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ORIGINAL ARTICLE

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; Gaggia *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 antihelminthics, 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 TCC GCT TAT TGA 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 10^9 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, $P < 0{\cdot}05$) 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 monocytoid 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, $P < 0{\cdot}05$) 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 unidentified. 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 *Achromobacter, Actinobacillus, Alcanivorax, Bacillus, Citrobacter, Clostridium, Corynebacterium, Desulfovibrio, Edwardsiella, Enterococcus, Eubacterium, Lactobacillus, Macrococcus*, *Ochromobactrum, Peptostreptococcus, Photobacterium, Pigmentiphaga, 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 99·54% identical to each other and with 96·3% 16S rDNA sequence identity to *Lactobacillus ceti*, 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 MMP077, 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 (Raftis *et al.* 2010).

Lactobacillus salivarius isolates were characterized using API 50 CHL strips. These biochemical test kits consist of 50 carbohydrate utilization/fermentation assays used to characterize lactobacilli and related micro-organisms. Substrate utilization results, summarized in Table 2, showed that dolphin-derived *Lact. salivarius* isolates MMP005, MMP006, MMP007 and MMP077 yielded identical biochemical and carbohydrate fermentation profiles and were positive for D-ribose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, D-sorbitol, *N*-acetylglucosamine, D-maltose, D-lactose, D-melibiose, D-saccharose (sucrose), D-trehalose and D-raffinose. These 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-adonitol, 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 MMP077, obtained from dolphin 'C' rectal swabs, strongly inhibited growth of the marine mammal-derived *Salm. enterica* serotype Enteritidis (strain MMP-3466467) and human pathogens

Table 1 Bacterial species recovered from dolphin samples and identified by 16S rDNA sequencing

Organism*	Rectal swab	Gastric fluid	Oral swab	Milk	Total (%)
Achromobacter denitrificans	1				1 (0·3%)
Actinobacillus scotiae	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 kullae	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	12 (3·9%)
Staphylococcus cohnii				1	1 (0·3%)
Staphylococcus delphini	1	8			9 (2·9%)
Staphylococcus epidermidis	4	6	2	10	22 (7·2%)
Staphylococcus hominis		2		8	10 (3·3%)
Staphylococcus pasteuri	2	4	1	3	10 (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.

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

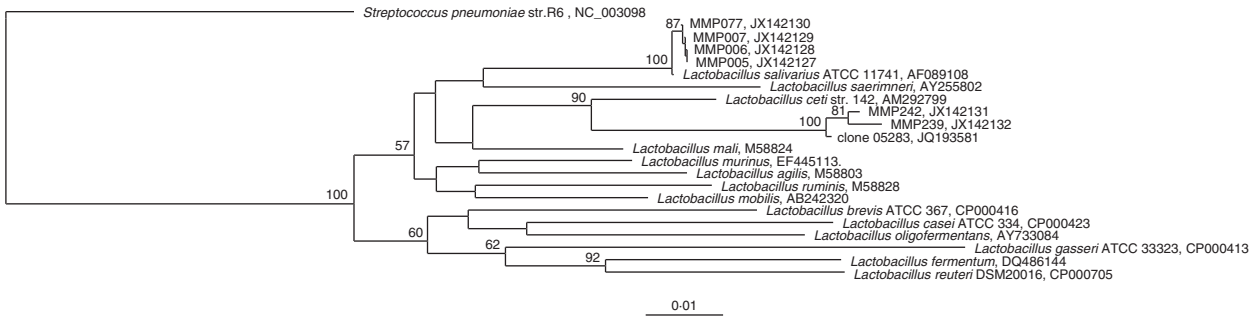

Figure 1 Phylogeny of *Lactobacillus* 16S rDNA sequences found in this study. *Lactobacillus* sequences found in this study (in bold) were compared with published sequences in a neighbour-joining tree with a Jukes-Cantor correction and a 1323-column filter. Numbers above branches refer to bootstrap values (in percentages out of 1000 trees; numbers below 50% are not shown). The scale bar represents evolutionary distance (1 substitution per 100 nucleotides). Sequence 05283 was obtained in a separate study of bacterial diversity in bottlenose dolphins using broad-range 16S rDNA PCR and clone library sequencing (E.M. Bik *et al.*, personal communication). Refer to Table S1 for the identity matrix of these sequences and to Table 2 for descriptions of MMP strains shown in this figure.

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	ND
MMP 241	Novel *Lactobacillus* sp.	Gastric fluid	Z	Brucella	Blood	ND	ND	ND
MMP 242	Novel *Lactobacillus* sp.	Gastric fluid	Z	Brucella	Blood	ND	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 (MAN), 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.

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).

Modulation of cytokine production

Secreted factors from *Lact. salivarius* isolates MMP005, MMP006, MMP007 and MMP077 were assayed for their ability to modulate TNF production by THP-1 human

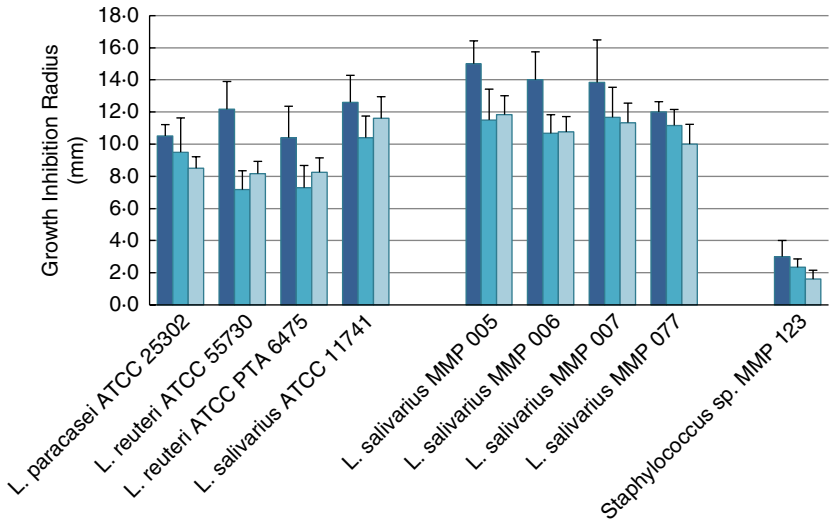

Figure 2 *Lactobacillus salivarius* isolates inhibit growth of selected marine mammal and human pathogens. Candidate probiotic strains were assayed for their ability to inhibit growth of selected marine mammal and human pathogens. Effector strain cultures (*Lact. salivarius* strains MMP005, MMP006, MMP007 and MMP077, *Staphylococcus* sp. MMP123 and reference strains *Lactobacillus paracasei* ATCC 25302, *Lact. salivarius* ATCC 11741, *Lactobacillus reuteri* ATCC 55730 and *Lact. reuteri* ATCC PTA 6475) were spotted onto de Man, Rogosa and Sharpe (MRS) agar and tested with indicator pathogen cell suspensions. Each pathogen growth inhibition zone radius was measured in millimetres (mm). *Lactobacillus salivarius* isolates obtained in this study from dolphin 'C' rectal swabs inhibited growth of marine mammal-derived *Salmonella enterica* serotype Enteritidis MMP-3466467 and human pathogens EHEC-JV.112 and ETEC-JV.3A5. Reference strains were also pathogen growth inhibitory (ANOVA, $P < 0{\cdot}05$). In contrast, *Staphylococcus* sp. MMP123 and other isolates from dolphin samples tested, including strains of *Bacillus thuringiensis, Edwardsiella ictaluri, Enterococcus casseliflavus, Escherichia/Shigella, Photobacterium damselae* and *Staphylococcus* spp. (not shown), were noninhibitory. MRS medium-only control experiments (not shown) confirmed that MRS had no effect on pathogen growth. Bars represent the mean radii of growth inhibition; error bars show standard deviations. (■) Salmonella; (■) EHEC and (■) ETEC.

monocytoid 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 micro-organisms 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

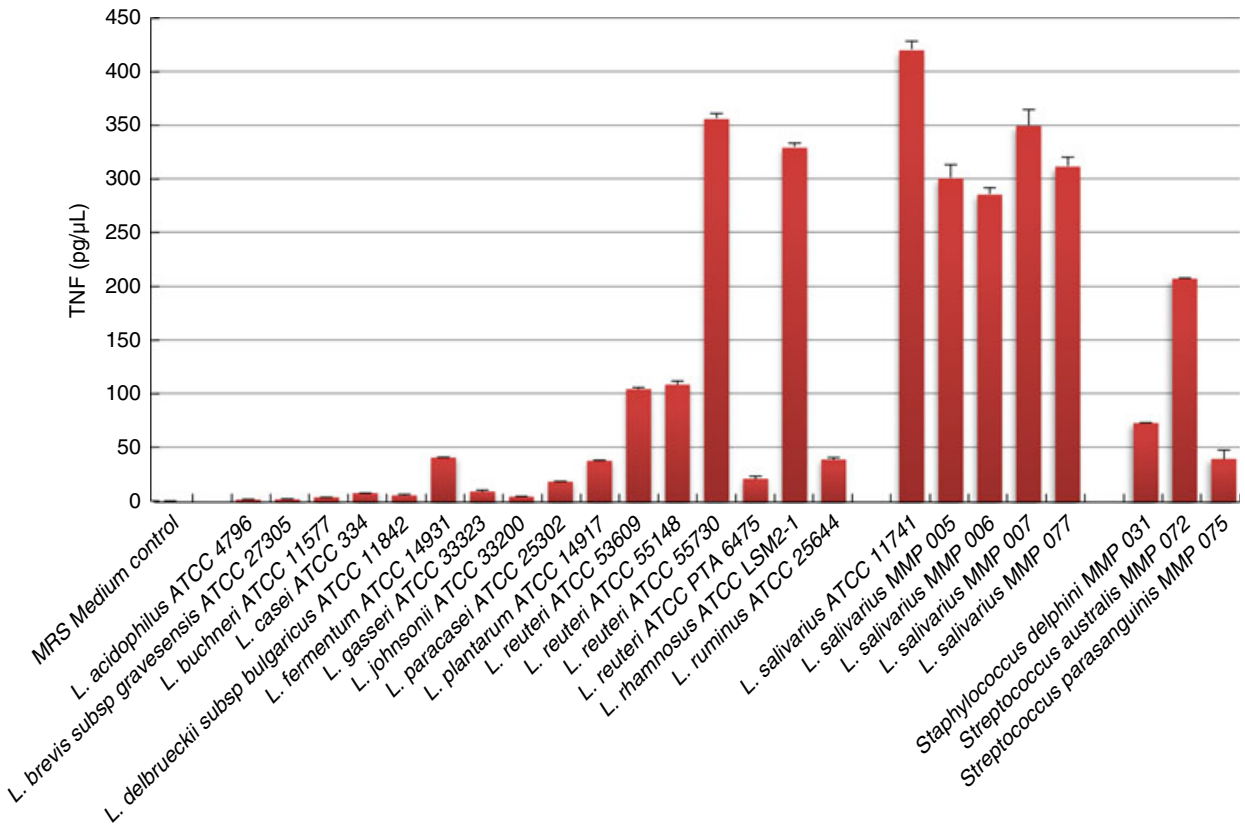

Figure 3 TNF production is stimulated by secreted factors from *Lactobacillus salivarius* isolates recovered from dolphins. *Lactobacillus salivarius* strains MMP005, MMP006, MMP007 and MMP077 were tested for their ability to modulate cytokine production in THP-1 human monocytoid cells. Conditioned media containing secreted factors were prepared by removing bacterial cells from late-log-phase cultures by filtration. Secreted factors were assayed for their ability to modulate TNF production by THP-1 human monocytoid cells. TNF was measured by quantitative ELISA. *Lactobacillus salivarius* strains MMP005, MMP006, MMP007 and MMP077, and reference strains *Lactobacillus rhamnosus* ATCC LSM2-1, *Lact. salivarius* ATCC 11741 and human-derived probiotic *Lactobacillus reuteri* ATCC 55730, significantly stimulated TNF production (ANOVA, $P < 0{\cdot}05$). de Man, Rogosa and Sharpe (MRS) medium-only control did not have an effect on TNF production. Results are presented as mean concentrations of TNF, with standard deviations represented by the error bars.

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 MMP077 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, antitumorigenic 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 (Valeur *et al.* 2004). Desired immunoprobiotic 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 immunoprobiotic 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.