SHORT RESEARCH ARTICLE

REVISED Amplicon pyrosequencing and ion torrent sequencing of wild duck eubacterial microbiome from fecal samples reveals numerous species linked to human and animal diseases [version 2; peer review: 3 approved with reservations]

Previously titled: Amplicon pyrosequencing of wild duck eubacterial microbiome from a fecal sample reveals numerous species linked to human and animal diseases

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Abstract

Our investigation into the composition of the wild duck, *Aythya americana*, eubacterial microbiome from a fecal sample using amplicon pyrosequencing revealed that the representative bacterial species were quite distinct from a pond water sample, and we were able to classify the major operational taxonomic units with *Fusobacterium mortiferum*, *Streptobacillus moniliformis*, *Lactobacillus intermedius*, *Actinomyces suimastitidis*, *Campylobacter Canadensis*, *Enterococcus cecorum*, *Lactobacillus aviarus*, *Actinomyces spp.*, *Pseudobutyrvibrio spp.* and *Helicobacter brantae* representing the majority of the eubacterial fecal microbiome. Bacterial species present in the analysis revealed numerous organisms linked to human and animal diseases including septicemia, rat bite fever, pig mastitis, endocarditis, malar masses, genital infections, skin lesions, peritonitis, wound infections, septic arthritis, urocystitis, gastroenteritis and drinking water diseases. In addition, to being known carriers of viral pathogens wild ducks should also be recognized as a potential source of a range of bacterial diseases.

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REVISED Changes from Version 1

Using ion torrent sequencing, we analyzed the eubacterial microbiome of 6 additional wild ducks of the *Ayantha americana* species. Our initial conclusion that the wild duck was a vector for bacterial disease was supported by the additional data and many of the initially reported pathogenic species were also present in the other samples. Statistical evaluation of the 7 samples revealed that exposure to wild duck feces equates to exposure to pathogenic bacteria and opportunistic bacterial pathogens.

See referee reports

Introduction

Throughout the history of medicine there has been an awareness of animal to human transmission of disease, and the etiological pathogens have been collectively described as zoonoses¹. Water fowl and wild birds have been identified as reservoirs for the virus Influenza A^{2,3}; a highly mutable and infectious pathogen that infects avian and mammalian species⁴. Ducks are observed in a multitude of fresh water sources including ponds, water fountains and pools where they can defecate; bacteria have been shown to be distributed through aerosols from ornamental fountains^{5,6} and reclaimed water dispensed through an irrigation system⁷. Humans may also have direct contact with ducks and their excrement through the recreational sport of duck hunting⁸. Ducks can also shed pathogens near chicken farms or other animals—such as pigs—that have access to outside areas. An avian influenza A virus (H7N7) epidemic in the Netherlands in 2003 thought to be initiated from a migratory water fowl resulted in the culling of 30 million poultry in an area of the country where free-range poultry farming was common⁹. Due to the migratory nature and unrestrained behavior of the wild duck, *Aythya Americana*, our study set out to investigate the bacterial microbiome of a wild duck and to identify its bacterial flora relative to the same bacterial species that have been reported to cause disease in farm animals and humans.

Methods

Amplicon pyrosequencing (bTEFAP) was originally described by Dowd *et al.*¹⁰ and has been used in describing a wide range of environmental and health related microbiomes including the intestinal populations of a variety of animals and their environments including cattle^{11–15}. Fecal samples obtained from wild ducks, *Aythya americana*, that were killed during duck hunting season (December 2012) by licensed hunters, were aseptically swabbed onto a Whatman FTA cards (GE Healthcare Life Sciences) using sterile swabs and gloves being careful to avoid environmental contamination. The flaps of the FTA cards were placed over the FTA paper and placed into a sterile pouch, and the FTA cards were stored at room temperature prior to DNA amplification. 2 mm punches were washed with FTA reagent and TE (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) according to the manufacturer's protocol, and the dried punches were used as template DNA for thermal cycling. DNA was also isolated from 50 mL of pond water as a negative comparison and sampled from a source of water visited by numerous avian species but not at the source of the fecal sampling but within the migratory range of *Aythya americana*. The pond water DNA was isolated using water RNA/DNA purification kit (0.45 µm) [Norgen Biotek Corp, Thorold, ON, Canada]. For thermal cycling and DNA

amplification we used the 16S universal Eubacterial primers 27f 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492r primer 5'-ACG-GCTACCTTGTTCAGACTT-3' (Integrated DNA Technologies). A single-step 30 cycle PCR using EconoTaq PLUS 2X Master Mix (Lucigen, Middleton, WI) were used under the following conditions: 94°C for 2 minutes, followed by 30 cycles of 95°C for 120 seconds; 42°C for 30 seconds and 72°C for 4 minutes; after which a final elongation step at 72°C for 20 minutes was performed. Following PCR, DNA products were resolved in a 1% agarose, 1X TAE gel stained with ethidium bromide and 1.5 Kb products were excised from the gel purified using a cyclo-prep spin column (Amresco, Solon, OH). All the DNA products were purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced using Roche 454 FLX titanium instruments and reagents following manufacturer's guidelines. The Q25 sequence data derived from the sequencing process was processed using a proprietary analysis pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX). Sequences were depleted of barcodes and primers. Next, short sequences < 200bp, sequences with ambiguous base calls, and sequences with homopolymer runs exceeding 6bp were removed. Sequences were then denoised and chimeras removed. Operational taxonomic units (OTUs) were defined after removal of singleton sequences, clustering at 3% divergence–97% similarity^{10,15}. OTUs were then taxonomically classified using BLASTn against a curated GreenGenes database^{16,17} and compiled into each taxonomic level into both “counts” and “percentage” files.

For ion torrent sequencing, the 16S rRNA gene V4 variable region PCR primers 515/806⁶⁰ were used in a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Ion Torrent PGM following the manufacturer's guidelines.

The prevalence of pathogenic bacteria was estimated by the means and 95% confidence intervals.

Results

Due to the aquatic nature of the animal, we initially expected that the biodiversity of bacterial species in the duck feces would reflect numerous bacterial species present in the pond water, and since we observed multiple species of aquatic birds in the pond we expected to find eubacteria in common. **Figure 1** is a modified heat map showing differences and similarities among the classes of eubacteria sequenced and identified. The figure demonstrates clear differences at the taxonomical level of Class with few common classes of bacteria namely Actinobacteria, Clostridia and Gammaproteobacteria.

However, similarities at the level of Genus and species included only *Agrobacterium tumefaciens* and a species of *Porphyromonas* and a species of *Ruminococcaceae* (**Figure 2**). This analysis indicated distinct differences between the eubacteria present in the duck fecal sample and the pond water sample, and it also indicated that our sampling of the duck feces was devoid of any obvious pond water eubacterial constituents.

Class	Duck Feces	Pond Water
Pedinophyceae	0.0000	0.0618
Ignavibacteria	0.0000	0.0071
Actinobacteria	3.1312	6.6874
Bacteroidia	0.1809	0.0335
Tm6 (candidate division)	0.0000	0.0071
Erysipelotrichi	0.0322	0.0000
Clostridia	3.6537	0.0653
Planctomycetacia	0.0000	0.2717
Epsilonproteobacteria	4.9419	0.0000
Prasinophyceae	0.0000	0.0935
Subsectionii	0.0000	1.2457
Coleochaetophyceae	0.0000	0.0053
Gloeobacterophycideae	0.0000	0.0335
Oscillatoriohycideae	0.0000	0.0053
Deltaproteobacteria	0.0020	0.0353
Betaproteobacteria	0.0000	1.2545
Holophagae	0.0000	0.0212
Thermoleophilia	0.0000	0.0141
Anaerolineae	0.0000	0.0106
Cytophagia	0.0000	0.0141
Flavobacteria	0.0000	0.1747
Coriobacteria	0.9647	0.0000
Nitrospira	0.0000	0.0053
Actinobacteria	6.0533	0.0000
Phycisphaerae	0.0000	0.0812
Fusobacteria	0.4361	0.0000
Gammaproteobacteria	0.2432	0.2064
Acidimicrobiia	0.0000	0.0159
Chloroflexi	0.0000	0.1006
Caldilineae	0.0000	0.0159
Fusobacteria	61.8031	0.0000
Bacilli	18.5478	0.0000
Alphaproteobacteria	0.0100	74.3780
Op8 (candidate division)	0.0000	0.0071
Synechococcophycideae	0.0000	11.4938
Sphingobacteria	0.0000	0.7728
Zygnemophyceae	0.0000	2.7402
Spartobacteria	0.0000	0.1412
Total	100	100

Figure 1. Comparison of Classes of Eubacteria present in the Duck to the Classes of Eubacteria present in pond water using a modified heat map. Darker colors represent a higher representation of the bacterial class.

The taxonomical classification of OTU at the level of genus and species was compiled in relation to percentages of the Eubacterial microbiome (Table 1). In Table 2, we referenced reported cases of diseases related to the bacteria sequenced from the duck's fecal sample reflecting the eubacterial microbiome's potential to cause disease in humans and other mammals. The largest representative bacterial species—relative to percentage—was *Fusobacterium mortiferum* at 31.6%. *Fusobacterium mortiferum* reports related to human disease are sparse, but *Fusobacterium* have been associated with rare but serious cases of bacteremia^{18,19}, and a 6 year study of “other gram-negative anaerobic bacilli” (OGNAB) isolated from anaerobic infections at the Wadsworth Clinical Anaerobic Clinical Anaerobic Bacteriology Research Laboratory in Los Angeles, CA reported that most strains of *Fusobacteria*—outside of *Fusobacterium nucleatum*—were resistant to erythromycin²⁰. The pathogen, *Fusobacterium nucleatum*, on the other hand, is well-known for its association with

Genus/species	Duck Feces	Pond Water
<i>Agrobacterium tumefaciens</i>	0.010	0.009
<i>Porphyromonas</i> spp.	0.111	0.004
<i>Ruminococcaceae</i> spp.	0.307	0.041

Figure 2. Bacterial species present in both duck feces and pond water.

Table 1. Taxonomical classification of operational taxonomic units into the Genus/species level with representative percentages of the Eubacterial Microbiome based on pyrosequencing data.

OTUs genus/species	% of Eubacterial microbiome
<i>Fusobacterium mortiferum</i>	31.609
<i>Streptobacillus moniliformis</i>	30.100
<i>Lactobacillus intermedius</i>	11.021
<i>Actinomyces suimastitidis</i>	4.474
<i>Campylobacter canadensis</i>	3.694
<i>Enterococcus cecorum</i>	3.585
<i>Lactobacillus aviarius</i>	2.792
<i>Actinomyces</i> spp.	1.966
<i>Pseudobutyrvibrio</i> spp.	1.811
<i>Helicobacter brantae</i>	1.248
<i>Coriobacteriaceae</i> spp.	0.928
<i>Actinomyces nasicola</i>	0.784
<i>Actinomyces odontolyticus</i>	0.699
<i>Lactobacillus aviarius</i>	0.627
<i>Roseburia</i> spp.	0.380
<i>Leptotrichia</i> spp.	0.364
<i>Ruminococcaceae</i> spp.	0.307
<i>Actinomyces turicensis</i>	0.295
<i>Bacillus</i> spp.	0.265
<i>Plesiomonas shigelloides</i>	0.239
<i>Fastidiosipila</i> spp.	0.213
<i>Actinomyces canis</i>	0.209
<i>Arcanobacterium pyogenes</i>	0.183
<i>Blautia</i> spp.	0.169
<i>Ruminococcus</i> spp.	0.157
<i>Veillonella ratti</i>	0.155
<i>Actinomyces europaeus</i>	0.139
<i>Atopobium vaginae</i>	0.121
<i>Lactobacillus</i> spp.	0.117
<i>Porphyromonas</i> spp.	0.111
<i>Parvimonas micra</i>	0.078
<i>Tessaracoccus</i> spp.	0.068
<i>Fusobacterium periodonticum</i>	0.058
<i>Atopobium rimae</i>	0.054
<i>Oscillibacter</i> spp.	0.054
<i>Helcococcus kunzii</i>	0.054
<i>Arthrobacter bergerei</i>	0.048
<i>Streptococcus macedonicus</i>	0.044
<i>Clostridium</i> spp.	0.044

OTUs genus/species	% of Eubacterial microbiome
<i>Peptostreptococcaceae</i> spp.	0.042
<i>Enterococcus</i> spp.	0.040
<i>Cetobacterium ceti</i>	0.038
<i>Veillonella magna</i>	0.036
<i>Cetobacterium</i> spp.	0.034
<i>Peptoniphilus asaccharolyticus</i>	0.034
<i>Flavonifractor</i> spp.	0.034
<i>Fusobacterium nucleatum</i>	0.030
<i>Actinomyces neuui</i>	0.026
<i>Bacteroides plebeius</i>	0.024
<i>Veillonella dispar</i>	0.024
<i>Streptococcus</i> spp.	0.020
<i>Dorea</i> spp.	0.018
<i>Allobaculum</i> spp.	0.016
<i>Porphyromonas gingivalis</i>	0.016
<i>Eubacterium sulci</i>	0.016
<i>Actinomyces lingnae</i>	0.016
<i>Bacteroides</i> spp.	0.016
<i>Collinsella</i> spp.	0.016
<i>Actinoplanes roseosporangius</i>	0.014
<i>Erysipelotrichaceae</i> spp.	0.014
<i>Lysinibacillus</i> spp.	0.014
<i>Corynebacterium freneyi</i>	0.014
<i>Myceligenans xiligouense</i>	0.012
<i>Actinomyces vaccimaxillae</i>	0.012
<i>Streptococcus suis</i>	0.012
<i>Anaerotruncus</i> spp.	0.012
<i>Sporosarcina</i> spp.	0.010
<i>IsotERICOLA variabilis</i>	0.010
<i>Olsenella</i> spp.	0.010
<i>Atopobium</i> spp.	0.010
<i>Agrobacterium tumefaciens</i>	0.010
<i>Microbispora rosea</i>	0.008
<i>Actinocorallia glomerata</i>	0.008
<i>Coprococcus</i> spp.	0.008
<i>Mobiluncus curtisii</i>	0.008
<i>Bacteroides coprocola</i>	0.008
<i>Prevotellaceae</i> spp.	0.006
<i>Sneathia</i> spp.	0.006
<i>Veillonella</i> spp.	0.006
<i>Gardnerella</i> spp.	0.006
<i>Varibaculum cambriense</i>	0.006
<i>Acinetobacter</i> spp.	0.004
<i>Actinomyces hongkongensis</i>	0.004
<i>Turicibacter</i> spp.	0.002
<i>Desulfovibrio</i> spp.	0.002
Total	100

disease and its ability to adhere to Gram-positive and Gram-negative bacteria in dental biofilms such as plaque²¹.

Streptobacillus moniliformis was also identified as a major constituent of the duck fecal eubacterial microbiome at 30.1%. Several well-studied and documented cases of disease are attributed to *S. moniliformis* including rat bite fever or Haverhill disease²², osteomyelitis²³, epidural abscesses²⁴, fever and polyarthralgia²⁵, bacteremia²⁶ and contaminated drinking water related disease²⁷.

Other organisms and their respective illnesses included *Lactobacillus intermedius* (11.02%) in a renal transplant infection²⁸, *Actinomyces suimastitidis* (4.47%) in pig mastitis²⁹ and *Campylobacter canadensis* (3.69%) in drinking water related disease²⁷. *Enterococcus cecorum* was another identified pathogen at 3.59% of the sequenced Eubacterial microbiome, and *E. cecorum* has been reported to cause disease in chicks^{30,31} and humans including aortic valve endocarditis³², empyema thoracis³³, septicemia in a malnourished adult³⁴ and recurrent bacteremic peritonitis in a patient with liver cirrhosis³⁵. *Actinomyces odontolyticus* (0.70%) has recently been reported to cause bacteremia in immunosuppressed patients³⁶, and members of the genus *Actinomyces* have been known to cause actinomycosis for some time. *A. odontolyticus* was reported by Michell, Hintz and Haselby in 1997 to be the cause of a malar mass in soft tissue in a human³⁷. A species of the genus *Leptotrichia* (0.36%) was also identified, a genus that has been associated with bacteremia in multiple myeloma patients receiving chemotherapy³⁸. Another *Actinomyces* present in the wild duck eubacterial microbiome was *Actinomyces turicensis* at 0.3%, a bacterium associated with a spectrum of diseases including genital infections, urinary tract infections, skin infections, post-operative wound infection, abscesses, appendicitis, ear and nose and throat infection and bacteremia³⁹. In addition, *Actinomyces europaeus* (0.14%) was reported in human abscesses⁴⁰, *Actinomyces neuui* (0.03%) was reported to cause endophthalmitis⁴¹ and periprosthetic infection⁴², *Actinomyces vaccimaxillae* (0.01%) was isolated from a cow jaw lesion⁴³ and *Actinomyces hongkongensis* (0.004%) was reported to cause high-mortality bacteremia in humans⁴⁴.

0.24% of the eubacterial population was composed of *Plesiomonas shigelloides* a well-documented pathogen associated with Travelers' diarrhea, dysentery and gastroenteritis⁴⁵⁻⁴⁸. *Arcanobacterium pyogenes* was also present (0.18%), a pathogen reported to cause soft tissue infections in humans⁴⁹. *Atopobium vaginae* (0.12%) was reported to cause bacterial vaginosis in a human⁵⁰ and *Varibaculum cambriense* (0.01%) was reported to cause complications with intra-uterine devices and vaginal infections in Hong Kong⁵¹. *Parvimonas micra* (0.08%) was associated with odontogenic infection⁵² and human bacteremia was reported with *Atopobium rima*⁵³, *Fusobacterium nucleatum*⁵⁴, *Corynebacterium freneyi*⁵⁵ and *Streptococcus suis*⁵⁶. Finally, *Veillonella dispar* (0.02%) was reported in a case of septic arthritis⁵⁷ and *Porphyromonas gingivalis* (0.02%) is a well-studied pathogen reported decades earlier and associated with periodontitis⁵⁸.

Six additional fecal samples from *Ayantha americana* were processed and sequenced using the ion torrent platform. The number of OTUs

Table 2. Diseases related to the eubacteria identified in the wild duck fecal microbiome.

Genus/Species	% of Biome	Disease	Reference
<i>Fusobacterium mortiferum</i>	31.61	Septicemia	18–20
<i>Streptobacillus moniliformis</i>	30.10	Rat bite fever/Haverhill, osteomyelitis, epidural abscess, fever and polyarthralgia, bacteremia, drinking water related disease	22–27
<i>Lactobacillus intermedius</i>	11.02	Renal transplant infection	28
<i>Actinomyces suimastitidis</i>	4.47	Pig mastitis	29
<i>Campylobacter canadensis</i>	3.69	Drinking water related disease	27
<i>Enterococcus cecorum</i>	3.59	Arthritis and osteomyelitis in chicks, enterococcal spondylitis (ES) chicks, Aortic valve endocarditis in humans, empyema thoracis, septicemia, recurrent bacteremic peritonitis	30–35
<i>Actinomyces odontolyticus</i>	0.70	Bacteremia in immunosuppressed patients, Malar mass	36,37
<i>Leptotrichia spp.</i>	0.36	Bacteremia	38
<i>Actinomyces turicensis</i>	0.30	Genital infections, urinary infections, skin infections, post-operative wound infection, abscess, appendicitis, ear and nose and throat infection, and bacteremia	39
<i>Plesiomonas shigelloides</i>	0.24	Travelers' diarrhea, dysentery, gastroenteritis	45–48
<i>Arcanobacterium pyogenes</i>	0.18	Human wound infections	49
<i>Actinomyces europaeus</i>	0.14	Human abscesses	40
<i>Atopobium vaginae</i>	0.12	Bacterial vaginosis	50
<i>Parvimonas micra</i>	0.08	Odontogenic infection	52
<i>Atopobium rimae</i>	0.05	Human Bacteremia	53
<i>Helcococcus kunzii</i>	0.05	Urocystitis in a sow	59
<i>Fusobacterium nucleatum</i>	0.03	Bacteremia	54
<i>Actinomyces neuui</i>	0.03	Endophthalmitis, periprosthetic infection	41,42
<i>Veillonella dispar</i>	0.02	Septic arthritis	57
<i>Porphyromonas gingivalis</i>	0.02	Periodontitis	32
<i>Corynebacterium freneyi</i>	0.01	Bacteremia	55
<i>Actinomyces vaccimaxillae</i>	0.01	Cow jaw lesion	43
<i>Streptococcus suis</i>	0.01	Meningitis, septicemia, endocarditis, arthritis, and septic shock in both pigs and human beings,	56
<i>Varibaculum cambriense</i>	0.01	Intrauterine devices and vagina	51
<i>Actinomyces hongkongensis</i>	<0.01	Bacteremia	44

increased from 85 to 163, and the rarefaction curve that included OTUs from only *Ayantha americana* indicated that additional OTUs can be expected due to the lack of a plateau on the graph (data not shown). However, the ion torrent sequencing data in Table 3 supported the 454 pyrosequencing data in that the *Anaerotruncus* genus was common along with *Bacteroides*, *Blautia*, *Campylobacter*, *Cetobacterium*, *Clostridium*, *Coprococcus*, *Erysipelotrichaceae*, *Faecalibacterium*, *Fusobacterium*, *Peptostreptococcaceae*, *Pseudobutyrvibrio*, *Roseburia*, *Ruminococcaceae*, *Streptobacillus* and *Veillonella*.

Forty six of the eubacterial OTUs have been reported as pathogens or opportunistic pathogens and on average they represented nearly 72% of the bacteria present, M=71.78, SD=5.96, 95% CI [57.19, 86.37]. *Anaerotruncus spp.*, *Campylobacter canadensis*, *Fusobacterium mortiferum*, *Helcococcus kunzii*, *Streptobacillus moniliformis*, *Streptococcus suis*, *Arcanobacterium pyogenes*, *Clostridium ramosum*, *Corynebacterium xerosis*, *Enterococcus faecium*, *Flavobacterium spp.* and *Veillonella magna* were recognized and evaluated as the most pathogenic representative bacteria. The twelve most pathogenic on average represented nearly 31% of the bacteria present, M=30.95, SD=9.70, 95% CI [7.19, 54.71]. The mean is not normally distributed for a small

sample of seven; consequently the confidence intervals were estimated using a t-distribution with six degrees of freedom.

Pyrosequencing of wild duck Oct 2013

1 Data File

<http://dx.doi.org/10.6084/m9.figshare.818973>

Discussion

Numerous pathogenic eubacterial species have been identified in the fecal samples obtained from the wild duck, *Aythya Americana*, using amplicon pyrosequencing and ion torrent sequencing, two widely accepted methods for analyzing the bacterial composition of microbial ecosystems. We were surprised to find that most of the species of eubacteria sequenced the duck feces were not present in a pond water sample from a water source that was known to be visited by numerous water fowl. Perhaps, the analyses of small samples from a pond or lake are not adequate when investigating the presence of avian contamination.

Table 3. Taxonomical classification of operational taxonomic units into the Genus/species level with representative percentages of the Eubacterial Microbiome based on Ion Torrent Sequencing Data.

	DAA-1	DAA-2	DAA-3	DAA-4	DAA-5	DAA-6
<i>Acetobacteraceae</i> spp.	0.2844	0.9695	0.1916	0.0000	0.0926	0.0000
<i>Actinobacillus rossii</i>	0.0000	0.0000	0.0958	0.0000	0.0000	0.0000
<i>Actinomadura</i> spp.	0.0000	0.0000	0.0000	0.0000	0.3704	0.0000
<i>Actinomyces canis</i>	0.0000	0.0000	0.0958	0.0000	0.0000	1.7157
<i>Actinomyces hyovaginalis</i>	0.0000	0.0000	0.0000	0.0000	0.4630	0.0490
<i>Actinomyces nasicola</i>	0.0000	0.0000	0.0000	0.0000	0.0000	2.6471
<i>Actinomyces odontolyticus</i>	0.0000	0.0000	0.0000	0.0000	0.0000	0.1471
<i>Actinomyces</i> spp.	0.0000	0.1385	0.0958	0.0000	0.0000	5.0980
<i>Actinomyces suimastitidis</i>	0.0000	0.0000	0.4789	0.0000	0.0000	5.0490
<i>Aeromonas</i> spp.	0.1896	0.1385	0.0958	0.0000	0.0000	0.0000
<i>Akkermansia</i> spp.	8.3412	3.1856	0.0000	0.0000	0.0000	0.0000
<i>Alistipes</i> spp.	0.1896	0.4155	0.0000	0.2548	0.0000	0.0000
<i>Allisonella</i> spp.	0.0000	0.0000	0.0000	0.7643	0.0000	0.0000
<i>Allobaculum</i> spp.	0.2844	0.1385	0.0000	0.1274	0.0926	0.0000
<i>Anaerobiospirillum</i> spp.	0.0948	1.6620	16.0920	0.0000	0.0000	0.0490
<i>Anaerobiospirillum thomasi</i>	0.0000	0.1385	1.9157	0.0000	0.0000	0.0000
<i>Anaerofilum</i> spp.	0.2844	0.0000	0.0000	0.0000	0.1852	0.0000
<i>Anaeroplasma</i> spp.	0.0000	0.5540	0.0000	0.0000	0.0000	0.0000
<i>Anaerosporbacter</i> spp.	0.0000	0.0000	0.0000	0.2548	0.0000	0.0000
<i>Anaerostipes</i> spp.	0.0000	0.0000	0.0000	0.3822	0.0000	0.0000
<i>Anaerotruncus</i> spp.	1.8009	1.9391	0.0958	7.7707	0.7407	0.0490
<i>Ancalomicrobium hirschii</i>	0.1896	0.2770	0.0958	0.0000	0.4630	0.0000
<i>Ancalomicrobium</i> spp.	0.0948	0.1385	0.0000	0.0000	0.0926	0.0000
<i>Andersenella</i> spp.	0.0000	0.0000	0.0000	0.5096	0.0000	0.0000
<i>Asanoa</i> spp.	0.0000	0.2770	0.2874	0.0000	0.1852	0.1471
<i>Atopobium parvulum</i>	0.0000	0.0000	0.0000	0.0000	0.1852	0.0000
<i>Atopobium rimae</i>	0.0000	0.0000	0.0000	0.0000	0.3704	1.2745
<i>Atopobium</i> spp.	0.0000	0.0000	0.0958	0.0000	0.0000	0.7353
<i>Atopobium vaginiae</i>	0.0000	0.0000	0.0000	0.0000	0.4630	0.5882
<i>Bacteroidales</i> spp.	0.6635	1.2465	0.1916	2.5478	0.0000	0.0000
<i>Bacteroides capillosus</i>	0.0948	0.0000	0.0000	0.0000	0.0926	0.0000
<i>Bacteroides coprocola</i>	1.4218	0.8310	0.0000	0.0000	0.0000	0.0000
<i>Bacteroides coprophilus</i>	0.2844	0.6925	0.1916	1.5287	0.0000	0.0000
<i>Bacteroides eggerthii</i>	0.6635	0.1385	0.0958	0.1274	0.0000	0.0000
<i>Bacteroides</i> spp.	11.0900	12.4654	0.8621	8.2803	1.4815	0.0000
<i>Barnesiella</i> spp.	0.0000	0.0000	0.0000	2.4204	0.0000	0.0000
<i>Beijerinckiaceae</i> spp.	0.0000	0.0000	0.0000	0.1274	0.2778	0.0000
<i>Blautia producta</i>	1.7062	0.6925	0.0000	0.7643	2.8704	0.0490
<i>Blautia</i> spp.	0.8531	0.4155	0.0000	1.9108	2.7778	0.0000
<i>Brachyspira innocens</i>	0.0000	0.0000	0.0000	0.1274	0.0000	0.0000
<i>Bulleidia solobacterium moorei</i>	0.0000	0.0000	0.0000	0.2548	0.1852	0.0000
<i>Campylobacter canadensis</i>	0.1896	12.8809	23.1801	4.5860	0.0000	0.0490
<i>Campylobacter upsaliensis</i>	0.0000	0.0000	0.5747	0.0000	0.0000	0.0000
<i>Candidatus_Alysiosphaera</i> spp.	0.0000	0.1385	0.0000	0.2548	0.0000	0.0000
<i>Candidatus_Phytoplasma</i>	0.0000	0.0000	0.0958	0.0000	0.0000	0.4902
<i>Cetobacterium somerae</i>	0.1896	0.0000	0.0958	0.3822	11.6667	0.0000
<i>Clostridium bartlettii</i>	0.0948	0.0000	0.4789	0.0000	0.0926	0.0000
<i>Clostridium bifermentans</i>	0.1896	0.4155	0.0958	0.0000	0.0000	0.0000

	DAA-1	DAA-2	DAA-3	DAA-4	DAA-5	DAA-6
<i>Clostridium celerecrescens</i>	0.0000	0.0000	0.0958	0.0000	0.0000	0.0490
<i>Clostridium colinum</i>	0.0000	0.0000	0.0000	0.0000	2.1296	0.0000
<i>Clostridium hiranonis</i>	0.0948	0.0000	0.4789	0.0000	0.0000	0.0000
<i>Clostridium lactatifermentans</i>	0.0948	0.4155	0.0000	0.0000	0.0000	0.0000
<i>Clostridium ramosum</i>	0.3791	0.0000	0.0000	0.0000	0.0000	0.0000
<i>Clostridium spiroforme</i>	3.3175	0.1385	0.1916	0.0000	1.5741	0.0000
<i>Clostridium spp.</i>	0.4739	0.1385	0.0000	0.3822	0.0000	0.0000
<i>Comamonadaceae spp.</i>	0.0000	0.0000	0.0000	0.3822	0.0000	0.0000
<i>Coprococcus spp.</i>	0.1896	0.0000	0.3831	3.1847	0.0926	0.0000
<i>Coriobacteriaceae spp.</i>	0.1896	0.0000	0.0958	0.0000	0.3704	0.9314
<i>Corynebacterium freneyi</i>	0.0000	0.0000	0.0000	0.0000	0.0000	1.5196
<i>Corynebacterium kroppenstedtii</i>	0.0000	0.1385	0.3831	0.0000	0.0000	0.0000
<i>Corynebacterium xerosis</i>	0.0000	0.0000	0.0000	0.0000	0.0000	1.0294
<i>Defluviicoccus spp.</i>	0.1896	0.1385	0.0000	0.0000	0.0000	0.0000
<i>Desulfovibrio spp.</i>	0.0000	0.5540	0.0000	0.0000	0.0000	0.0980
<i>Dorea spp.</i>	0.0000	0.0000	0.0000	1.6561	0.0000	0.0000
<i>Enterobacter spp.</i>	0.0000	0.0000	0.0000	0.0000	0.0000	0.1471
<i>Enterococcus cecorum</i>	0.0000	0.0000	0.0000	0.0000	0.0000	0.3922
<i>Enterococcus faecium</i>	0.0000	0.0000	0.0000	0.0000	0.5556	0.0000
<i>Erysipelothrix spp.</i>	0.0000	0.1385	0.0000	0.0000	0.3704	0.0000
<i>Erysipelotrichaceae anaerorhabdus furcosa</i>	0.0948	0.0000	0.0000	0.0000	1.6667	0.0000
<i>Erysipelotrichaceae spp.</i>	1.3270	0.8310	0.0000	7.7707	6.8519	0.0000
<i>Erythrobacter unidentified</i>	0.0000	0.5540	0.0000	0.0000	0.0000	0.0000
<i>Escherichia_shigella spp.</i>	2.3697	0.4155	18.4866	0.0000	0.0000	0.0000
<i>Faecalibacterium prausnitzii</i>	7.8673	11.2188	0.6705	0.0000	0.0926	0.0000
<i>Faecalibacterium spp.</i>	5.7820	2.6316	1.3410	4.2038	0.3704	0.0490
<i>Fastidiosipila spp.</i>	0.0000	0.0000	0.0000	0.0000	1.2963	0.0000
<i>Flavobacterium spp.</i>	0.0000	0.0000	0.0000	0.1274	0.0000	0.0000
<i>Flavonifractor spp.</i>	0.0948	0.0000	0.0000	0.3822	0.4630	0.0000
<i>Fusobacterium mortiferum</i>	7.6777	5.6787	1.2452	1.4013	0.0000	5.4902
<i>Fusobacterium nucleatum</i>	0.0000	0.0000	0.6705	0.0000	0.0000	0.0000
<i>Fusobacterium spp.</i>	0.2844	0.4155	0.0958	0.7643	0.0000	0.0000
<i>Geobacillus pallidus</i>	0.0000	0.0000	0.0000	0.0000	0.0000	0.0980
<i>Helcococcus kunzii</i>	0.0000	0.0000	0.0958	0.0000	0.0000	0.3922
<i>Helcococcus ovis</i>	0.0000	0.0000	0.0000	0.0000	0.0000	0.1471
<i>Helcococcus sueciensis</i>	0.0000	0.0000	0.0000	0.0000	0.5556	0.0000
<i>Helicobacter brantae</i>	0.0000	0.1385	0.0000	0.1274	0.0000	1.0784
<i>Helicobacter mustelae</i>	0.0000	0.0000	0.0000	0.0000	0.0000	0.0490
<i>Hydrogenophaga spp.</i>	0.0000	0.0000	0.0000	0.7643	0.0000	0.0000
<i>Insolitispirillum insolitospirillum peregrinum</i>	0.0000	0.1385	0.1916	0.0000	0.0000	0.0000
<i>Lactobacillus intermedius</i>	0.0000	0.0000	0.0000	0.0000	0.1852	0.0980
<i>Lactobacillus salivarius</i>	0.0000	0.0000	0.0000	0.0000	0.0926	1.5196
<i>Lactobacillus spp.</i>	0.0000	0.0000	0.0000	0.0000	0.0000	0.0980
<i>Leptotrichia goodfellowii</i>	0.0000	0.0000	0.0000	0.0000	0.0000	0.5392
<i>Meiothermus silvanus</i>	0.0000	0.2770	0.0000	0.0000	0.0000	0.0000
<i>Methylobacterium spp.</i>	0.2844	3.3241	0.0000	7.2611	0.0000	0.0000
<i>Microbacterium aurum</i>	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
<i>Microbispora rosea</i>	0.0000	0.2770	0.0000	0.0000	0.5556	0.2451
<i>Mitochondria spp.</i>	0.0000	0.0000	0.0000	0.0000	0.0926	0.0000
<i>Moryella spp.</i>	0.1896	0.0000	0.0958	0.0000	0.0926	0.0000

	DAA-1	DAA-2	DAA-3	DAA-4	DAA-5	DAA-6
<i>Mycobacterium</i> spp.	0.0000	0.2770	0.0000	0.0000	0.1852	0.0000
<i>Nostoc commune</i>	0.0000	0.4155	0.0000	0.0000	0.0000	0.0000
<i>Nostoc linckia</i> var. <i>arvense</i>	0.0000	0.8310	0.0000	0.0000	0.0000	0.0000
<i>Novispirillum</i> spp.	0.0000	0.0000	0.4789	0.0000	0.0000	0.0000
<i>Novosphingobium subterraneum</i>	0.0000	0.0000	0.2874	0.0000	0.0000	0.0000
<i>Oribacterium</i> spp.	0.8531	0.8310	0.0000	0.0000	5.5556	0.0000
<i>Oscillospira</i> spp.	0.0000	0.0000	0.0000	0.5096	0.0926	0.0000
<i>Paludibacter</i> spp.	0.0000	0.0000	0.0000	0.5096	0.0000	0.0000
<i>Parabacteroides</i> spp.	0.7583	0.0000	0.0958	0.2548	0.0000	0.0000
<i>Paraprevotella</i> spp.	0.0000	0.0000	0.0000	0.3822	0.0000	0.0000
<i>Parvimonas micra</i>	0.0000	0.0000	0.0958	0.0000	1.3889	0.0000
<i>Pasteurella stomatis</i>	0.0000	0.0000	0.3831	0.0000	0.0000	0.0000
<i>Peptoniphilus asaccharolyticus</i>	0.0000	0.0000	0.0000	0.0000	0.8333	0.0980
<i>Peptostreptococcaceae</i> spp.	5.1185	2.9086	6.5134	5.7325	1.7593	0.2451
<i>Planctomycetaceae</i> spp.	0.0948	0.1385	0.0000	7.0064	0.0000	0.0000
<i>Porphyromonas canoris</i>	0.0000	0.0000	0.3831	0.0000	0.0000	0.0000
<i>Porphyromonas gingivalis</i>	0.0000	0.0000	1.0536	0.0000	0.0000	0.0000
<i>Prevotella</i> spp.	0.0000	0.0000	0.0000	0.5096	0.0000	0.0000
<i>Prevotellaceae</i> spp.	0.0948	0.1385	0.0000	0.0000	0.0000	0.0000
<i>Prochlorococcus marinus</i> mit 9313	0.0000	0.0000	0.0000	0.1274	0.0000	0.0000
<i>Propionibacterium acnes</i>	0.0000	0.0000	0.0000	0.5096	0.0000	0.0000
<i>Proteiniphilum</i> spp.	0.1896	0.4155	0.0000	0.0000	0.0000	0.0000
<i>Pseudobutyrvibrio</i> spp.	1.8009	0.2770	0.0000	0.7643	0.2778	0.0000
<i>Pseudonocardia alaniniphila</i>	0.0000	0.0000	0.0000	0.0000	0.2778	0.0980
<i>Pseudonocardia xinjiangensis</i>	0.0000	0.2770	0.0000	0.0000	0.1852	0.1961
<i>Ralstonia pickettii</i>	0.0000	0.0000	0.0000	1.5287	0.0000	0.0000
<i>Rhodobacter</i> spp.	0.3791	0.6925	0.0000	0.0000	0.1852	0.0000
<i>Rhodobacteraceae</i> spp.	0.5687	0.6925	0.0958	0.0000	0.0000	0.0000
<i>Rhodospirillaceae</i> spp.	0.4739	0.4155	0.0000	0.0000	0.0000	0.0000
<i>Roseburia eubacterium rectale</i>	0.0000	0.0000	0.2874	0.0000	0.0000	0.0000
<i>Roseburia</i> spp.	0.0948	0.0000	0.0958	0.0000	0.1852	0.2941
<i>Roseococcus</i> spp.	0.3791	0.0000	0.0000	0.0000	0.0000	0.0000
<i>Roseomonas gilardii</i>	0.2844	0.0000	0.0000	0.0000	0.0000	0.0000
<i>Roseomonas lacus</i>	0.5687	0.4155	0.0000	0.0000	2.5000	0.0000
<i>Roseomonas</i> spp.	0.2844	0.1385	0.0958	0.0000	0.0926	0.0000
<i>Roseomonas terrae</i>	0.0000	0.0000	0.0000	0.0000	1.0185	0.0000
<i>Rubellimicrobium</i> spp.	0.0948	0.0000	0.0000	0.0000	0.0926	0.0000
<i>Rubribacterium</i> spp.	0.1896	0.1385	0.0958	0.0000	0.0000	0.0000
<i>Ruminococcaceae human gut metagenome</i>	0.0000	0.0000	0.0000	0.0000	0.1852	0.0000
<i>Ruminococcaceae</i> spp.	24.5498	15.7895	3.0651	12.2293	36.5741	5.1471
<i>Ruminococcus gnavus</i>	0.0000	0.0000	0.0000	0.3822	0.0000	0.0000
<i>Ruminococcus</i> spp.	0.0000	0.0000	0.0000	1.0191	0.0000	0.0000
<i>Saccharofermentans</i> spp.	0.1896	1.1080	0.0000	0.0000	0.0000	0.1961
<i>Sandaracinobacter</i> spp.	0.2844	0.0000	0.0000	0.0000	0.0000	0.0000
<i>Saprospiraceae</i> spp.	0.0000	0.0000	0.1916	0.0000	0.0000	2.5000
<i>Sciscionella</i> spp.	0.0000	0.2770	0.0000	0.0000	0.1852	0.0980
<i>Selenomonas</i> spp.	0.0000	0.0000	0.0000	0.3822	0.0926	0.0000
<i>Slackia human intestinal bacterium_dze</i>	0.0000	0.0000	0.0000	0.0000	0.1852	0.0000
<i>Sneathia</i> spp.	0.0000	0.0000	0.0000	0.0000	0.0000	1.9118
<i>Solobacterium</i> spp.	0.2844	0.2770	0.0000	0.0000	0.3704	0.0000

	DAA-1	DAA-2	DAA-3	DAA-4	DAA-5	DAA-6
<i>Spirochaeta</i> spp.	0.0000	0.4155	0.0000	1.0191	0.0000	0.0000
<i>Streptobacillus moniliformis</i>	0.2844	0.8310	14.4636	0.0000	1.2963	56.6176
<i>Streptococcus macedonicus</i>	0.0948	0.0000	0.0000	0.0000	3.7963	0.0000
<i>Streptococcus suis</i>	0.0000	0.0000	0.0000	0.0000	0.0926	0.0000
<i>Subdoligranulum</i> spp.	0.6635	1.2465	0.0958	3.6943	0.5556	0.0000
<i>Succinivibrio wallaby gut_metagenome</i>	0.0000	0.0000	1.1494	0.0000	0.0000	0.0000
<i>Sutterella</i> spp.	0.2844	0.4155	0.0000	0.3822	0.0000	0.0000
<i>Synechococcus elongatus</i>	0.0000	0.0000	0.0000	0.3822	0.0000	0.0000
<i>Syntrophomonas</i> spp.	0.1896	0.2770	0.0000	0.0000	0.7407	0.0000
<i>Tessaracoccus</i> spp.	0.0000	0.0000	0.0000	0.0000	0.0926	0.0980
<i>Thalassospira</i> spp.	0.8531	2.4931	0.0000	0.8917	0.1852	0.0490
<i>Thermoanaerobacterium saccharolyticum</i>	0.0000	0.1385	0.0000	0.0000	0.0000	0.0000
<i>Treponema</i> spp.	0.0000	0.0000	1.0536	0.0000	0.0000	0.0000
<i>Veillonella</i> spp.	0.0000	0.1385	0.0000	0.0000	0.3704	0.0980
<i>Xylanibacterium pachnodae</i>	0.0000	0.1385	0.0000	0.0000	0.0926	0.2451
Total	100.0000	100.0000	100.0000	100.0000	100.0000	100.0000

The summary in [Table 2](#) indicates that many of the bacteria that are listed are clinically important causing severe diseases such as bacteremia and septicemia. The potential to cause disease can be appreciated when one considers that wild-duck feces can contaminate food, drinking water and open wounds. In addition, bird feces can easily contaminate pond fountains and ornamental fountains—where aerosols are produced—and the aerosols can carry the bacteria in a similar way to what has been reported for *Legionella pneumophila*⁴⁷. It is possible that many of the bacterial entities when disseminated to humans and other animals could also cause subclinical respiratory illnesses that are not reported due to patient resolution and the empiric treatment of respiratory illnesses with antibiotics without diagnostic testing.

It is only prudent to recommend that immunocompromised humans, the elderly and animals should limit their exposure to environments where ducks are abundant and may have polluted the water source—this includes ponds with fountains, outdoor pools and ornamental fountains that are not properly maintained. That realization also supports the practice of adequately chlorinating or sanitizing artificial pools and fountains to prevent opportunistic infections through aerosols or breaks in the skin.

Duck hunters should also be aware of the risk of bacterial contamination in addition to the risk posed by the influenza virus. Additionally, reclaimed water poses a threat to the elderly and other immunocompromised humans who might be exposed to aerosols that are produced when the reclaimed water is used as a source of irrigation such as in golf courses and gardens, a common practice that might warrant further inquiry.

When determining the cause of disease it is difficult—if not impossible—to identify the source of infection, and whether it has indeed originated from an animal that is migratory or aquatic in nature. Many of the bacterial species that were cited to cause infections among humans were also found in the excrement of a migratory and aquatic bird that is unrestrained and defecates in

water supplies and defecates around farm animals that are raised as “free-range” and exposed to the environment.

Our analysis of the wild duck was focused on *Ayantha americana*, a wild duck common to Florida and the Southeastern region of the United States. The statistical analysis of seven fecal samples indicated that the wild duck eubacterial microbiome contained numerous bacteria that were pathogenic or opportunistic pathogens, and the wild duck should be recognized as a vector for bacterial contamination and disease to humans and farm animals.

Author contributions

TS carried out the majority of the molecular biology techniques in the laboratory, AG was instrumental in obtaining the wild ducks specimens. SD provided expertise in pyrosequencing and bioinformatics. DM statistically analyzed the data. JC conceived the study and wrote the first draft of the manuscript. All authors were involved in the revision of the draft and have agreed to the final content. The study was an active learning exercise that helped bridge the understanding of Medical Microbiology with field research, molecular biology and bioinformatics for graduate students seeking their Masters (MS) degree in Biomedical Sciences under the guidance of Dr. Coffman.

Competing interests

No competing interests were disclosed.

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Version 2

Reviewer Report 23 May 2014

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Jorge Santo Domingo

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After a reassessment of the paper, and after reading the other reviewers' comments and authors' responses, I am still very skeptical about the interpretation on using relatively small fragments of the 16S rDNA to assign identities to the species level. The Ion Torrent sequences were at best 251 bp. While it is not clear what is the average pyrosequencing read length of the, we often cannot get more than 400 bp as the quality of base calling is significantly poor after 300 bp. I could not get which primers were used for pyrosequencing and therefore the read length for the pyrosequences (yes they indicated the primers used to amplify the entire 16S but which primer was used for the sequencing was not indicated; perhaps it was the 27F?).

I would agree that it is possible for some of the sequences to be nearly identical to some reported pathogens, but the sequences were not shared or deposited in a public database so this reviewer (or the readers for that matter) can't confirm these results. This is important as the reported levels of pathogens in the feces was very high (>70%), which will strongly suggest that these duck species are important emerging vectors of human disease. These claims have important public health implications and therefore more evidence will be needed to further substantiate this.

It is also curious that the authors tried to make a link between duck feces and water risk exposure. The evidence here is also weak and is based on a few sequences that are shared by the two types of samples. I'm assuming that there are other fecal potential sources impacting these waters; it is then possible that the primary vectors will be the other hosts.

In general, there was an effort to add more fecal samples to the study which was a concern brought up by all the reviewers. However, the authors' responses to the reviewers' comments was not as comprehensive as I would have liked to see. Yet, there are still missing some pieces of information on the methods, and other information such as how many sequences they obtained, and how they arrive to the identity of environmental sequences that makes it difficult to evaluate this study. Again, by not sharing representative sequences it is difficult to know if they are referring to identities at the genus level and their interpretation has to do with genera that contain pathogenic species. It would be good to place the sequences within a phylogenetic

context to further confirm the clades they belong to.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 09 May 2014

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Jonas Waldenström

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I have now re-read the paper after the revision, and also the comments from [Benjamin Kirkup](#) which were very insightful and to the point. I think that a careful rephrasing of parts of the manuscript should be done, more clearly saying that the findings of 16S rRNA gene sequences from these bacteria doesn't necessarily mean that they are pathogenic to humans, as this trait will be dependent on many factors that are not covered with the current approach. I still think an inventory of bacterial species from waterfowl is interesting, as our current understanding of what microorganisms that are present in the environment and in wild animals is rudimentary. My expertise is stronger in avian biology than in phylogenomics and I think that the detailed comments from [Benjamin Kirkup](#) are very good for strengthening this paper.

However, there were some points I raised in my initial review that I still think should merit attention. Specifically, I think that the biology of the study organism is left out more or less completely. Why was this duck species chosen? A few lines about its distribution, feeding behavior and tendencies to live close to humans would be helpful for your readers, especially those that do not have a background in ornithology. All ducks are not the same: they differ in ecological traits as well in their capacity to be infected and perpetuate pathogens. For instance, the influenza A virus which is mentioned in the paper show clear differences in prevalence and subtypes among different species of waterfowl, as well as displaying both spatial and temporal variation in virus abundance, subtype predominance and genetic lineages. At present, the paper is written as if the study species represents all ducks at all timepoints, and including some more details about the specific duck species could easily amend this. For instance, if one raises the potential risk arising from duck hunting it would be good to know that the studied species is a significant part of the hunting bag (my guess would be that dabbling ducks are more often shot than diving ducks, such as the *Aythya americana*). Further, is this species common in urban environments, i.e. are people really exposed?

Small comments:

- Consider whether water fowl or waterfowl should be used.
- In the introduction and elsewhere, *Aythya Americana* should be *Aythya americana*. Please also add the common name when the species first is mentioned.
- The end of the first paragraph in the results should be Gammaproteobacteria
- Does reference 27 really state that *Campylobacter canadensis* is linked to water related disease? I think this is not the case, but cannot access the paper in question at the moment.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 13 December 2013

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Benjamin Kirkup

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Strong *et al.* is a characterization of the fecal microbiome from *Aythya americana*. Wild waterfowl have been implicated in zoonotic disease transmission and can confound water quality studies which track fecal contamination through waterways. Adult ducks culled during hunting and pond water were both sampled. The relationship between the pond and the ducks is unclear in the text. The text suggests that the pond was free from *Aythya americana*, but both within the range of *Aythya americana* and visited by other waterfowl.

This article is not only an exploration of a relevant sample type but also a teaching exercise for master's degree students. This fundamentally worthy goal presents recognized limitations.

One fecal sample was sequenced by Roche 454 FLX pyrosequencing; six additional samples were sequenced by Ion Torrent sequencing. One water sample was sequenced by 454 pyrosequencing. After quality control of the reads, singletons were removed, the reads were clustered into OTUs at 3% divergence and the centroids of each OTU assigned to taxa by BLASTn against a GreenGenes database.

The major conclusion of the article - in the title, abstract, results and discussion - represents the major problem with the article. There is a significant gap in evidence between a representative

read from an operational taxonomic unit based on clustering a partial 16S rRNA sequence at the 3% level and an inference of virulence. This point can be pressed from both sides. The connection between taxonomy and 16S rRNA sequences is not generally precise to the species level (Chan *et al.*, 2012 as one example). In certain cases, curated databases have allowed such a connection (Conlan *et al.*, 2012). The 16S rRNA has a non-obvious relationship with ecology (Tikhonov & Wingreen, 2013; Preheim *et al.*, 2013; and Vandewalle *et al.*, 2012); small changes can reflect large differences, larger differences can reflect no difference at all.

This comes around to the other side: even within an MLST sequence type within a named species, virulence can be highly variable (Weissman *et al.*, 2012). Using a larger sample of the genome is not necessarily an improvement over the 16S rRNA. It depends on which sections of the genome are selected (Polz *et al.*, 2006; Preheim *et al.*, 2011; etc). As a result, it is perhaps technically correct to call a species “linked to human and animal diseases” but without any statement about how likely a duck feces isolate is to belong to a human, domestic animal, or even waterfowl pathogen subtype, the claim is frankly unsound and even alarmist (“Duck hunters should also be aware of the risk...”). Thus, the focus of this article on the pathogenic potential of the duck feces microbiome is problematic. This issue is widespread in the literature, unfortunately, and contributes to the issues which Jonathan Eisen raises (<http://phylogenomics.blogspot.com/2013/08/overselling-microbiome-award-swiss.html>) in his ‘overselling the microbiome.’

Major experiments would be required to substantiate the claims of pathogenic potential by introducing duck fecal microbes into animal models of disease. Another possibility is the use of deep shotgun metagenomics, perhaps combined with long read or single cell sequencing to permit genome assembly, to enable a bioinformatic approach. The authors state that it is not possible to provenance a pathogen, but should they have whole genome sequence from cases (including ill waterfowl, domestic animals or humans) and from waterfowl feces, there could be strong grounds for such a claim.

The claim that the bacteria present in the feces are not those in the pond water is unsustainable (‘devoid of any obvious pond water eubacterial constituents’). Not only is this contradicted in Figure 2, but the depth of sequencing was insufficient to demonstrate that the organisms are not present in both samples. That the dominant microbes are not the same is a reasonable claim. However, few microbiologists would have expected the duck feces or the water to be representative of each other. In addition, with a single water sample, one might claim that the water does not fall within the range of the duck samples; but the inverse claim cannot be sustained statistically for lack of multiple samples. This analysis can be performed under R using packages developed for microbiome analysis (ie phyloseq McMurdie & Holmes, 2013, and HMP La Rosa *et al.*, 2012).

There are other analyses which could fruitfully be included in the manuscript without any additional sequencing. For example, there is no graphical or statistical comparison of the 7 fecal samples. These samples – including the pond sample - are not compared to any of the water samples from the earth microbiome project databases, IMG/j, or MG-RAST databases. The comparison might be limited because the methods of DNA extraction and sequencing were not standard for these other projects, but there are 824 aquatic samples and 6 bird samples in the IMG/j database, samples from birds in the MG-RAST database, and bird and freshwater associated samples in the EMP.

Overall, in fulfillment of the obligations of formal review, the data appears to be collected appropriately and the methods described adequately. The data is included in the publication. The paper itself requires revision with regard to the conclusions and could be substantially enhanced by some additional bioinformatics.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 13 Dec 2013

Jonathan Coffman, Barry University, Hollywood, USA

Thank you for your extensive review and recommendations for improvement and further analysis. Taking the time to review our article is much appreciated. Thank you for the additional references to phyloseq and HMP and the MG-RAST database.

The intent was not to be an alarmist but to bring awareness. In South Florida, we have many elderly and AIDS patients who are susceptible to opportunistic infections. Numerous fountains in S. Florida are found in ponds and lakes that are visited by water fowl, and aerosols are created as the fountains blast the non-treated water into the air (not to mention irrigation systems on golf courses). Most respiratory infections in our system are empirically treated with antibiotics so its difficult to obtain laboratory on many respiratory infections. As an instructor in Medical Virology, I have been well aware of the avian flu and water fowl as the major reservoir for the virus--an animal that shows no signs of illness when carrying the most pathogenic strains to humans. I have also been privy to unreported cases of human illnesses and hunting dog illnesses (vomiting and diarrhea) following duck hunts or when wild ducks are processed/cleaned for food. That sparked our interest in the bacterial component.

What minimal doses of bacteria will cause disease in an elderly human, AIDS patient or immunocompromised patient? It would be interesting to perform a study with T cell deficient CD-1 nude mice and duck fecal aerosols, but that type of study is beyond the capabilities of our lab and university.

Competing Interests: No competing interests were disclosed.

Version 1

Reviewer Report 13 November 2013

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Jonas Waldenström

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The novel sequencing techniques now allow for assessing a much larger fraction of the microorganisms a host is carrying. In the past, an investigation of the gastrointestinal microbiota of an animal would rely either on the culturable fraction of microorganisms, or laborious cloning and sequencing. The paper by Strong *et al.* uses amplicon sequencing of the 16sRNA gene for detecting and characterizing the microbiome of a wild duck fecal sample. The resulting data is compared to a water sample from another pond, and referenced to the existing literature to identify possible human and animal pathogens.

I have some concerns with this paper. First, naturally, is the very limited sample size. With only a single sample from each of the two entities, it is problematic to tackle within-host and between host variability, and any temporal variation present in the data. Furthermore, the duck and the pond water sample were collected by different methods, which also may affect template DNA purity and yield, and the samples are not from the same site.

The authors focus the majority of the paper on what potential human pathogens the duck is carrying. This is interesting, as many human pathogens have animal reservoirs and identifying them may lead us to better understand their epidemiology. There is a range of human pathogens identified, and the authors make recommendations based on these findings. However, recommendations need to be thoroughly justified by data, and in at least one case I did not find the link to human infection proven. This is *Campylobacter canadensis* that in table 2 is said to cause drinking water related disease. However, the reference cited for this claim does not discuss *C. canadensis*, but waterborne outbreaks in general.

The methods/result sections could be made more informative. Every method has its cons and pros, and it would help the reader to illustrate how it was made sure the primers and protocol used were suitable for bird fecal microbiota. Have they been validated on avian fecal samples before? It is not presented clearly how many sequences that were obtained, how many that were possible to bin to different species/genera etc.

Surprisingly, the duck host is not presented in any detail at all. I am not familiar with *Aythya americana*, as it is restricted to North America, but there are a number of *Aythya* species in Europe, all of which are diving ducks, often feeding to a large extent on bivalves and other mollusks, and not really dabbling in the surface water. This will likely influence the potential exposure of *A. americana* feces to humans, and should be taken into account in the recommendations given in the paper. As a biologist, I am also curious to know why these bacteria are in the bird, and what they do there – are they commensals, or pathogens? What has been found in other bird species? This is not discussed in the present version.

Taken together, the data should be considered with some caution as it is based on such a small

sample set and conclusions from comparisons should therefore be toned down. Preferentially, the study should be extended to include more avian samples.

Minor things:

- **Title:** Very little in this article actually refers to animal disease, which makes the title slightly misleading.
- **Introduction:** It would be good if the host could be put in a larger perspective here, and that previous work on ducks as carriers of human-associated bacterial pathogens are cited to a larger extent. The ornamental/decorative fountains are unlikely significant duck habitats.
- **Figure 1:** Are the numbers referring to percentages? And is it not more a table than a figure?
- Please check bird names and bacterial names and make sure species names are not starting with capital letters (e.g. *Aythya americana*, not *Americana* etc).

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 13 Dec 2013

Jonathan Coffman, Barry University, USA

Thank you for your comments and recommendations. We are continuing our study with additional samples to increase our N.

Competing Interests: No competing interests were disclosed.

Reviewer Report 06 November 2013

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Jorge Santo Domingo

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The article of Strong *et al.* focuses on the description of the bacterial community of a duck fecal

sample. In the study the authors analyzed 16S rDNA pyrosequencing data to determine the identity and diversity of the bacteria present in the sample. They also collected water samples and performed similar studies in order to determine if the water was influenced by the duck staging over the area where the sample was collected. The presence of pathogenic bacteria in avian feces is well documented, although most of the work has been conducted with a relatively limited number of species. It is also known that birds can influence the microbial quality of surface waters. Thus any additional data on the occurrence and relative abundance of potential pathogens from birds and their relationship to human health risks is welcomed.

The study, as presented, suffers from some relevant limitations and in my opinion the data should be considered preliminary in nature. Perhaps the most important one is the low number of samples analyzed ($n=1$) for the fecal and water samples. Thus in this regard it would be difficult to determine if this sample is representative of the duck fecal microbiota or not. It is also not clear how many sequences were analyzed in this study and which area of the 16S rRNA gene was used in the analysis. The authors mentioned that they amplified the entire 16S rRNA gene but normally such large amplicons are not used for next generation sequencing. To this reviewer it seems that there is some details missing on the method, e.g. perhaps the sequencing facility performed a second amplification with primers that generated a smaller PCR product which was compatible with the sequencing technology. There are many other details on the methods used that are not clear, such as how long was the fecal sample at room temperature before it was processed, holding time for water sample before processed, the total volume of water filtered and how it was filtered. They also collected and analyzed one water sample on one data, which again it would be difficult to be representative of the pond. Typically, more samples would be needed to arrive at any conclusions.

There is also the issue of using relatively short fragments of the 16S rRNA to accurately identify a bacterium at the species levels. What was the length of the sequences used in each of the cases and what was the sequence identity for each of the sequences that were identified as closely related to the identified pathogens? The authors would benefit from performing additional assays to further confirm the presence of potentially pathogenic populations. 16S rDNA-based assays might be available for some of the "pathogens" identified but preferably functional genes should be used if considered for such an exercise. The authors should also be considered to compare this dataset (albeit limited in scope) with other studies in which 16S rDNA sequencing information has been used to describe avian fecal microbiota.

As a minor comment, there are two data points in Figure 1 (which actually is a table) for *Fusobacteria*.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 06 Nov 2013

Jonathan Coffman, Barry University, USA

The method for pyrosequencing including the variable regions of the 16S rRNA gene was referenced and previously described by:

Scot E. Dowd, Yan Sun, Randy D. Wolcott, Alexander Domingo, and Jeffery A. Carroll. **Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) for microbiome studies: bacterial diversity in the ileum of newly weaned Salmonella-infected pigs.** *Foodborne Pathogens and Disease*. August 2008, 5(4): 459-472.

We have obtained additional samples from *Aythya americana* and sequenced the samples using ion torrent sequencing and made a rarefaction graph.

We understand we had a limited sample size and are trying to increase it, despite being hindered by rather limited resources and time constraints.

The Whatman FTA card information (manufacturer's website):

Advantages and benefits

- Capture nucleic acid in one easy step.
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- Suitable for virtually any cell type.

Competing Interests: No competing interests

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