lactobacillus* spp. from bottlenose dolphin specimens. *Lactobacillus salivarius* isolates were recovered from dolphin rectal swab samples, and a novel *Lactobacillus*...
species was recovered from dolphin gastric fluid. *Lactobacillus*-like 16S rDNA sequences were also detected in dolphin gastric fluid and rectal swab samples by broad-range rDNA PCR and clone sequencing (E.M. Bik et al., personal communication), with some of the amplified sequences being 99-8% identical to the 16S rDNA sequences of the novel *Lactobacillus* strains that were cultured in this study. The 16S rDNA sequences of the novel *Lactobacillus* are 96-3% identical to the sequence of a cultivated, marine mammal-derived *Lact. ceti* (Vela et al. 2008). Isolates of the novel *Lactobacillus* species were fastidious and grew too slowly to perform standard biochemical assays.

Our focused search for lactic acid bacteria yielded isolates from genera other than *Lactobacillus* and may include organisms with beneficial features. Given the limited information available on marine mammal indigenous gastrointestinal microbiota (Venn-Watson et al. 2008), with existing studies primarily addressing their role in the digestion of food (Olsen et al. 1994), the isolation and identification of a diverse set of members of the bottlenose dolphin indigenous microbiota, including lactobacilli, should significantly contribute to the understanding of the dolphin microbiome. Future studies characterizing the dolphin gastrointestinal microbiota will help elucidate the contribution of these organisms to their host’s health.

Pathogens that affect gastrointestinal dolphin health have been previously reviewed elsewhere (Baskin 2006; Venn-Watson et al. 2008) and include astroviruses (Rivera et al. 2009), *Campylobacter* spp. (Foster et al. 2004; Stoddard 2005; Goldman et al. 2011), *Clostridium* spp. (Buck et al. 1987), *Edw. tarda* (Coles et al. 1978), *Giardia* and *Cryptosporidium* species (Hughes-Hanks et al. 2005), *Helicobacter* (Harper et al. 2000, 2002) and *Salmonella* spp. (Foster et al. 1999; Smith et al. 2002).

The action and effectiveness of probiotics can be evaluated by a variety of screening techniques, which include assessment of antimicrobial activities, modulation of cytokine production, host colonization and resistance to acid or bile stress (Rastall et al. 2005; Guarner et al. 2012). Antagonistic properties of probiotic organisms have been demonstrated *in vitro* and *in vivo* in bifidobacteria, lactobacilli and combinations of probiotics (Neville and O’Toole 2010; Jara et al. 2011; Langa et al. 2012). Adaptation of *Lact. salivarius* to the gastrointestinal niche has entailed the capacity to inhibit pathogens and to tolerate the host’s antimicrobial defences, granting it considerable interest as a promising probiotic species (Neville and O’Toole 2010). *Lactobacillus salivarius* have been shown to exhibit antagonistic properties against *Listeria* (Barrett et al. 2007; O’Shea et al. 2011), *Salmonella* (Pascual et al. 1999; Casey et al. 2007), *Campylobacter* (Robyn et al. 2012) and other pathogenic bacteria (Corr et al. 2007; Neville and O’Toole 2010) and have been utilized to eradicate *Helicobacter* infection in humans and other mammals (Aiba et al. 1998; Canducci et al. 2002). The capacity of *Lact. salivarius* to prevent infection has been attributed to a number of features including the production of salivaricin (Barrett et al. 2007) and other bacteriocins (Corr et al. 2007; O’Shea et al. 2011), prevention of colonization (Kabir et al. 1997), interference with cytokine induction and virulence gene expression (Ryan et al. 2009), and modulation of human intestinal cells (O’Hara et al. 2006). In addition, standard *Lactobacillus* metabolism produces antimicrobials such as hydrogen peroxide, lactic acid and other organic acids that can inhibit pathogen growth by chelating essential nutrients or sensitizing bacteria to antimicrobial assault (Neville and O’Toole 2010) and can decrease pathogen toxin production, such as Shiga toxin by EHEC (Carey et al. 2008). In this study, we evaluated the probiotic potential of dolphin-associated *Lact. salivarius* isolates by testing their ability to inhibit the growth of several pathogens *in vitro*. The strong inhibition of selected marine mammal and human pathogens by dolphin-derived *Lact. salivarius* isolates MMP005, MMP006, MMP007 and MMP007 confirms studies carried out in humans and other animal models and supports the selection of these organisms for further consideration as candidate probiotics for marine mammals.

*Lactobacillus salivarius* have been used for the capacity to modulate both humoral and cell-mediated immunity in humans and various animal models (Neville and O’Toole 2010). In effect, *Lact. salivarius* have been shown to increase antibody production and reduce cell-mediated immune responses in intestinal cells (Brisbin et al. 2011), to evoke intestinal immunomodulatory responses (Walsh et al. 2008) and to modulate cytokine induction by pathogens (Ryan et al. 2009). The production of exopolysaccharides contributes to its immunomodulatory, anti-tumorogenic and prebiotic properties and is valued in the food industry (Neville and O’Toole 2010; Raftis et al. 2010). The functional immunoassays we performed to study TNF modulation *in vitro* have been validated in various *in vivo* studies (Pena et al. 2005; Lin et al. 2008; Oksaharju et al. 2013) and are useful to screen for bacteria with immunomodulatory capacities (Thomas et al. 2012). In this study, we determined that TNF production by human monocytoid cells is stimulated by secreted factors from *Lact. salivarius* strains isolated from dolphins. This is in contrast to other well-characterized *Lact. salivarius* strains, reported to induce an anti-inflammatory environment in the host (Neville and O’Toole 2010). Reduction in TNF expression has been shown to be of benefit to the host in cases involving chronic inflammatory conditions such as IBD, IBS and autoimmune diseases such as rheumatoid arthritis, Crohn’s disease and...
psoriasis (Jones and Versalovic 2009; Thomas et al. 2012), as TNF contributes to the pathogenesis of inflammatory diseases. However, the TNF signalling pathway plays a crucial role in the activation of the innate immune response to pathogens (Secher et al. 2009) and has been proven necessary for protection against intracellular microbes such as Salmonella and Listeria, Mycobacterium tuberculosis and other Gram-positive and Gram-negative organisms (Dinarello 2003). TNF induction has been shown to be beneficial to the host in specific contexts, such as protection against pathogens (Secher et al. 2009) and neoplasia (Calzascia et al. 2007; Iyer et al. 2008). The benefits of induction of pro-inflammatory cytokine secretion by probiotic lactobacilli have been well documented for established immunostimulatory probiotic Lact. reuteri 55730 (Value et al. 2004). Desired immunoprotective traits should be chosen in accordance with specific treatment needs. Hence, Lact. salivarius-enhanced stimulation of host immunity by TNF induction can be beneficial to dolphins by contributing to the dolphin-derived Lact. salivarius strains’ anti-infective properties, a salient beneficial feature contingent upon clinical trial validation. Immunomodulatory compounds secreted by dolphin-derived Lact. salivarius strains can be characterized as previously described for other probiotic lactobacilli (Thomas et al. 2012), ideally using dolphin cell lines, and thus facilitate understanding of cytokines and pathways that are activated in the cells of the natural host.

The Human Genome Sequencing Center (HGSC) at Baylor College of Medicine has engaged in a study to sequence the genomes of 24 mammals. The genome of the bottlenose dolphin has been sequenced from DNA obtained from a healthy female dolphin from the Navy Marine Mammal Program (http://www.hgsc.bcm.tmc.edu/). Comprehensive studies of the marine mammal microbiome and genome should allow us to better understand the co-evolution of these unique hosts with their microbiomes and environment (Muegge et al. 2011; Spor et al. 2011). With a more complete molecular survey of the dolphin microbiota, and the use of functional metagenomics, a more targeted, customized approach for identifying and culturing probiotic bacteria in dolphins will be greatly facilitated. We hope to further evaluate the isolates obtained in this study by monitoring colonization and effects on the dolphin microbiota, characterizing the active immunoprotective compounds secreted by dolphin lactobacilli, assessing immune pathway activation and production of other indicators of immune function such as IL-6, IL-10 and IL-12, using dolphin host cell lines, and examining the contributions to health outcomes. Additional characterization of these lactobacilli will aid in the understanding of their biology and function within their cetacean hosts.

In conclusion, we isolated and characterized Lactobacillus spp. indigenous to the dolphin microbiota. To the best of our knowledge, this is the first study to isolate Lactobacillus spp. from the bottlenose dolphin. Dolphin-derived Lact. salivarius strains appear to be capable of stimulating TNF production by mammalian cells and inhibiting the growth of select marine mammal and human pathogens. As has been the case for other Lact. salivarius strains in other host species, these dolphin-associated isolates deserve further consideration as candidate probiotics in future clinical studies. Further studies are needed to characterize these isolates fully, as well as to optimize culturing of the novel Lactobacillus sp. and establish whether it might also have beneficial properties and applications. The introduction of candidate probiotic strains indigenous to marine mammals will offer new possibilities for the prevention and treatment of infectious diseases and gastrointestinal disorders in these unique animals.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

Table S1 *Lactobacillus* similarities matrix.