SHORT RESEARCH ARTICLE

Amplicon pyrosequencing of wild duck eubacterial microbiome from a fecal sample reveals numerous species linked to human and animal diseases [version 1; referees: 1 approved with reservations, 1 not approved]

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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*, *Actimomyces spp*., *Pseudobutyrivibrio 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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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 zoonoses[1]. 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 species[4]. 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 system[7]. Humans may also have direct contact with ducks and their excrement through the recreational sport of duck hunting[8]. 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 common[9]. 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]. A fecal sample obtained from a wild duck, *Aythya americana*, that was killed during duck hunting season (December 2012) by a licensed hunter, was aseptically swabbed onto a Whatman FTA card (GE Healthcare Life Sciences) using a sterile swab and gloves being careful to avoid environmental contamination. The flap of the FTA card was placed over the FTA paper and placed into a sterile pouch, and the FTA card was 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 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´-ACGGCTACCTTGTTACGACTT-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.

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 Gammaproteobactera.

However, similarities at the level of Genus and species included only *Agrobacterium tumefaciencs* 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.

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 erythromycin[20]. The pathogen, *Fusobacterium nucleatum*, on the other hand, is well-known for its association with disease and its ability to adhere to Gram-positive and Gram-negative bacteria in dental biofilms such as plaque[21].

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 disease[22], osteomyelitis[23], epidural abscesses[24], fever and polyarthralgia[25], bacteremia[26] and contaminated drinking water related disease[27].

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
Oscillatoriophycideae	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.

Other organisms and their respective illnesses included *Lactobacillus intermedius* (11.02%) in a renal transplant infection[28], *Actinomyces suimastitidis* (4.47%) in pig mastitis[29] and *Campylobacter canadensis* (3.69%) in drinking water related disease[27]. *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 endocarditis[32], empyema thoracis[33], septicemia in a malnourished adult[34] and recurrent bacteremic peritonitis in a patient with liver cirrhosis[35]. *Actinomyces odontolyticus* (0.70%) has recently been reported to cause bacteremia in immunosuppressed patients[36], and members of the genus *Actinomyces* have been known to cause actinomycosis for some time. *A. odonolyticus* was reported by Michell, Hintz and Haselby in 1997 to be the cause of a malar mass in soft tissue in a human[37]. A species of the genus *Leptotrichia* (0.36%) was also identified, a genus that has been associated with bacteremia in multiple myeloma

Genus/species	Duck Feces	Pond Water
Agrobacterium tumefaciens	0.010	0.009
Porphyromonas spp.	0.111	0.004
Ruminococcaceae 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.

OTUs genus/species	% of Eubacterial microbiome
Fusobacterium mortiferum	31.609
Streptobacillus moniliformis	30.100
Lactobacillus intermedius	11.021
Actinomyces suimastitidis	4.474
Campylobacter canadensis	3.694
Enterococcus cecorum	3.585
Lactobacillus aviarius	2.792
Actinomyces spp.	1.966
Pseudobutyrivibrio spp.	1.811
Helicobacter brantae	1.248
Coriobacteriaceae spp.	0.928
Actinomyces nasicola	0.784
Actinomyces odontolyticus	0.699
Lactobacillus aviarius	0.627
Roseburia spp.	0.380
Leptotrichia spp.	0.364
Ruminococcaceae spp.	0.307
Actinomyces turicensis	0.295
Bacillus spp.	0.265
Plesiomonas shigelloides	0.239
Fastidiosipila spp.	0.213
Actinomyces canis	0.209
Arcanobacterium pyogenes	0.183
Blautia spp.	0.169
Ruminococcus spp.	0.157
Veillonella ratti	0.155
Actinomyces europaeus	0.139
Atopobium vaginae	0.121
Lactobacillus spp.	0.117
Porphyromonas spp.	0.111
Parvimonas micra	0.078
Tessaracoccus spp.	0.068
Fusobacterium periodonticum	0.058
Atopobium rimae	0.054
Oscillibacter spp.	0.054
Helcococcus kunzii	0.054
Arthrobacter bergerei	0.048
Streptococcus macedonicus	0.044
Clostridium spp.	0.044
Peptostreptococcaceae spp.	0.042

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

patients receiving chemotherapy[38]. 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 bacteremia[39]. In addition, *Actinomyces europaeus* (0.14%) was reported in human abscesses[40], *Actinomyces neuii* (0.03%) was reported to cause endophthalmitis[41] and periprosthetic infection[42], *Actinomyces vaccimaxillae* (0.01%) was isolated from a cow jaw lesion[43] and *Actinomyces hongkongensis* (0.004%) was reported to cause high-mortality bacteremia in humans[44].

0.24% of the eubacterial population was composed of *Plesiomonas shigelloides* a well-documented pathogen associated with Travelers' diarrhea, dysentery and gastroenteritis[45–48]. *Arcanobacterium pyogenes* was also present (0.18%), a pathogen reported to cause soft tissue infections in humans[49]. *Atopobium vaginae* (0.12%) was reported to cause bacterial vaginosis in a human[50] and *Varibaculum cambriense* (0.01%) was reported to cause complications with intrauterine devices and vaginal infections in Hong Kong[51], *Parvimonas micra* (0.08%) was associated with odontogenic infection[52] 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 arthritis[57] and *Porphyromonas gingivalis* (0.02%) is a well-studied pathogen reported decades earlier and associated with periodontitis[58].

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 sample obtained from the wild duck, *Aythya Americana*, using amplicon pyrosequencing, a widely accepted method 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.

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 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

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

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

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.

It is only prudent to recommend that immunocompromised humans and animals should limit their exposure to environments where ducks may have polluted the water source—this includes outdoor pools and fountains. 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 defecates in water supplies and around other animals. However, since our analysis was limited to the careful analysis of a single, wild duck's eubacterial microbiome, the disease potential was relative to that animal only and cannot be extrapolated to all ducks of the same species. Thus, the disease

potential is relative to this studied microbiome and further statistical studies will be needed to determine the global risks associated with duck excrement among different species.

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. 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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Open Peer Review

Current Referee Status: ✗ ?

Version 1

Referee Report 13 November 2013

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? **Jonas Waldenström**
Centre for Ecology and Evolution in Microbial model Systems (EEMiS), Linnæus University, Kalmar, Sweden

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).

I have read this submission. I 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.

Competing Interests: No competing interests were disclosed.

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.

Referee 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 were 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 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.

I have read this submission. I 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.

Competing Interests: No competing interests were disclosed.

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.
- Captured nucleic acid is ready for downstream applications in less than 30 minutes.
- DNA collected on FTA Cards is preserved for years at room temperature.
- FTA Cards are stored at room temperature before and after sample application, reducing the need for laboratory freezers.
- Suitable for virtually any cell type.

Competing Interests: No competing interests