Gut microbiota and age shape susceptibility to clostridial enteritis in lorikeets under human care

David Minich[1*] (minichdavid@gmail.com)
Christopher Madden[1*] (madden.80@osu.edu)
Mauricio A. Navarro[2,3] (mnavarrob@ucdavis.edu)
Leo Glowacki[4] (justgowacky@gmail.com)
Kristen French-Kim[1] (french-kim.1@osu.edu)
Willow Chan[5] (chan.882@osu.edu)
Morgan V. Evans[1,6] (morgvevans@gmail.com)
Kilmer Soares[1,7] (kilmerzootec2010@hotmail.com)
Ryan Mrofchak[1] (mrofchak.5@osu.edu)
Rushil Madan[1] (madan.48@buckeyemail.osu.edu)
Gregory A. Ballash[1] (ballash.4@osu.edu)
Krista LaPerle[1,8] (la-perle.1@osu.edu)
Subhadeep Paul[4] (paul.963@osu.edu)
Yael Vodovotz[5] (vodovotz.1@osu.edu)
Francisco A. Uzal[2] (fauzal@ucdavis.edu)
Margaret Martinez[1,9] (maggiemartinez0106@gmail.com)
Jennifer Hausmann[10] (JHausmann@denverzoo.org)
Randall E. Junge[11] (randy.junge@columbuszoo.org)
Vanessa L. Hale[1] (hale.502@osu.edu)

[1]Ohio State University College of Veterinary Medicine, Columbus, OH, USA
[2]California Animal Health & Food Safety Lab, University of California, Davis, San Bernardino, CA, USA
[3]Instituto de Patología Animal, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Chile
[4]Ohio State University College of Arts and Sciences, Columbus, OH, USA
[5]Ohio State University College of Food, Agricultural, and Environmental Sciences, Columbus, OH, USA
[6]Ohio State University College of Public Health, Columbus, OH, USA
[7]Department of Animal Science, College of Agricultural Sciences (CCA), Federal University of Paraiba (UFPB), Areia, PB, Brazil
[8]Ohio State University Comparative Pathology & Digital Imaging Shared Resource, Columbus, OH, USA
[9]The Marine Mammal Center, Sausalito, CA, USA
[10]Denver Zoo, Denver, CO, USA
[11]Columbus Zoo and Aquarium, Columbus, OH, USA

*contributed equally

Corresponding author:
Vanessa L. Hale, MAT, DVM, PhD
Veterinary Preventive Medicine Department
Ohio State University College of Veterinary Medicine

1900 Coffey Rd.
Columbus, OH 43210
hale.502@osu.edu

Gut microbiota and age shape susceptibility to clostridial enteritis in lorikeets under human care

David Minich[1*], Christopher Madden[1*], Mauricio A. Navarro[2,3], Leo Glowacki[4], Kristen French-Kim[1], Willow Chan[5], Morgan V. Evans[1,6], Kilmer Soares[1,7], Ryan Mrofchak[1], Rushil Madan[1], Gregory A. Ballash[1], Krista LaPerle[1,8], Subhadeep Paul[4], Yael Vodovotz[5], Francisco A. Uzal[2], Margaret Martinez[1,9], Jennifer Hausmann[10], Randall E. Junge[11], Vanessa L. Hale[1]

[1]Ohio State University College of Veterinary Medicine, Columbus, OH, USA
[2]California Animal Health & Food Safety Lab, University of California, Davis, San Bernardino, CA, USA
[3]Instituto de Patología Animal, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Chile
[4]Ohio State University College of Arts and Sciences, Columbus, OH, USA
[5]Ohio State University College of Food, Agricultural, and Environmental Sciences, Columbus, OH, USA
[6]Ohio State University College of Public Health, Columbus, OH, USA
[7]Department of Animal Science, College of Agricultural Sciences (CCA), Federal University of Paraiba (UFPB), Areia, PB, Brazil
[8]Ohio State University Comparative Pathology & Digital Imaging Shared Resource, Columbus, OH, USA
[9]The Marine Mammal Center, Sausalito, CA, USA
[10]Denver Zoo, Denver, CO, USA
[11]Columbus Zoo and Aquarium, Columbus, OH, USA

*contributed equally

Abstract

Background

Enteritis is a common cause of morbidity and mortality in lorikeets that can be challenging to diagnose and treat. In this study, we examine gut microbiota in two lorikeet flocks with enteritis (Columbus Zoo and Aquarium – CZA; Denver Zoo - DZ). Since 2012, the CZA flock has experienced repeated outbreaks of enteritis despite extensive diet, husbandry, and clinical modifications. In 2018, both CZA and DZ observed a spike in enteritis. Recent research has revealed that the gut microbiota can influence susceptibility to enteropathogens. We hypothesized that a dysbiosis, or alteration in the gut microbial community, was making some

lorikeets more susceptible to enteritis, and our goal was to characterize this dysbiosis and determine the features that predicted susceptibility.

Results

We employed 16S rRNA sequencing to characterize the cloacal microbiota in lorikeets (CZA n = 67, DZ n = 24) over time. We compared the microbiota of healthy lorikeets, to lorikeets with enteritis, and lorikeets susceptible to enteritis, with "susceptible" being defined as healthy birds that subsequently developed enteritis. Based on sequencing data, culture, and toxin gene detection in intestinal contents, we identified *Clostridium perfringens* type A (CZA and DZ) and *C. colinum* (CZA only) at increased relative abundances in birds with enteritis. Histopathology and immunohistochemistry further identified the presence of gram-positive bacilli and *C. perfringens*, respectively, in the necrotizing intestinal lesions. Finally, using Random Forests and LASSO models, we identified several features (young age and the presence of *Rhodococcus fascians* and *Pseudomonas umsongensis*) associated with susceptibility to clostridial enteritis.

Conclusions

We identified *C. perfringens* type A and *C. colinum* associated with lorikeet necrohemorrhagic enteritis at CZA and DZ. Susceptibility testing of isolates lead to an updated clinical treatment plan which ultimately resolved the outbreaks at both institutions. This work provides a foundation for understanding gut microbiota features that are permissive to clostridial colonization and host factors (e.g. age, prior infection) that shape responses to infection.

Keywords

Clostridium, enteritis, lorikeets, gut microbiota, trypsin

Background

Enteritis is one of the most common causes of morbidity and mortality in captive and free-living lorikeets and lories, and outbreaks have been reported at multiple zoological institutions [1-5]. In an informal survey of 12 North American zoos that house 10 or more lorikeets, 11 of the 12 zoos reported a history of enteritis in their flocks, and 50% of the zoos reported recurrent outbreaks of enteritis (Junge, Hausmann, unpublished data). In at least five of these zoos, repeated cultures failed to identify an etiologic agent, and a combination of broad-spectrum antimicrobials were employed as empiric therapy. This approach increases the risk of promoting antimicrobial resistance [6]; moreover, in many cases, it also failed to resolve the outbreak or prevent recurrences of enteritis.

The microbiome is a collection of bacteria, Archaea, viruses, and microbial eukaryotes that live on or in hosts such as lorikeets. The microbiome is increasingly being realized as a source for biomarkers that predict disease and clinical outcomes [7-9] and serve as targets for therapeutics [10] (e.g. probiotics, prebiotics, phage therapy, dietary modification, microbiota transplants). Over the past decade, studies on human and animal microbiomes have increased exponentially [11], and we have learned that these microbial communities are critical to host health. The gut microbiome, for example, plays an important role in nutrient acquisition and metabolism [12], immune development [13], and pathogen defense [14, 15]. There are a growing number of studies on avian [16-18] and wildlife microbiota [19-21], and these studies are providing key insights into health and disease using minimally or non-invasive sampling techniques [22-25]. Microbiome studies are also being used to inform conservation efforts in wildlife species [26-28]. Recent research has further revealed that the gut microbiota influences susceptibility to enteropathogens. For example, *Clostridium*, *Campylobacter*, and *Salmonella* species, all of which are common agents in avian enteritis [1, 2, 5, 29-34], are adept at invading

dysbiotic (or already disrupted) microbial communities but not at colonizing healthy microbial communities [35-37].

In this study, we examined the microbiota over time in two lorikeet flocks (Columbus Zoo and Aquarium [CZA], Denver Zoo [DZ]) that experienced one or more outbreaks of enteritis. Between 2012 and 2018, the CZA lorikeet flock experienced repeated outbreaks of enteritis despite extensive efforts to resolve these issues through nutrition, sanitation, medication, and habitat and husbandry modifications [38]. In 2018, lorikeet morbidity and mortality events spiked, and necropsy reports consistently identified severe and necrotizing enteritis in these lorikeets, but bacterial cultures were frequently negative or variable (e.g. *Escherichia coli, Enterococcus spp.*). Around the same time, DZ was also managing an outbreak of enteritis in their lorikeet flock. We took a novel approach to lorikeet enteritis and sampled healthy and sick birds at CZA and DZ over 10 months. We hypothesized that a dysbiosis, or alteration in the gut microbial community, was making some lorikeets more susceptible to enteritis. Hence, our goal was to characterize this dysbiosis and determine if and what features predicted susceptibility.

Results

Columbus Zoo and Aquarium lorikeet enteritis

We sampled a total of 67 lorikeets at the CZA between November 2018 and September 2019. During this time, 34 lorikeets developed enteritis one or more times while the remaining birds (n = 33) never developed enteritis (**Table 1**). Birds with enteritis were identified through clinical signs (diarrhea, lethargic, fluffed). “Enteritis” samples collected from the same bird within two weeks of the initial enteritis sample were considered a single case. Enteritis samples collected

beyond 2 weeks from the initial sample in the same bird were counted as a second case of enteritis. There were no significant differences by sex in the number of lorikeets that did or did not develop enteritis ($\chi^2 = 5.7$, $p = 0.06$; χ^2 between males and females only, excluding "unknown" = 0.12, $p = 0.73$). However, there was a significant difference by age ($\chi^2 = 9.7$, $p = 0.02$) and by species (χ^2 with all species = 7.2, $p = 0.07$; χ^2 between rainbow and coconut lorikeets only (the two dominant species in this flock) = 6, $p = 0.01$), with enteritis occurring more in younger birds (< 2 years old) and in coconut lorikeets. Birds that survived a previous episode of enteritis also had a 2.2 times increased risk of developing future enteritis; although, this was only marginally significant [95% CI: 0.99-5.29; p=0.051]. We also observed an increase in enteritis cases between December 2018 and March 2019 (**Fig. 1a**).

Gut microbiota by demographics and season

We did not observe significant differences in microbial composition or diversity (Shannon, Observed Features) by sex or species; although, by age, some differences in composition were detected via the Unweighted, but not the Weighted UniFrac metric (**Additional File 4**, alpha-diversity, all Kruskal-Wallis $p > 0.05$; **Additional File 5**). There were also significant differences in microbial composition and diversity by season (November 2018, February 2019, September 2019) with diversity declining significantly over time (**Fig. 1b,c,** Weighted UniFrac, PERMANOVA $p = 0.001$; Observed Features Kruskal-Wallis, all $p < 0.001$, **Additional File 6**).

Gut microbiota changes with enteritis

As compared to healthy lorikeets, we observed a significant decrease in microbial diversity and altered microbial composition in lorikeets with enteritis or lorikeets that died or were euthanized

as a result of enteritis (denoted "post-mortem" lorikeets) (**Fig. 2,** Observed Features Kruskal-Wallis all $p < 0.0005$, Weighted UniFrac PERMANOVA $p = 0.001$; **Additional File 7**). The top differentially abundant microbe between healthy birds and birds with enteritis was *Clostridium perfringens*, which was significantly increased in lorikeets with enteritis (**Fig. 2c**; ANCOM W=1098, no post-mortem birds included in this analysis). There were three other differentially abundant clostridia, including *C. colinum, C. neonatale,* and *C. paraputrificum* which were also all increased in birds with enteritis (**Additional File 8**). We then performed this analysis on a subset of birds (n = 25) for which we had matched samples at both healthy and enteritis time points and we again observed a significantly increased abundance of clostridia including *C. perfringens* and *C. neonatale* in these birds when they had enteritis (**Fig. 2c**, ANCOM W = 788, **Additional File 9**).

Culture, genotyping, and susceptibility testing

After identifying clostridial DNA in our sequencing data, we employed anaerobic culture of lorikeet intestinal contents to look for the presence of viable clostridia in the gut. Intestinal content was collected from a total of 13 lorikeets that died or were euthanized due to enteritis. Contents were cultured on TSA agar with 5% sheep blood. All colonies with unique morphology were picked between 24 - 48 hours and underwent MALDI-TOF identification. We identified clostridia in 7 out of the 13 intestinal content samples including *C. perfringens* in 6 of these 7 samples (**Table 2**). Other microbes that were identified in culture included: *Escherichia coli* (in 3 out of 13 samples), and *C. paraperfringens* or *C. baratii*, *C. paraputrificum*, and *C. disporicum* each in 1 out of 13 samples. We did not culture *C. colinum* in any sample.

We then performed toxinotyping on the 6 *C. perfringens* isolates. All isolates contained the *cpa* gene (encoding alpha toxin) and were identified as *C. perfringens* type A. Five out of 6 isolates also contained the *cpb2* gene (encoding beta 2 toxin) (**Table 2**). Susceptibility testing was performed on 2 isolates. Both isolates were susceptible to metronidazole and penicillin, and one was also susceptible to clindamycin.

Pathology of necrotizing enteritis

We next evaluated the intestinal histopathology of lorikeets with enteritis to determine whether we could identify *C. perfringens* in intestinal lesions. To do this, we examined formalin-fixed paraffin-embedded (FFPE) blocks of lorikeet intestinal tissue from a total of 37 CZA lorikeets that were submitted to The Ohio State University College of Veterinary Medicine between 2015 and 2019. Twenty-eight of these lorikeets had necrotizing enteritis while 7 died or were euthanized due to unrelated causes (encephalitis-2, trauma-1, non-enteric mycobacteriosis-2, air sacculitis-1, proventriculitis-1) (**Table 3**).

There were no significant differences by sex or species in the number of lorikeets that did or did not have enteritis (by sex: Freeman-Halton extension of Fisher's Exact Test $p = 0.73$; between males and females only, excluding "unknown", $p = 0.06$; by species: Fisher's Exact Test $p = 0.11$). There was also no significant difference in average age between birds with and without enteritis (Kruskal Wallis $p = 0.13$); although, birds with enteritis were generally younger. We also examined the number of enteritis deaths by month and observed an increase in cases during summer (June-September) (**Fig. 3a**).

The majority of lorikeets had a clinical history of sudden weight loss, diarrhea, or sudden death. Most often, gross findings consisted of marked muscle wasting with a prominent keel (**Fig. 3b**), and multiple severely dilated loops of intestines with thin walls and scant or watery contents and/or gas (**Fig. 3c**). Other gross findings included tan opaque viscous coelomic fluid, intestinal segments impacted with friable dark red contents, and thickened tan mesentery with multiple small, white, firm to soft nodules within the intestines as well as on the serosa (**Fig. 3d**).

By histopathology, the most common finding (96 % of cases) was an intraluminal coagulum comprised of red blood cells, bacterial colonies and sloughed necrotic mucosa (**Fig 3e**). Approximately two thirds (64 %) of the cases had full thickness heterophilic and/or granulomatous enteritis with ulceration (**Fig 3f**). Approximately two thirds of the cases (61 %) had marked intestinal loop dilation, with over one third (39 %) having villous fusion and/or blunting. Thirty six percent had fibrosis within the intestinal wall, most often at sites of transmural inflammation and necrosis. The most commonly associated lesion was granulomatous and/or heterophilic coelomitis (86 %) with half of those cases having intracoelomic bacteria, most frequently bacilli or mixed bacteria. Other common lesions associated with necrotizing enteritis included mild to moderate renal tubular necrosis and/or mineralization most likely due to dehydration or septicemia (50 %) and marked extramedullary hematopoiesis within the liver (61 %). Evaluation of intestinal segments with modified Brown-Hopps Gram stain demonstrated mixed bacteria within the necrohemorrhagic coagulum and/or necroulcerative intestinal lesions. Eighty-nine percent of cases had large gram-positive bacilli present within the necrohemorrhagic coagulum and these gram-positive bacilli were also the most abundant bacteria present, followed by gram-positive cocci (71%), and gram-negative bacilli (54%) (**Fig. 3g**), whereas 57 % of the

transmural enteritis lesions contained large gram-positive bacilli, followed by 25 % with gram-positive cocci and 21 % with gram-negative bacilli.

There was a strong correlation between the histopathologic characterization of lesions as chronic versus acute with the clinical history of repeated versus first-time enteritis cases, respectively (Fisher's exact test, p=0.033; Relative risk 2.95, 95% CI = [0.86 – 10.08]; Sensitivity 0.875, 95% CI = [0.62 – 0.98]). The chronicity of the lesions was assessed based on the presence of fibrosis and mononuclear inflammatory infiltrates. There was no association between chronicity and the presence of *C. perfringens* (χ^2 = 0.0009, p = 0.98) or *C. colinum* (χ^2 = 0.36, p = 0.55).

Clostridium identification and toxinotyping in FFPE lorikeet intestinal samples

We then used immunohistochemistry (IHC) and an anti-*C. perfringens* antibody to determine whether the gram-positive bacilli present in intestinal lesions were *C. perfringens*. We identified *C. perfringens* in 22 out of the 28 (79%) enteritis-positive FFPE intestinal samples and in 1 out of 7 (14%) samples that had no enteritis (**Table 4, Fig. 3h,i,j**). PCR Toxinotyping of the FFPE intestinal tissue identified the *C. perfringens* alpha toxin gene (*cpa*) in 13 of the 28 (46%) enteritis-positive samples and 1 of the 7 (14%) samples with no enteritis lesions. No FFPE intestinal samples were positive for any of the following toxin genes: *cpb* (beta), *etx* (epsilon), *itx* (iota), *cpe* (enterotoxin), or netB (necrotic B-like). Additionally, although the gene encoding *cpb2* (beta-2 toxin) was identified in intestinal isolates, we did not find *cpb2* in the FFPE enteritis-positive samples (**Table 4**). *C. colinum* was identified by PCR in 18 of the 28 (84%) enteritis-positive intestines and 1 (14%) of the samples with no lesions.

Denver Zoo lorikeet enteritis

To determine whether *C. perfringens* could also be found in lorikeets with enteritis at other institutions, we collected cloacal swabs and intestinal content from lorikeets at the DZ between November 2018 and May 2019. We identified 12 birds that died or were euthanized due to enteritis, and sampled these birds at necropsy ("post-mortem"). These birds were then age-, sex-, and species-matched as closely as possible to 12 healthy lorikeets that were sampled during a flock survey in May 2019 when all birds were reported to be healthy (**Additional File 10**). Similar to the CZA lorikeets, we observed decreased microbial diversity and altered microbial composition in the post-mortem lorikeets with enteritis as compared to healthy birds (Observed Features Kruskal-Wallis $p < 0.0005$, Weighted UniFrac PERMANOVA $p = 0.001$, **Fig. 4; Additional File 11**). *C. perfringens* was also significantly increased in relative abundance in the post-mortem birds (**Fig. 4c,d**; ANCOM W = 67). However, none of the DZ samples contained *C. colinum* 16S reads nor did they test positive for *C. colinum* via PCR. Type A *C. perfringens* was also cultured from eight of the DZ birds with enteritis.

Susceptibility to clostridial enteritis

Odds and risk ratios associated with C. perfringens and C. colinum presence in healthy birds

After identifying clostridial enteritis in two separate lorikeet flocks, we then used the CZA lorikeet data to explore factors that could be linked to susceptibility. First, we examined 16S rRNA reads for the presence of *C. perfringens* and *C. colinum* in healthy birds to determine whether they were predictors for developing future enteritis. Healthy CZA birds that had *C. perfringens* (n = 24, 36%) or *C. colinum* (n = 7, 11%) present in their gut were not at increased odds or risk of developing future enteritis (*C. perfringens*: OR = 0.59, 95% CI = [0.21-1.62], p =

294 0.31; RR = 0.76, 95% CI = [0.44-1.32], p = 0.33; *C. colinum*: OR = 1.37, 95% CI = [0.28-6.71],
295 p = 0.69; RR = 1.16, 95% CI = [0.58-2.32], p = 0.67). Notably, *C. perfringens* and *C. colinum*
296 were only present at low relative abundances (<1%) in healthy birds, if present at all.
297
298 *Gut microbial composition and diversity of susceptible birds*
299 We then divided all healthy CZA birds into two groups: True Healthy and Susceptible. True
300 Healthy birds remained healthy throughout the entire sampling period (Nov. 2018 - Sept. 2019)
301 and never developed enteritis. Susceptible birds were healthy birds that went on to develop *C.*
302 *perfringens* enteritis at least during the sampling period. We compared the microbial
303 communities of these groups and observed significantly increased microbial diversity and altered
304 microbial composition in Susceptible birds as compared to True Healthy birds or birds with
305 enteritis (Observed Features Kruskal-Wallis $p < 0.005$, Weighted UniFrac PERMANOVA p =
306 0.001, **Fig. 5; Additional File 12).**
307
308 *Predicting susceptibility using Random Forests and LASSO models*
309 Next, we constructed Random Forests (RF) and LASSO models to compare True Healthy and
310 Susceptible birds based on samples collected during the February 2019 CZA flock survey (all
311 healthy birds). A total of 1503 microbial taxa (amplicon sequence variants - ASVs) were
312 included in these models along with demographic variables including lorikeet age and species.
313 Sex was not included as it was unknown for 31% of the birds. Seventy-five percent of the
314 samples were used as a training set and 25% of the samples were used as a test set. The RF
315 model identified the relative importance of variables as predictors (**Additional File 12,c**) while
316 the LASSO model identified whether a variable was associated with susceptibility or true health

317 (**Additional File 12,d**). We then collated variables that were identified in both the RF and
318 LASSO models (**Fig. 5c**). The top 26 variables included 23 taxa associated with Susceptible
319 birds and 1 taxon (family Peptostreptococcaceae) associated with True Healthy birds. Rainbow
320 lorikeets (as opposed to coconut lorikeets) were also associated with health while the
321 "WasQuarantined TRUE" variable was associated with susceptibility. This variable represented
322 young lorikeets (< 1 year old) that were transferred from another institution; these birds
323 underwent an initial quarantine prior to integration with the CZA flock. Some of the taxa
324 predictive of susceptibility included: *Rhodococcus fascians*, *Kocuria* spp., *Pseudomonas*
325 *umsongensis*, two taxa in the family Enterobacteriacea and an *Aeromonas* spp.
326
327 *Dietary analysis for trypsin inhibitors*
328 Finally, we examined lorikeet diets in relation to *C. perfringens* susceptibility. Several *C.*
329 *perfringens* toxins, including *cpa*, *cpb*, *pfo*, and *cpb2* (the toxin observed in 5 CZA *C.*
330 *perfringens* isolates) are sensitive to the host-produced protease trypsin [39]. However, trypsin
331 inhibitors present in the diet can block the activity of trypsin and thereby increase the risk of *C.*
332 *perfringens* toxin-mediated enteritis. As lorikeets are nectivores and their main diet under human
333 care consists of reconstituted powdered nectar, we opted to test trypsin inhibitor levels in six
334 commercial nectar brands including brands used at CZA and DZ. The range of trypsin inhibition
335 for the nectars was 0-1.79 trypsin inhibitor units (TIU)/mg dry nectar, denoting relatively low
336 inhibition (**Fig. 6**). For reference, raw soybeans, which have been linked to *C. perfringens* toxin-
337 mediated enteritis in poultry, contain approximately 46 TIU/mg, and soy protein concentrate
338 contains 9.45 TIU/mg [40]. As such, the low levels of trypsin inhibition detected in commercial
339 nectars are unlikely to be playing a major role in susceptibility to *C. perfringens* enteritis in

lorikeets; although we cannot rule out the possibility that other supplementary food items (e.g. sweet potatoes or legumes) may have contributed to toxin-mediated clostridial enteritis. Notably, *C. colinum* toxins have yet to be characterized; therefore, the role of trypsin and dietary trypsin inhibitors on *C. colinum* pathogenesis is unknown.

Discussion

Our initial goal in this study was to characterize the gut microbiota of sick and healthy lorikeets with the hypothesis that a dysbiosis was driving susceptibility to enteritis. While we did identify gut microbial alterations associated with susceptibility, we also ended up identifying the probable etiologic agents of enteritis in both the CZA and the DZ lorikeet flocks. Specifically, we observed increased relative abundances of *C. perfringens* and *C. colinum* (CZA lorikeets only) in the 16S rRNA sequencing data. We then cultured lorikeet intestinal contents and identified, genotyped, and susceptibility-tested multiple *C. perfringens* isolates. A histopathologic examination of intestinal tissues further revealed inflammation, necrosis, and ulcerative lesions that also contained gram-positive bacilli consistent with clostridial enteritis and specifically *C. colinum* or *C. perfringens*. IHC and toxinotyping of intestinal tissues confirmed the presence of *C. perfringens* in lorikeets with enteritis. PCR testing also confirmed the presence of *C. colinum* in CZA lorikeets with enteritis. We then compared the gut microbiota of healthy CZA lorikeets that developed enteritis to healthy CZA lorikeets that never developed enteritis during our 10-month sampling period, and we identified several features associated with susceptibility to enteritis including: age (younger birds are more susceptible), and increased relative abundances of *Rhodococcus fascians*, *Pseudomonas umsongensis*, two taxa in the family Enterobacteriacea, and an *Aeromonas* spp., among others. This work allowed us to identify the

363 probable causative agents of lorikeet enteritis at two zoos, develop an optimal treatment plan
364 based on genotyping and susceptibility testing, and profile healthy birds at high risk of clostridial
365 enteritis.
366
367 **Demographics of lorikeet enteritis**
368 *C. perfringens* has been linked to enteritis in multiple mammal and bird species [34, 39, 41-44],
369 including in lorikeets and other psittacines [5, 32, 33, 45-47]. In this study, young lorikeets (< 2
370 years old) were more likely to develop clostridial enteritis. We also observed some differences in
371 microbial composition by age (**Additional File 5**), and age emerged as a predictor of
372 susceptibility in the Random Forests model (**Additional File 12,c**). Previous reports in other
373 avian species note that the immunological naivete of young birds may increase susceptibility to
374 *C. perfringens* while adult birds are more resistant [48, 49]. We also found that coconut lorikeets
375 were more likely to develop enteritis as compared to other lorikeet species. It is less clear what
376 may be driving species differences in clostridial enteritis; although, there were more coconut
377 lorikeets than any other species at CZA, and *C. perfringens* has been associated with necrotic
378 enteritis in coconut lorikeets at other institutions [47]. While type A was the dominant *C.*
379 *perfringens* toxinotype reported in the previous study on lorikeets, toxinotype C was also
380 common. Toxinotypes B, D, E, F, and G were also observed but less common [47]. Sex has also
381 been reported as a factor that influences susceptibility to necrotic enteritis in birds [41]; although,
382 we did not observe differences by sex in this study. Taken together, our results suggest that both
383 microbial and immunological factors may contribute to clostridial enteritis in young lorikeets.
384
385 **Seasonal changes in healthy lorikeet gut microbiota**

We observed flock-wide shifts in gut microbiota between the three flock surveys in November 2018, February 2019, and September 2019. In previous studies, seasonal alterations in avian microbiota have been linked to diet, migration, and breeding status [21, 50-52]. Lorikeets in the CZA flock have a consistent diet and environment year-round, and they do not migrate - thus mitigating these factors as drivers for the observed seasonal changes. While breeding status could be influencing these gut microbial alterations, it is also possible that flock-wide prophylactic antimicrobial use during enteritis outbreaks and between flock surveys drove these shifts. Notably, microbial diversity decreased significantly from November 2018 to September 2019, which is a shift that could be consistent with antimicrobial use across host species including in birds [53-58].

Lorikeet enteritis: microbes to host

Lorikeets with enteritis demonstrated microbial community shifts and histopathological changes as compared to healthy lorikeets. Based on 16S rRNA sequencing, culture, and genotyping, we confirmed the presence and increased relative abundance of *C. perfringens* type A in CZA and DZ lorikeets with enteritis. In the CZA lorikeets, we further determined that *C. perfringens* was directly associated with necroulcerative intestinal enteritis via IHC and multiple intestinal isolates of *C. perfringens* contained the *cpb2* toxin. *C. colinum* was also found at increased relative abundances in CZA but not DZ birds based on 16S sequences and PCR. In both CZA and DZ birds, we observed decreased microbial diversity and altered microbial composition in lorikeets with enteritis as compared to healthy lorikeets. *C. perfringens* and *C. colinum* have been implicated previously in necroulcerative enteritis in birds including lories, lorikeets, and poultry [47, 59-63]. Microbial community alterations have also been reported in chickens

409 infected with *C. perfringens* [64-67]. While several clostridial species are considered normal
410 flora in some avian species including poultry, in psittacines, clostridial species are rarely found
411 in the intestines of healthy birds, and taxa such as *C. colinum* and *C. perfringens* are considered
412 pathogenic [4, 31, 59, 68, 69]. Previous studies have linked the *C. perfringens* beta-2 toxin
413 (*cpb2*) identified in CZA birds with enteritis in psittacines [33], storks [70], pigs [71], and
414 poultry [72]; however, the cpb2 toxin has also been identified in healthy individuals (poultry,
415 horses, dogs, and other avian species) and its role enteritis is not clear [73-77]. *C. colinum*-linked
416 enteritis has only been reported in avian species, but virulence factors, toxins, and disease
417 pathogenesis for *C. colinum* have yet to be fully elucidated [59].
418
419 Our results suggest that *C. perfringens* was the driver of enteritis in the DZ birds, while in the
420 CZA birds, either *C. colinum*, or *C. perfringens*, or both acting synergistically, could have been
421 driving the infections or creating a dysbiotic environment that allowed the other to thrive.
422 Synergistic *C. perfringens* / *C. colinum* co-infections have been reported previously in poultry
423 [78], while other clostridial co-infections (*C. perfringens* / *C. difficile*) have been reported in
424 humans, foals, and dogs [79-81]. Phylogenetically related bacterial taxa can share functional
425 traits and fill similar metabolic niches [82]; thus, it is feasible that an environment permissive to
426 one type of clostridia may also be permissive to another clostridia.
427
428 **Susceptibility to enteritis**
429 Besides age, seasonality has also been linked to necrotic enteritis in birds. In poultry, enteritis
430 cases occur more frequently in late winter and early spring [83-85], which is what we observed
431 in the CZA lorikeets; although historically, CZA reported enteritis cases across all seasons. DZ

cases were also clustered during winter months, however this was the only occurrence of an enteritis outbreak DZ had experienced. Additionally, birds that had a history of enteritis were at 2.2 times increased risk of developing future enteritis. Gross pathology and histopathology data further linked signs of chronic lesions (fibrosis, mucosal atrophy, villus blunting/fusion, wasting) to birds with repeated cases of enteritis. Repeated bouts of enteritis in some CZA lorikeets likely resulted in permanent intestinal damage that hindered absorption and peristalsis, leading to malnutrition and intestinal stasis, which are known risk factors for *C. perfringens* enteritis [61, 86, 87]. While some lorikeets survived one or even 2 cases of enteritis, no bird lived beyond a third episode.

Diet – and specifically dietary trypsin inhibitors – can also be a risk factor for *C. perfringens* enteritis – depending on the toxinotype [45, 87, 88]. As such, we assessed trypsin inhibition in 6 commercial nectars including the nectars used at CZA and DZ. All nectars contained very low levels of trypsin inhibitors and are unlikely to be contributing to enteritis incidence. However, supplementary diet items, such as cooked sweet potatoes, which were briefly part of the DZ lorikeet diet during the enteritis outbreaks, could potentially have contained higher levels of trypsin inhibitors. Notably, trypsin inhibitor levels vary widely across sweet potato cultivars [89-91], and we did not evaluate trypsin inhibition in any supplementary foods.

Finally, we examined microbial communities in healthy birds that later went on to develop enteritis (Susceptible) or remained healthy throughout the entire study period (True Healthy). Susceptible birds displayed minor but significant differences in microbial community diversity (Observed Features), composition, and differentially abundant taxa. These microbial community

differences could be linked to age as young birds were also the most susceptible. Although we found no significant difference in microbial diversity or composition in healthy lorikeets by age, birds in the youngest age group (< 2 years old) had the greatest microbial diversity (**Additional File 4, c,f**) which was also true in the Susceptible birds (**Fig. 5a**). Increased microbial diversity has also been observed in young chickens susceptible to *C. perfringens* infection as opposed to those that were more resistant [92]. Moreover, differences in microbial composition by age (Unweighted UniFrac, but not Weighted UniFrac) were significant in healthy birds ($p = 0.01$ **Additional File 5**). This suggests that age may influence lorikeet microbial community structure, and with a larger sample size, this may have been more apparent.

Whether shaped by age or not, the microbial community differences observed in Susceptible birds suggest that a lorikeet's pre-existing microbial community structure could potentially influence the ability of a clostridial pathogen (e.g. *C. perfringens* or *C. colinum*) to colonize the intestinal tract. This could be achieved through alterations in the metabolic environment that create a more favorable niche for clostridia to expand. The presence of primary bile acids, for example, can act as a germinant for *Clostridium* species, while the presence of secondary bile acids (produced by bacteria that convert primary to secondary bile acids), can inhibit *C. perfringens* proliferation [93, 94]. Minor alterations in pre-existing microbial community composition and differentially abundant microbes have also been reported in chickens susceptible to *C. perfringens* [92].

We identified several microbes that were associated with susceptibility including *Rhodococcus fascians*, *Pseudomonas umsongensis*, an *Aeromonas* spp., and two taxa in the family

Enterobacteriacea. *Rhodococcus fascians* has been found at increased abundances in juvenile birds (sparrows, < 1 year old) as compared to older birds and could be an age-related taxa [21]. A single human case report also highlights a co-infection between *R. fascians* and a clostridial species (*C. difficile*) [95], which leads to the intriguing question as to whether these 2 species interact in ways that may support each other's growth. However, this co-infection was in an immunocompromised individual, so the relevance is unclear. Both *Pseudomonas* and *Aeromonas* species have been independently associated with enteritis in birds [86, 96, 97]. In a previous study that employed a subcutaneous abscess model, the addition of *Pseudomonas aeruginosa* or various Enterobacteriaceae species enhanced the growth of *C. perfringens* [98] suggesting that interactions between these taxa may indeed facilitate clostridial infections. Our RF and LASSO models also identified several other microbial taxa associated with susceptibility; although, the potential role these taxa may be playing in clostridial infections or enteritis is undetermined and requires additional study.

This study had several limitations: We identified both *C. colinum* and/or Type A *C. perfringens* in lorikeets with enteritis; however, the mechanisms by which these bacteria caused disease remain unclear. For example, while *C. colinum* has been empirically and experimentally linked to ulcerative enteritis in birds, its virulence factors have yet to be elucidated [60]. Second, although both *C. perfringens* alpha toxin (*cpa*) and beta-2 toxin (*cpb2*) have been associated with enteritis in multiple host species including birds, the role of these toxins in enteritis pathogenesis is ambiguous, and both of these toxin genes have been found in the intestines of healthy animals [39, 60, 99]. It is possible that neither *cpa* nor *cpb2* are key virulence factors in Type A. *C. perfringens* and that other unidentified virulence factors played a role in lorikeet enteritis.

Additionally, toxin gene presence (e.g. PCR, used in this study) does not necessarily equate to toxin gene expression. However, clostridia and its respective toxin genes are considered aberrant in healthy psittacines [4, 31, 59, 68, 69], suggesting that they are playing a role in enteritis even if their virulence factors are not fully defined. The surrounding gut microbiota and metabolites could also be mediating *C. perfringens* pathogenesis including colonization and toxin expression as has been demonstrated in *Clostridiodes difficile* [35, 36]

Conclusions

In this study, we systematically examined gut microbiota and susceptibility to clostridial enteritis in two lorikeet flocks under human care. A few of our key take-aways: 1) Clostridia can be challenging to detect via culture in lorikeet cloacal swabs, but anaerobic culture of intestinal contents yielded *C. perfringens* in 6 out of 13 isolates from CZA, and 16S sequencing allowed ready identification of *C. perfringens* and *C. colinum* in birds with enteritis. As clostridia are not normal inhabitants in psittacines, this was a significant finding. 2) Clostridial isolates then underwent genotyping and susceptibility testing, which allowed us to update the lorikeets' clinical treatment plans to more targeted therapies, aligned with antimicrobial stewardship practices (DZ – metronidazole, CZA - florfenicol and clindamycin in clinically affected birds and prophylactic flock-wide application of bacitracin). Since June of 2019, and as of this writing, there have been no new cases of enteritis in lorikeets at either CZA or DZ. 3) Young age (potentially linked with immunological naivete, limited exposures, or lower trypsin activity [100]), prior enteritis, and specific microbes including *R. fascians*, *P. umsongensis*, and Enterobacteriacea taxa are linked with susceptibility to enteritis, and these microbes could be promoting clostridial infections by establishing a niche conducive to colonization in a yet-to-be

524 determined manner. 4) Diet – including trypsin inhibitors – can also influence susceptibility to
525 clostridial enteritis. Although, commercial nectars were low in trypsin inhibitors, we cannot rule
526 out the possibility that other supplementary food items (e.g. sweet potatoes or legumes) could
527 have contributed to toxin-mediated enteritis. Clostridial enteritis, and *C. perfringens* in
528 particular, not only affects lorikeets, but can also cause devastating losses in the poultry industry
529 (commonly Type G *C. perfringens* with NetB toxin), and lead to gastrointestinal disease in
530 humans and other mammals – depending on the toxinotype. This work provides a foundation for
531 understanding gut microbiota features that are permissive to clostridial colonization and host
532 factors (e.g. age, prior infection) that shape responses to infection.
533
534 **Methods**
535
536 **Sample Collection – Columbus Zoo and Aquarium**
537 Cloacal swabs were obtained from all healthy lorikeets at the CZA (n=67 birds) during routine
538 flock health surveys at 3 timepoints (November 2018, February 2019, September 2019) (See
539 **Additional File 1**, **a** for experimental design). The flock was composed of four species of lories
540 and lorikeets including: rainbow lorikeets (*Trichoglossus moluccanus*), coconut lorikeets
541 (*Trichoglossus haematodus*), marigold lorikeets (*Trichoglossus capistratus*), and lorys
542 (*Trichoglossus*). Each bird was weighed and body condition scored during these surveys. Cloacal
543 swabs, intestinal tissue, and/or intestinal contents were collected opportunistically from lorikeets
544 (n = 34 birds) that presented with enteritis or died / were euthanized due to enteritis between
545 November 2018 and September 2019.
546

A total of 246 samples were collected from birds at CZA – 172 samples from healthy birds and 74 samples from birds with enteritis. Twenty-eight additional samples were also collected from the environment and included samples of freshly prepared nectar, water, fruit, and swabs of the aviary floor and perches. Swabs from live birds that presented with enteritis were collected prior to initiation of antimicrobial therapy and, in some cases, throughout treatment. For necropsies performed at CZA, intestinal contents were milked directly into a 2 ml screw-top tube without buffer. Swabs (Puritan, Guilford, ME) and intestinal contents were immediately transferred to a -80°C freezer and stored until sample processing.

Sample Collection – Denver Zoo

Cloacal swabs were collected from the entire lorikeet flock during a flock survey in May 2019. At this time, all lorikeets were reported to be healthy. Cloacal swabs, intestinal tissue, and intestinal contents were collected opportunistically from lorikeets (n=12) that died or were euthanized due to enteritis between November 2018 and May 2019 (see **Additional File 1**, **b** for experimental design). These birds were then age-, sex-, and species-matched to 12 healthy lorikeets. Upon collection, all swabs and intestinal contents were immediately transferred to a -80°C freezer and stored until sample processing and DNA extraction.

DNA extraction, 16S rRNA amplification, and sequencing

Bacterial DNA isolation from cloacal swabs and intestinal contents (approximately 200 mg) was performed using QIAamp PowerFecal DNA Kits (Qiagen, Venlo, Netherlands). For cloacal swabs, powerbeads and lysis buffer were added directly to the screw top tubes containing the swabs. A bead beating step (6 m/s for 40 sec.) was used to replace the vortex step from the

manufacturer's protocol. The remainder of the isolation was executed according to the protocol. DNA isolation from formalin-fixed paraffin-embedded (FFPE) intestinal tissues collected at necropsy was performed using the QIAamp DNA FFPE Tissue Kit following the manufacturer recommendations. Following DNA isolation, DNA concentration was measured using a Qubit Fluorometer 4 (Invitrogen, Carlsbad, CA, USA), and purity was assessed with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Samples were submitted for library preparation and sequencing at Argonne National Laboratory. Earth Microbiome Project primers 515F and 806R were used to amplify the V4 hypervariable region of the bacterial 16S rRNA gene. Amplicons were sequenced on an Illumina MiSeq in 2 x 250 paired-end mode.

16S rRNA sequence processing

The 16S rRNA sequences were processed, filtered, and analyzed using QIIME 2 version 2020.11 [101] and DADA2 [102]. Taxonomic assignment of amplicon sequence variants (ASVs) was performed using the Greenegenes 13_8 database with 99% sequence identity cutoff. (Note: We also performed taxonomic assignment with SILVA 132 and found that, in this case, Greengenes provided more specific taxonomic assignments, particularly in the Clostridia taxa.) A total of 246 samples from CZA and 30 samples from DZ were submitted for sequencing. Samples with fewer than 1000 reads were removed from analyses including 23 CZA samples and 6 DZ samples. This resulted in a total of 223 CZA samples and 24 DZ samples that were used in our analyses. After filtering, we obtained a total of 3,236,674 reads from the CZA samples (average: 13,911 reads per sample; range: 1003 to 68,537 reads) and 264,769 reads from the DZ samples (average: 9,026 reads per sample; range: 1922 to 26,766 reads). Sequences identified as mitochondria, chloroplasts, or eukaryotic reads were removed. Based on an examination of negative controls,

we also identified the following taxa as contaminants and removed them from analyses: a taxa in the order RF39 (Mollicutes phyla); a taxa in the genus *Allobaculum*, a taxa in the genus *Massilia*; *Haemophilus parainfluenzae*; *Prevotella copri*; a taxa in the genus *Sphingomonas*; a taxa in the genus *Bradyrhizobium*; *Pseudomonas viridiflava;* and a taxa in the genus *Thermicanus*.

Culture, bacterial identification, and genotyping

Lorikeet intestinal contents collected at necropsy were plated on reduced Trypticase Soy Agar (TSA II) with 5% sheep blood (BD BBL, Franklin Lakes, NJ) and incubated anaerobically (5% CO_2, 5% H_2, 90% N_2) at 37ºC until growth was seen (24 - 48 hours). As needed, bacteria was sub-cultured on *C. perfringens* selective agar, tryptose sulfite cycloserine (TSC), (Sigma Aldrich, St. Louis, MO). Bacterial colony identification was performed via Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF). *C. perfringens* isolates were submitted for toxinotyping via multiplex PCR at the Ohio Department of Agriculture Animal Disease Diagnostic Laboratory (ODA ADDL). This PCR included primers for toxin types A – E (*cpa*, *cpb*, *cpb2*, *cpe*, *etx*, and *iota A*). The same primer sets and thermal cycler parameters were used to analyze DNA extracted from FFPE tissues for comparison.

CZA Lorikeets submitted to pathology

Between 2015 - 2019, naturally deceased or humanely euthanized CZA lorikeets (due to severe clinical disease) underwent a complete macroscopic postmortem examination necropsied by veterinarians at the Columbus Zoo and Aquarium. Intestinal tracts were collected from lorikeets with enteritis (n=28) and with unremarkable intestines (n=7). Various organs, including the intestinal tracts, were placed in 10% neutral buffered formalin for fixation, stored at room

616 temperature, and submitted to The Ohio State University College of Veterinary Medicine for
617 evaluation of the formalin fixed organs and histopathology.
618
619 **Histopathology**
620 Intestinal tracts, as well as other collected and formalin fixed tissues, were routinely trimmed,
621 paraffin embedded, and stained with hematoxylin and eosin (H&E) for initial evaluation by the
622 Comparative Pathology & Digital Imaging Shared Resource (CPDISR). Intestines were
623 evaluated by two veterinary pathologists board-certified by the American College of Veterinary
624 Pathologists (FU and MM) to characterize cases as necrotizing enteritis (n=28) vs
625 control/unremarkable intestines (n=7). All sections with necrotizing enteritis were subsequently
626 stained with the modified Brown-Hopps gram stain applied to identify and characterize
627 intralesional bacteria, and a Masson's Trichrome stain to characterize chronicity of the lesions
628 through the presence of fibrosis.
629
630 **Immunohistochemistry**
631 We then looked for the presence of *Clostridium perfringens* within intestinal sections from
632 lorikeets with (n=28) or without enteritis (n=7) via immunohistochemistry (IHC) using a
633 polyclonal rabbit anti-*Clostridium* perfringens antibody, OASA07164, Aviva Systems Biology,
634 San Diego, CA). The IHC protocol is described in **Additional File 2**.
635
636 ***Clostridium perfringens* toxinotyping**
637 Toxinotyping on DNA extracted from FFPE lorikeet intestinal tissues was performed at the San
638 Bernardino branch of the California Animal Health and Food Safety (CAHFS) Laboratory,

University of California-Davis, using a previously established method[47]. Additional testing for the *C. perfringens* alpha toxin (*cpa*) and beta-2 toxin (*cpb2*) was performed on CZA intestinal isolates at The Ohio State University using the following primers: cpaF (5'-GCTAATGTTACTGCCGTTGA -3'), cpaR (5'- CCTCTGATACATCG GTAAG -3'), cpb2F (5'- AGATTTTAAATATGATCCTAACC -3') and cpb2R (5'-CAATACCCTTCACCAAATACTC -3'). PCR conditions for *cpa* and *cpb2* testing were as follows: PCR was performed in a total volume of 25 µL containing 0.75 µL of each primer (0.3 µM), 2 µL of extracted DNA, 12.5 µL of HiFi Hot Start Master Mix (Kapa Biosystems) and 9 µL of nuclease-free water. Thermocycler profiles were as follows: 95 °C for 3 min, 45 cycles of 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 60 s, and a final extension step at 72 °C for 5 min. Positive controls included DNA extracted from known, toxin typed, *C. perfringens* isolates.

***Clostridium colinum* PCR**

PCR for *C. colinum* was also performed at CAHFS. Scrolls from (FFPE) sections of small intestine from the lorikeets were deparaffinized and the DNA extracted using a commercial kit (QIAamp DNA FFPE tissue kit; Qiagen) following the manufacturer's instructions. The extracted DNA was used as a template for PCR amplification of a ~192-bp fragment of the 16S rRNA gene of *C. colinum*, using the primers CcolF (5′- CGGCTGGATCACCTCCTTTC-3′) and CcolR (5′-ACATTTTTGTCTGGCTCACGA-3′). PCR was performed in a total volume of 25 µL containing 0.5 µL of each primer (0.5 µM), 3 µL of extracted DNA, 7 µL of nuclease-free water, and 14 µL of Platinum™ II Hot-Start Green PCR Master Mix (2X) (Invitrogen). Thermocycler profiles were as follows: 95 °C for 7 min, 35 cycles of 95 °C for 60 s, 60 °C for 60 s, and 72 °C for 60 s, and a final extension step at 72 °C for 7 min. Samples were held at 4 °C. Positive

controls included DNA extracted from a commercial bacterial strain (ATCC 27770) and from FFPE sections of quail disease cases in which *C. colinum* had been isolated. DNA extracted from DZ samples also underwent *C. colinum* PCR.

Measurement of trypsin inhibitor levels in nectar

We measured trypsin inhibitor levels in six commercial lorikeet feeds commonly used at zoological institutions and aviaries around North America, including at CZA and DZ. Lorikeet feeds included: Mazuri Softbill Diet for Iron-Sensitive Birds (Mazuri Exotic Animal Nutrition, St. Louis, MO), Blessing's Gourmet Blend Lory Nectar (Blessing's Pet Food Products, Murrieta, CA), Mazuri Lorikeet Diet (Formula: 5AB4, Mazuri Exotic Animal Nutrition, St. Louis, MO), Rainbow Landing Nectar (Berwick Productions, Inc. Escondido, CA), Roudybush Lory Nectar 15 (Roudybush, Woodland, CA), and Higgins Intune Lory Food (Higgins Premium Pet Foods, Miami, FL).

Nectar preparation

To eliminate assay interference due to free fatty acids, all nectars were first defatted through a hexane (Thermo Fisher Scientific, Waltham, MA) extraction. Nectars were combined with three times their volume of pure hexane and mixed for one minute. The samples were then allowed to sit for 10 minutes to allow for a separation of layers, and the top hexane-fat layer was removed. This process was repeated a total of three times for each nectar. Defatted nectars were then allowed to dry overnight in a fume hood. Once dry, 1 g of defatted nectar was added to 50 g of 0.01M NaOH (Thermo Fisher Scientific, Waltham, MA). The mixture was stirred slowly on a

684 stir plate for 3 hours. Extracts were then centrifuged at 4696 x g, and the supernatant was
685 decanted to produce the final extract.
686
687 *Trypsin inhibitor assay*
688 Trypsin inhibitor assays were carried out based on standard American Association of Cereal
689 Chemists (AACC) methods [103], with modifications as proposed by Liu (2019) [40]. Reagent
690 preparation is described in **Additional File 3**. Nectar solutions were diluted to various levels by
691 combining 0 - 1 mL of nectar extract with enough deionized water to yield 2 mL total.
692 Concentration ranges chosen were based upon preliminary trials, with the aim of yielding
693 absorbance data points that fell in the range of 30 – 70 % inhibition. The 2 mL of diluted nectar
694 were added to 15-mL centrifuge tubes and combined with 5 mL benzoyl-DL-arginine-p-
695 nitroanilide hydrochloride (BAPA) solution. The mixture was incubated in a 37 °C water bath to
696 bring it up to temperature, and then the assay reaction was initiated by adding 2 mL pre-warmed
697 trypsin solution (0.02 mg trypsin/mL) to each tube. The tubes were allowed to react for exactly
698 10 minutes at 37 °C, then the reaction was stopped with the addition of 1 mL 30 % acetic acid
699 solution. Samples were allowed to cool to room temperature before measuring absorbance at 410
700 nm with an HP 8453 UV-Vis spectrophotometer (Hewlett Packard, Palo Alto, CA). Absorbance
701 readings were corrected with nectar blanks by mixing all reagents, but adding trypsin solution
702 after the acetic acid to ensure the enzyme was inactive. A positive control sample was also made
703 using 2 mL water in place of nectar and running the assay as delineated above.
704
705 *TIU calculation*

With the definition that 1 TIU = a decrease in 0.01 absorbance compared to a positive control sample, TIU/mg could be calculated as follows:

$$\text{TIU} = (\text{positive control sample absorbance} - \text{nectar sample absorbance}) / 0.01$$

$$\text{TIU/mg} = \text{TIU}/ [\text{Nectar concentration(mg/mL)} \times 10 \text{ mL assay solution}]$$

To compare trypsin inhibitor concentrations (TIU/mg) between nectars, we applied a one-way ANOVA followed by pairwise Tukey's tests.

Statistical analyses

We compared the number of lorikeets that ever had enteritis versus the number of lorikeets that never developed enteritis by age, sex, and species using a χ^2 test [104]. In cases where groups had a frequency less than 5, we use the Yates' χ^2 correction. For cases in which a group contained zero individuals (e.g. 0 females), we used the Freeman-Halton extension of the Fisher's exact test. To compare average age across groups, we used a Kruskal-Wallis test after testing for normality using a Shapiro-Wilk test. For microbial community analyses, alpha diversity was compared between groups using observed features, the Shannon diversity metric, and the Kruskal-Wallis test. Beta diversity was evaluated using permutational multivariate analysis of variances (PERMANOVAs) between groups on Bray-Curtis distance matrices. All alpha and beta diversity *p*-values were corrected for multiple comparisons using the Benjamini Hochberg false discovery rate (FDR) correction. A p-value < 0.05 was considered significant. Differential abundances of microbes by status (healthy, enteritis, susceptible) or season (November 2018, February 2019, September 2019) were tested using an ANCOM [105].

Machine learning methods were used to assess susceptibility to enteritis based on microbial relative abundances and other demographic factors. Specifically, microbial composition was used in a supervised setting for classifying birds into True Healthy and Susceptible groups. True Healthy birds never developed enteritis throughout the sampling period (Nov. 2018 - Sept. 2019) while Susceptible birds developed enteritis at least once during this time. Random forests (RF)[106] and logistic regression with appropriate regularization (LASSO)[107, 108] were employed to differentiate these groups. The predictive accuracy was then assessed through cross-validation using an area under the receiver-operating characteristics curve (ROC).

Declarations

Ethics approval and consent to participate

IACUC approval through The Ohio State University (# 2019A00000028).

Consent for publication

Not applicable.

Availability of data and material

Sequencing data is available at NCBI Bioproject PRJNA722436.

Competing Interests

Not applicable.

Funding

Funding for this project provided by the following:

Columbus Zoo/Ohio State Cooperative Grants Program

National Institutes of Health Training Grant (T35)

The Ohio State University Infectious Diseases Institute

The Ohio State University College of Veterinary Medicine

American Association of Zoo Veterinarians' Wild Animal Health Fund

The Comparative Pathology & Digital Imaging Shared Resource is supported in part by grant P30 CA016058 from the National Cancer Institute, Bethesda, MD.

Authors' contributions

RJ and JH were involved in identification of the clinical syndrome, project conceptualization, sample collection, and clinical data.

DM, CM, and VH were involved in project development, DNA extraction, sequencing data analysis and interpretation, manuscript writing, and figure preparation.

VH also obtained project support and oversaw/managed the project and performed demographic / epidemiological analyses. RMrochak, RMadan, KS, and CM were involved in toxinotyping on intestinal isolates. ME was involved in sequencing data filtering and processing.

LG and SP were involved in Random Forests and LASSO analysis and interpretation, manuscript writing, and figure preparation. KF-K, MM, KLP, GB, and FU were involved in histopathology analysis, staining, and interpretation. MM and FU were also involved in manuscript writing and figure preparation. KF-K and MM were involved in gross pathology interpretation and figure preparation. FU performed histopathology, IHC analysis, interpretation, and figure preparation.

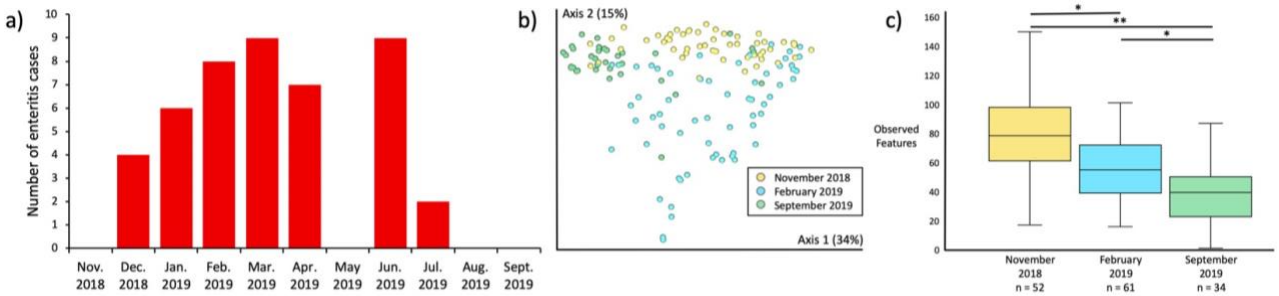
MN performed toxinotyping on FFPE blocks, *C. colinum* PCR, and was involved in manuscript writing and table preparation. WC, KS, and YV were involved in trypsin inhibitor testing and data interpretation. WC and YV were also involved manuscript writing and figure preparation.

Acknowledgements

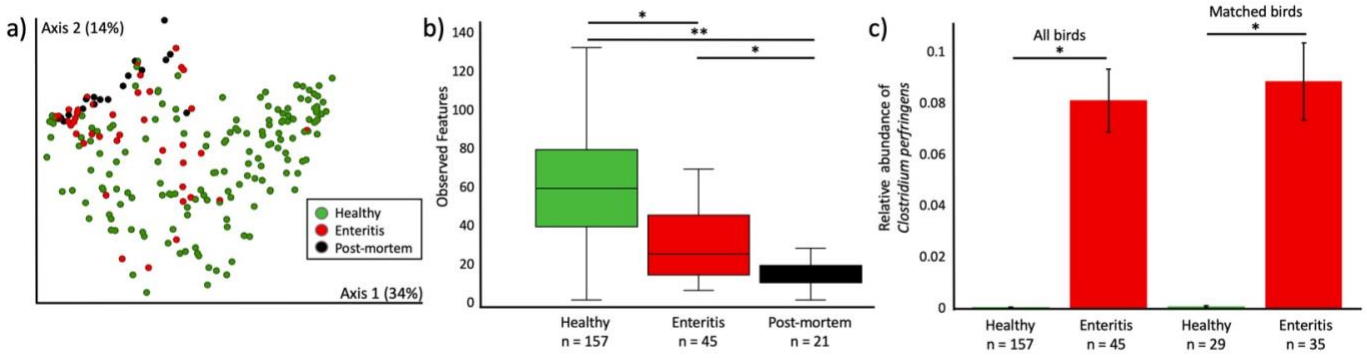
We would like to acknowledge all of the keepers and veterinary staff at the Columbus Zoo and Aquarium and the Denver Zoo for facilitating sample collection and providing detailed health, medication, and dietary record. We also thank Dr. Michael Martinez for assistance with gross pathology photos, and we gratefully acknowledge Drs. Tamara Kruse and Helena Mendes-Soares for early discussions that shaped this project.

Figure Legends

Figure 1. Seasonality in enteritis and gut microbiota in healthy lorikeets a) Number of enteritis cases by month in Columbus Zoo and Aquarium lorikeets. Enteritis samples collected from the same bird within two weeks of the initial enteritis sample were considered a single case. Enteritis samples collected beyond 2 weeks from the initial sample in the same bird were counted as a second case of enteritis. Columbus Zoo & Aquarium healthy birds only: **b)** Microbial community composition (Weighted UniFrac) and **c)** diversity (Observed Features) by season (November 2018, February 2019, September 2019). There were significant shifts in microbial composition by season (PERMANOVA $p = 0.001$), and microbial diversity decreased significantly between November 2018 and September 2019 (Kruskal-Wallis $*p < 0.001$, $**p < 0.0001$). (Also see **Additional File 6.**)



794 **Figure 2. Microbial community analysis in CZA lorikeets with enteritis.** Microbial
795 composition and diversity in healthy lorikeets, lorikeets with enteritis, and lorikeets that died or
796 were euthanized due to enteritis (postmortem). **a)** Microbial composition (Weighted UniFrac)
797 was significantly altered (PERMANOVA $p = 0.001$) and **b)** microbial diversity (Observed
798 Features) was significantly decreased (Kruskal-Wallis $*p < 0.0005$, $**p < 1 \times 10^{-9}$) in lorikeets
799 with enteritis or postmortem lorikeets. (Also see **Additional File 7**.) **c)** The relative abundance of
800 *Clostridium perfringens* was significantly increased lorikeets with enteritis across all birds
801 (ANCOM, W = 1098) and across matched birds (ANCOM, W = 788). "All birds" included
802 samples from healthy birds that never got enteritis. "Matched birds" included only 25 birds that
803 had both enteritis and healthy samples.

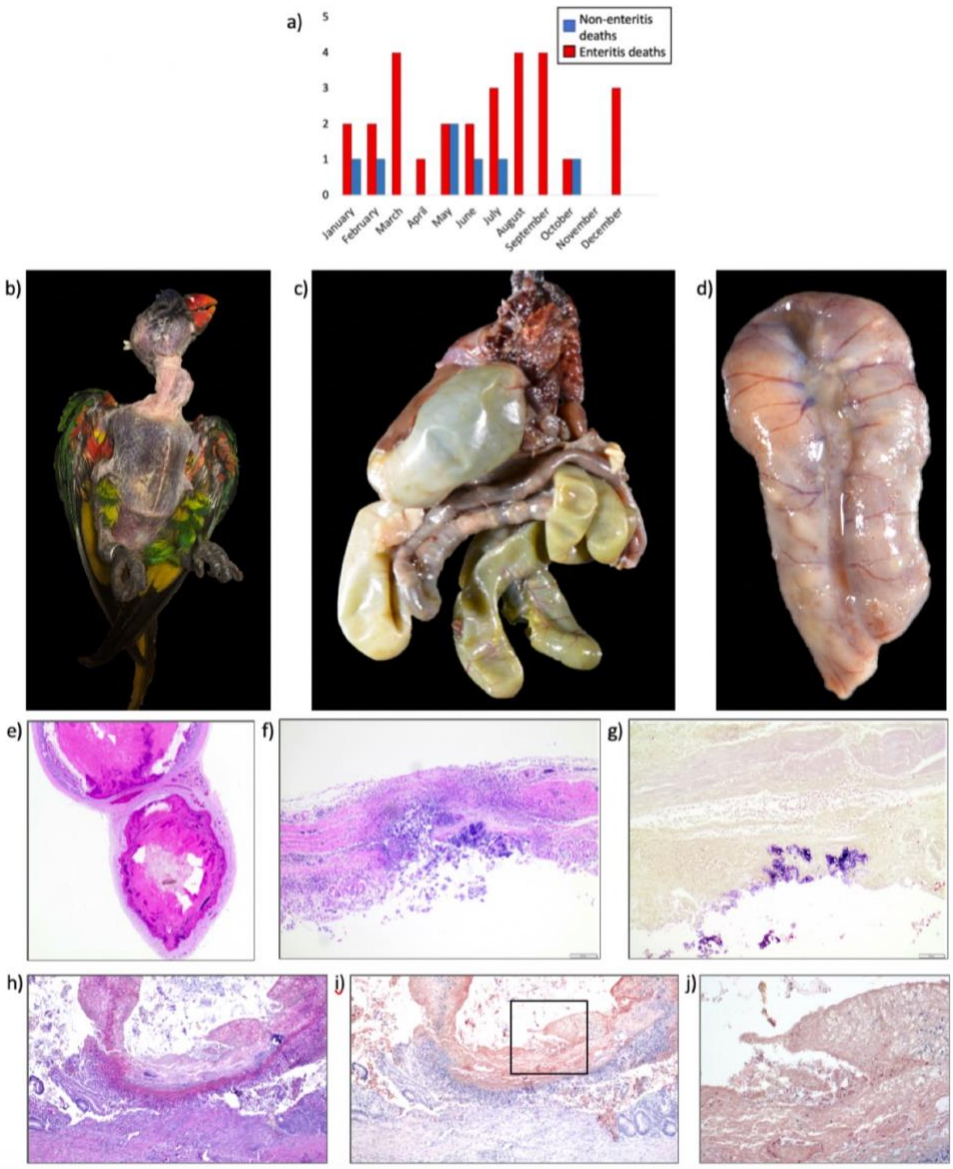


804

805

806 **Figure 3. Lorikeet enteritis gross and histopathology. a)** Number of lorikeet enteritis versus
807 non-enteritis deaths by month. Cases from CZA were diagnosed histologically over a 5-year
808 period (2015-2019). An increase in enteritis cases were observed during the summer months
809 (June-September). (**b-d**) Gross necropsy findings typical of lorikeets with enteritis. **b)** The
810 feathers have been plucked to demonstrate the decreased pectoral muscle mass of affected birds
811 leading to a prominent keel. **c)** There are multiple severely dilated loops of intestines, including
812 the paired ceca, with thin intestinal walls. Also present are segments of thickened and nodular

intestines mottled dark tan and pale tan. Tissues are formalin fixed. **d)** Formalin fixed loop of small intestine from an affected lorikeet. There are multiple soft to firm pale tan nodules within the wall and on the serosa of the intestines. The mesentery is also thickened by pale tan tissue and similar nodules. **(e-g)** Histologic findings from lorikeets with enteritis. **e)** Hematoxylin and eosin stain (H&E) of a chronic case of necrotizing enteritis in a lorikeet. Two loops of intestine are markedly dilated with thinning of the intestinal wall and replacement with fibrosis. The lumens are impacted with a coagulum of degenerate red blood cells, bacterial colonies and sloughed mucosa that often compress the remaining atrophied and blunted intestinal villi. There is also inflammation on the serosa and adjacent mesentery. **f)** H&E of acute transmural necrotizing and ulcerative enteritis in a lorikeet. The sparse remaining mucosa is characterized by a large central ulcer, inflammatory cells including macrophages and heterophils throughout the intestinal wall centered around the ulcer and overlying large colonies of bacteria. **g)** Modified Brown-Hopps of same intestinal section as in **Fig 3f**. The superficially adhered bacterial colonies within the lesion are monomorphic large gram-positive bacilli. **h)** H&E of necrotizing and ulcerative enteritis in a lorikeet. There is little remaining mucosa with a large central focus of ulceration, numerous heterophils and macrophages, followed by a layer of fibrin and degenerate red blood cells with admixed large bacterial colonies and sloughed necrotic mucosal epithelium. **i)** Immunohistochemistry (IHC) against *Clostridium perfringens* from the same intestinal section as in **Fig 3h**. Box indicates region in **Fig 3j** under higher magnification. **j)** IHC against *C. perfringens* within the indicated region from **Fig3i**. The light brown staining is non-specific labeling of the sloughed necrotic mucosa and hemorrhage. The punctate dark brown staining indicates immunolabeling of bacteria within the necrohemorrhagic coagulum and focus of ulceration.



836

837

838 **Figure 4. Altered microbial community diversity and composition in Denver Zoo (DZ)**

839 **lorikeets with enteritis.** Microbial composition and diversity in healthy lorikeets and lorikeets

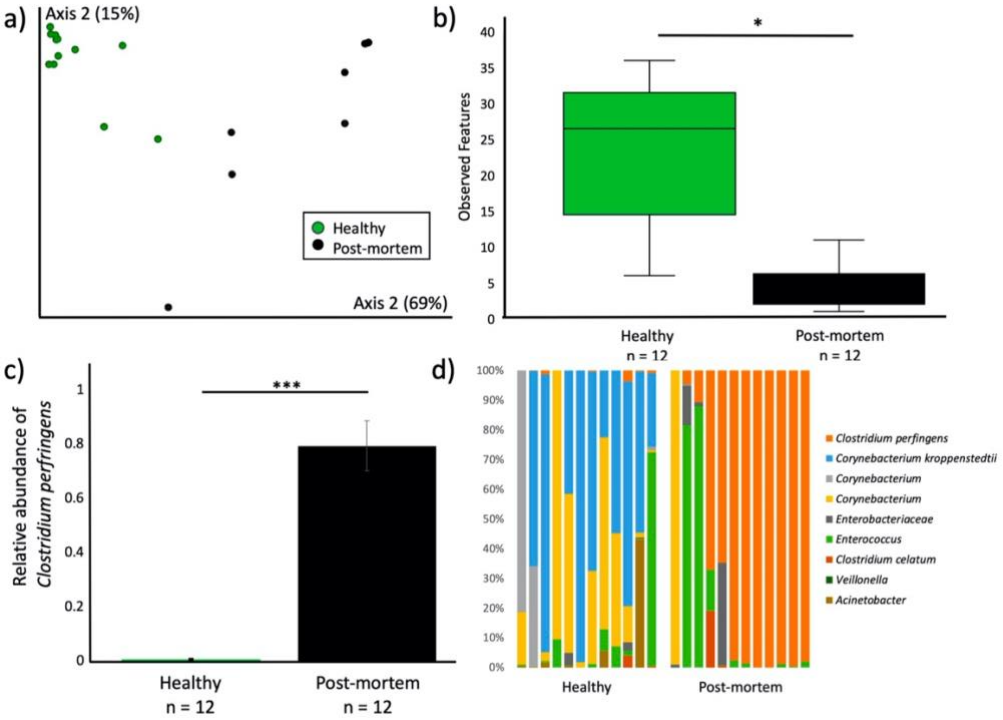
840 that died or were euthanized due to enteritis ("post-mortem" lorikeets). **a)** Microbial composition

841 (Weighted UniFrac) was significantly altered (PERMANOVA $p = 0.001$) and **b)** microbial

842 diversity (Observed Features) was significantly decreased (Kruskal-Wallis $*p < 0.0005$) in post-

843 mortem lorikeets. (Also see **Additional File 11**.) **c)** The relative abundance of *C. perfringens*

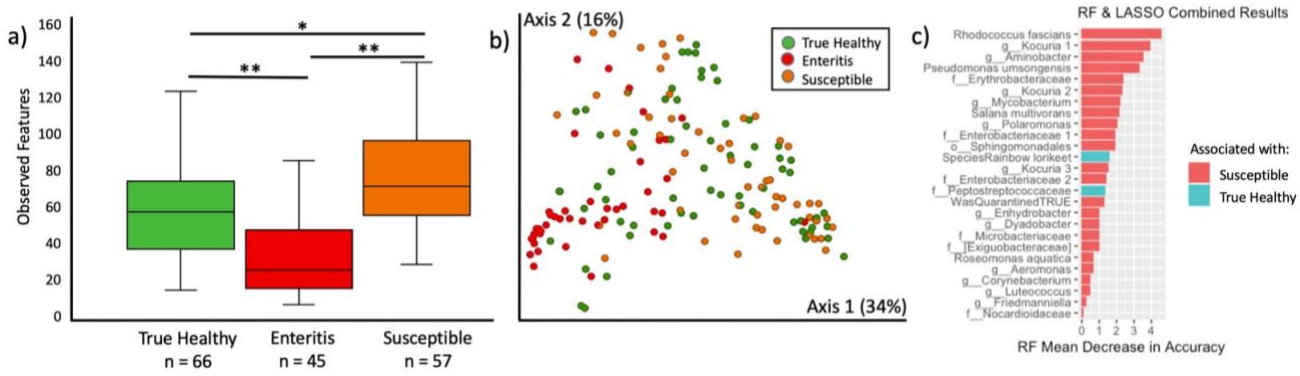
844 was also significantly increased in post-mortem lorikeets as compared to healthy lorikeets
845 (ANCOM, W = 67). **d)** Taxa bar plots showing taxonomic distributions within healthy and post-
846 mortem lorikeets with enteritis. Post-mortem lorikeet microbial communities were dominated by
847 *C. perfringens*.



848
849

850 **Figure 5. Susceptible CZA lorikeets have increased microbial diversity and altered**
851 **microbial composition that predicts enteritis.** Healthy lorikeets that never developed enteritis
852 throughout the sampling period were identified as "True Healthy" while healthy birds that
853 developed enteritis at least once during the sampling period were identified as "Susceptible."
854 "Enteritis" represents birds with enteritis that were sampled while they were clinically ill. No
855 post-mortem samples are included in this figure. **a)** Microbial diversity (Observed Features,
856 Kruskal-Wallis, all p <0.005) was increased and **b)** microbial composition was altered

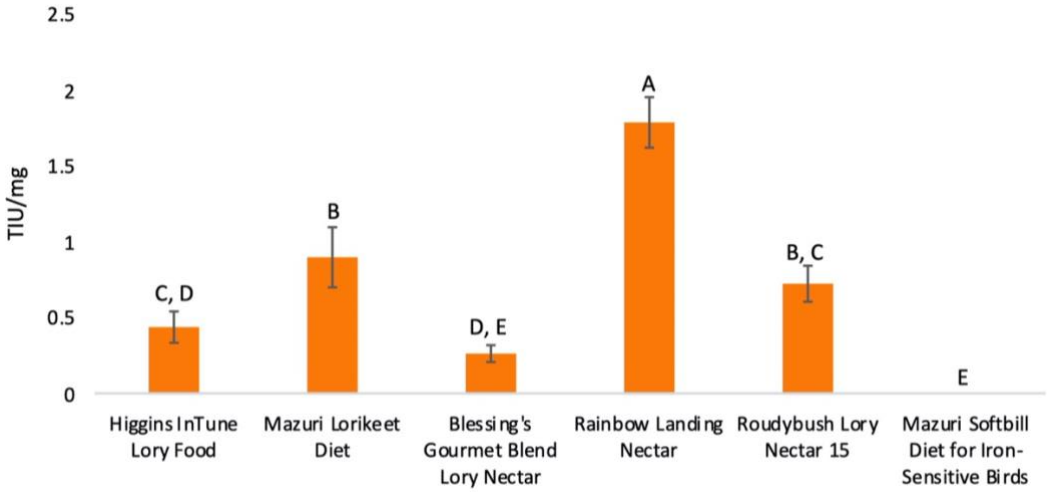
857 (PERMANOVA p =0.001) in Susceptible birds. **c)** Twenty-six variables including 24 microbial
858 taxa and two demographic variables were identified in Random Forests and LASSO models as
859 predictive of susceptibility or true health. The size of the bars represents the effect size of each
860 variable as predicted by the RF model. (Also see **Additional File 12**.)



861

862

863 **Figure 6. Trypsin inhibitor measurements in commercial nectars.** Trypsin inhibitor units
864 (TIU) per milligram nectar were measured in 6 commercial nectars and concentrations were
865 compared using a one-way ANOVA and Tukey's Test (α = 0.05). Bars that share a letter are not
866 significantly different.



867

868

869 **Table Legends**

870

871 **Table 1. Columbus Zoo and Aquarium (CZA) Lorikeet Demographics.** Number and percent

872 of Columbus Zoo & Aquarium lorikeets that ever or never had enteritis between Nov. 2018 and

873 Sept. 2019 by sex, age, and lorikeet species.

Table 1. Columbus Zoo and Aquarium (CZA) Lorikeet Demographics.

	Lorikeets that had **one or more episodes of enteritis** between Nov. 2018-Sept. 2019	Lorikeets that **never had enteritis** between Nov. 2018-Sept. 2019	***p*-value**
Sex (n, %)			
Male	9 (13.4 %)	14 (20.9 %)	p = 0.06 (all) p = 0.73 (male vs. female only)
Female	11 (16.4 %)	14 (20.9 %)	
Unknown	14 (20.9 %)	5 (7.5 %)	
Age (n, %)			
0-2 years	19 (28.3%)	7 (10.4%)	p = 0.02
3-5 years	7 (10.4%)	16 (23.9 %)	
6-10 years	6 (9.0%)	6 (9.0%)	
>11 years	2 (3.0 %)	4 (6.0 %)	
Species (n, %)			
Rainbow	8 (11.9 %)	17 (25.4 %)	p = 0.07 (all) p = 0.01 (rainbow vs. coconut only)
Coconut	23 (34.3 %)	13 (19.4 %)	
Marigold	1 (1.5 %)	0 (0 %)	
Lory	2 (3 %)	3 (4.5 %)	

874

875

876 **Table 2. Intestinal Content Isolates from CZA Lorikeets**

Table 2. Intestinal content isolates from CZA lorikeets

Bird ID	Intestinal contents, growth in culture?	MALDI identification	*cpa* (alpha toxin)	*cpb2* (beta2 toxin)	Inferred *C. perfringens* toxinotype	Selected for susceptibility testing?
104073	No	None				
113096	Yes	*Clostridium perfringens and Clostridium disporicum*	+	+	A	
115057	No	None				
115069	Yes	*Enterococcus faecium*				
115094	No	None				
116046	Yes	*Esherichia coli*				
118064	Yes	*Clostridium perfringens*	+	+	A	
118071	Yes	*Clostridium perfringens, Escherichia coli* , and *Clostridum paraperfringens* or *Clostridum baratii*	+	+	A	yes
118075	Yes	*Clostridium perfringens*	+	+	A	yes
118089	Yes	*Clostridium perfringens, Esherichia coli,* an organism with no ID	+	+	A	
118115	No	None				
118118	Yes	*Clostridium perfringens* and *Clostridium disporicum*	+	-	A	
118119	Yes	*Clostridium paraputrificum*				

Table 3. Lorikeet Demographics for CZA lorikeets submitted to pathology. The Freeman-Halton extension of the Fisher's Exact test was used to calculate p-values for sex and species. A Kruskal-Wallis test was used to calculate the p-value for age.

Table 3. Lorikeet Demographics for CZA lorikeets submitted to pathology

	Lorikeets with enteritis	Lorikeets with no enteritis	*p*-value
Sex (n, %)			
Male	12 (34.3%)	5 (14.3%)	$p = 0.73$ (all) $p = 0.06$ (male vs. female only)
Female	12 (34.3%)	0 (0%)	
Unknown	4 (11.4%)	2 (5.7%)	
Age (Mean ± SD)	4.6 ± 4.7	9.3 ± 6.1	$p = 0.13$
Species (n, %)			
Rainbow	7 (20%)	5 (14.2%)	$p = 0.11$
Coconut	14 (40%)	1 (2.9%)	
Other	7 (20%)	1 (2.9%)	

884 **Table 4. Toxin profiles, IHC, and *C. colinum* PCR on lorikeet intestines submitted to**

885 **pathology**

Table 4. Toxin profiles, IHC, and *C. colinum* PCR on lorikeet intestines submitted to pathology

Sample ID	Age (yrs)	Sex	Species	Acute / Chronic	Status	*C. perfringens* IHC	*cpa*	*cpb*	*etx*	*itx*	*cpe*	*NetB*	*C. colinum* PCR
15-1444	3	F	Rainbow	acute	enteritis	+	-	-	-	-	-	-	+
15-1455	14.7	F	Rainbow	acute	enteritis	+	+	-	-	-	-	-	+
15-1456	5.4	M	Coconut	acute	enteritis	-	+	-	-	-	-	-	+
15-1636	11	M	Rainbow	acute	enteritis	+	-	-	-	-	-	-	+
15-2050	0.8	F	Lorikeet	acute	enteritis	-	-	-	-	-	-	-	-
18-1004	0.8	F	Coconut	acute	enteritis	+	+	-	-	-	-	-	+
18-1304	6	M	Rainbow	acute	enteritis	+	-	-	-	-	-	-	-
18-1442	2	M	Rainbow	acute	enteritis	+	+	-	-	-	-	-	+
18-1698	15.5	F	Coconut	acute	enteritis	+	+	-	-	-	-	-	+
18-950	2.1	F	Lory	acute	enteritis	+	-	-	-	-	-	-	-
19-1164	1	M	Coconut	acute	enteritis	-	-	-	-	-	-	-	+
19-1220	1.3	F	Coconut	acute	enteritis	+	+	-	-	-	-	-	-
19-15	8.6	F	Coconut	acute	enteritis	+	-	-	-	-	-	-	-
19-183	0.8	F	Coconut	acute	enteritis	+	+	-	-	-	-	-	-
19-185	3.9	F	Coconut	acute	enteritis	+	-	-	-	-	-	-	-
19-427	0.6	unk	Lorikeet	acute	enteritis	+	+	-	-	-	-	-	+
19-428	0.9	unk	Coconut	acute	enteritis	+	+	-	-	-	-	-	+
19-488	15.6	M	Rainbow	acute	enteritis	-	-	-	-	-	-	-	+
19-526	1	unk	Coconut	acute	enteritis	no tissue	-	-	-	-	-	-	-
15-1681	4.8	M	Coconut	chronic	enteritis	+	+	-	-	-	-	-	+
15-1834	8.9	M	Lorikeet	chronic	enteritis	-	-	-	-	-	-	-	+
16-82	7	M	Lorikeet	chronic	enteritis	+	+	-	-	-	-	-	+
18-1561	2.8	M	Violet necked	chronic	enteritis	+	-	-	-	-	-	-	+
19-1241	1.3	unk	Coconut	chronic	enteritis	+	+	-	-	-	-	-	+
19-1242	3	F	Marigold	chronic	enteritis	+	+	-	-	-	-	-	-
19-863	5	M	Rainbow	chronic	enteritis	+	-	-	-	-	-	-	-
15-1228	0.2	M	Coconut	chronic active	enteritis	+	-	-	-	-	-	-	+
15-1230	0.2	F	Coconut	chronic active	enteritis	+	-	-	-	-	-	-	+
15-1226	11	M	Rainbow	NA	No lesions	+	+	-	-	-	-	-	-
16-426	0.2	M	Lorikeet	NA	No lesions	-	-	-	-	-	-	-	-
16-653	1	unk	Coconut	NA	No lesions	-	-	-	-	-	-	-	+
17-150	12	M	Rainbow	NA	No lesions	-	-	-	-	-	-	-	-
17-1689	13	M	Rainbow	NA	No lesions	-	-	-	-	-	-	-	-
17-988	13	unk	Rainbow	NA	No lesions	-	-	-	-	-	-	-	-
19-1244	14.9	M	Rainbow	NA	No lesions	-	-	-	-	-	-	-	-

886

887

888 **Additional Files**

889

890 **Additional File 1: Experimental design.** Lorikeet sampling by season and opportunistically

891 during cases of enteritis at the a) Columbus Zoo and Aquarium and the b) Denver Zoo.

892

Additional File 2: *Clostridium perfringens* IHC protocol from California Animal Health & Food Safety Laboratory

Additional File 3: Reagent preparation for measurement of Trypsin Inhibitor Levels in nectar

Additional File 4: Microbial community diversity in healthy CZA birds by sex, species, and age. Healthy birds across all time points: Microbial diversity (Shannon Diversity Index and Observed Features) did not differ significantly by **a**,**d**) sex, **b**,**e**) species, or **c**,**f**) age (Kruskal Wallis: Shannon sex $p = 0.77$, species $p = 0.25$, age $p = 0.89$; Observed Features sex $p = 0.93$, species $p = 0.18$, age $p = 0.08$).

Additional File 5: CZA Microbial community composition by sex, species, and age.

Additional File 6: Seasonality in gut microbiota in healthy CZA lorikeets. a) Microbial community composition (Unweighted UniFrac) and **b)** diversity (Shannon Diversity Index) by season (November 2018, February 2019, September 2019). There were significant shifts in microbial composition by season (PERMANOVA $p = 0.001$), and microbial diversity decreased significantly between November 2018 and September 2019 (Kruskal-Wallis $^*p < 0.001$). (Also see **Fig. 1**.)

Additional File 7: Altered microbial diversity and composition in CZA lorikeets with enteritis. Microbial composition and diversity in healthy lorikeets, lorikeets with enteritis, and

lorikeets that died or were euthanized due to enteritis (post-mortem). **a)** Microbial composition (Unweighted UniFrac) was significantly altered (PERMANOVA $p = 0.001$) and **b)** microbial diversity (Shannon Diversity Index) was significantly decreased (Kruskal-Wallis $*p < 0.005$, $**p < 0.0005$, $***p < 0.00001$) in lorikeets with enteritis or post-mortem lorikeets. (Also see **Fig. 2.**)

Additional File 8: Differentially abundant microbes by health status. Based on an ANCOM, Clostridia were significantly increased in relative abundance in lorikeets with enteritis as compared to healthy lorikeets. This analysis included 157 healthy samples and 45 enteritis samples from a total of 67 birds. No post-mortem samples were included in this analysis. Clostridia are in **bold** text.

Additional File 9: Differentially abundant microbes by health status in 25 birds with matched healthy / enteritis samples. Based on an ANCOM, Clostridia were significantly increased in relative abundance in lorikeets with enteritis as compared to healthy lorikeets. This analysis included 29 healthy samples and 35 enteritis samples from a total of 25 birds. No post-mortem samples were included in this analysis. Clostridia are in **bold** text.

Additional File 10: Denver Zoo (DZ) Lorikeet Demographics. The p-value for sex were based on a χ^2 test. A Kruskal-Wallis test was used to calculate the p-value for age.
The Freeman-Halton extension of the Fisher's Exact test was used to calculate a p-value for species.

938 **Additional File 11: Altered microbial composition and diversity in Denver Zoo lorikeets**
939 **with enteritis.** Microbial composition and diversity in healthy lorikeets and lorikeets that died or
940 were euthanized due to enteritis (post-mortem). **a)** Microbial composition (Unweighted UniFrac)
941 was significantly altered (PERMANOVA $p = 0.001$) and **b)** microbial diversity was significantly
942 decreased (Shannon, Kruskal-Wallis *$p < 0.005$) in post-mortem lorikeets. (Also see **Fig. 4**.)
943
944 **Additional File 12: Susceptible CZA lorikeets have altered microbial composition that**
945 **predicts enteritis.** Healthy lorikeets that never developed enteritis throughout the sampling
946 period were identified as "True Healthy" while healthy birds that developed enteritis at least
947 once during the sampling period were identified as "Susceptible." "Enteritis" represents birds
948 with enteritis that were sampled while they were clinically ill. No post-mortem samples are
949 included in this figure. **a)** Microbial diversity (Shannon, Kruskal-Wallis, $p < 0.005$) was
950 increased in Susceptible and True Healthy birds as compared to birds with enteritis and **b)**
951 microbial composition (Unweighted UniFrac) was altered in Susceptible birds. Variables
952 associated with susceptibility or health were then predicted by a **c)** Random Forest (RF) or **d)**
953 LASSO model. The RF model has a sensitivity of 0.75, a specificity of 0.571, and an overall
954 accuracy of 0.557. This model identifies the relative importance of each variable but not whether
955 the variable is associated with susceptibility or health. The LASSO model has a sensitivity of
956 0.875, a specificity of 0.571, an overall accuracy of 0.733, and generates an area under the curve
957 (AUC) of 0.72. This model (LASSO) identifies whether a variable is associated with
958 susceptibility or health but not the relative importance of the variable.
959
960
961 **References**

1. Ward MP, Ramer JC, Proudfoot J, Garner MM, Juan-Sallés C, Wu CC: **Outbreak of Salmonellosis in a Zoologic Collection of Lorikeets and Lories (Trichoglossus, Lorius, and Eos spp.)**. *Avian Diseases* 2003, **47**(2):493-498.
2. Shima AL, Osborn KG: **An Epornitic of Salmonella typhimurium in a Collection of Lories and Lorikeets**. *Journal of Zoo and Wildlife Medicine* 1989, **20**(3):373-376.
3. Mackie JT, Black D, Prior H: **Enteritis associated with adeno-virus-like particles in captive lorikeets**. *Australian Veterinary Journal* 2003, **81**(5):293-295.
4. Ferrell ST, Tell L: **Clostridium tertium Infection in a Rainbow Lorikeet (Trichoglossus haematodus haematodus) with Enteritis**. *Journal of Avian Medicine and Surgery* 2001, **15**(3):204-208.
5. Karunakaran N, Nagarajan K, Prathiba A, Soundararajan C, Bharathi SV: **Incidence, Diagnosis and Treatment of Clostridial Enteritis in Lories and Lorikeets**. *Intas Polivet* 2018, **19**:385+.
6. Melander RJ, Zurawski DV, Melander C: **Narrow-Spectrum Antibacterial Agents**. *Medchemcomm* 2018, **9**(1):12-21.
7. Sze MA, Schloss PD: **Leveraging Existing 16S rRNA Gene Surveys To Identify Reproducible Biomarkers in Individuals with Colorectal Tumors**. *mBio* 2018, **9**(3):e00630-00618.
8. Bhatt AP, Redinbo MR, Bultman SJ: **The role of the microbiome in cancer development and therapy**. *CA: a cancer journal for clinicians* 2017, **67**(4):326-344.
9. Wen C, Zheng Z, Shao T, Liu L, Xie Z, Le Chatelier E, He Z, Zhong W, Fan Y, Zhang L *et al*: **Quantitative metagenomics reveals unique gut microbiome biomarkers in ankylosing spondylitis**. *Genome Biology* 2017, **18**(1):142.
10. Lemon KP, Armitage GC, Relman DA, Fischbach MA: **Microbiota-Targeted Therapies: An Ecological Perspective**. *Science Translational Medicine* 2012, **4**(137):137rv135.
11. Young VB: **The role of the microbiome in human health and disease: an introduction for clinicians**. *BMJ* 2017, **356**:j831.
12. Hacquard S, Garrido-Oter R, González A, Spaepen S, Ackermann G, Lebeis S, McHardy Alice C, Dangl Jeffrey L, Knight R, Ley R *et al*: **Microbiota and Host Nutrition across Plant and Animal Kingdoms**. *Cell Host & Microbe* 2015, **17**(5):603-616.
13. Round JL, Mazmanian SK: **The gut microbiota shapes intestinal immune responses during health and disease**. *Nat Rev Immunol* 2009, **9**(5):313-323.
14. Kamada N, Chen GY, Inohara N, Núñez G: **Control of Pathogens and Pathobionts by the Gut Microbiota**. *Nature immunology* 2013, **14**(7):685-690.
15. Pickard JM, Zeng MY, Caruso R, Núñez G: **Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease**. *Immunol Rev* 2017, **279**(1):70-89.
16. Waite DW, Deines P, Taylor MW: **Gut Microbiome of the Critically Endangered New Zealand Parrot, the Kakapo (Strigops habroptilus)**. *PLOS ONE* 2012, **7**(4):e35803.
17. Xenoulis PG, Gray PL, Brightsmith D, Palculict B, Hoppes S, Steiner JM, Tizard I, Suchodolski JS: **Molecular characterization of the cloacal microbiota of wild and captive parrots**. *Vet Microbiol* 2010, **146**:320-325.
18. Ryu H, Grond K, Verheijen B, Elk M, Buehler DM, Santo Domingo JW: **IIntestinal Microbiota and Species Diversity of *Campylobacter* and *Helicobacter spp.* in**

Migrating Shorebirds in Delaware Bay. *Applied and Environmental Microbiology* 2014, **80**(6):1838.

19. Hale VL, Tan CL, Niu K, Yang Y, Knight R, Zhang Q, Cui D, Amato KR: **Diet Versus Phylogeny: a Comparison of Gut Microbiota in Captive Colobine Monkey Species**. *Microbial Ecology* 2018, **75**(2):515-527.
20. Bolnick DI, Snowberg LK, Hirsch PE, Lauber CL, Knight R, Caporaso JG, Svanbäck R: **Individuals' diet diversity influences gut microbial diversity in two freshwater fish (threespine stickleback and Eurasian perch)**. *Ecology Letters* 2014, **17**(8):979-987.
21. Escallón C, Belden LK, Moore IT: **The Cloacal Microbiome Changes with the Breeding Season in a Wild Bird**. *Integrative Organismal Biology* 2019, **1**(1).
22. Kueneman JG, Woodhams DC, Harris R, Archer HM, Knight R, McKenzie VJ: **Probiotic treatment restores protection against lethal fungal infection lost during amphibian captivity**. *Proceedings Biological sciences* 2016, **283**(1839):20161553.
23. Amato KR, Metcalf JL, Song SJ, Hale VL, Clayton J, Ackermann G, Humphrey G, Niu K, Cui D, Zhao H *et al*: **Using the gut microbiota as a novel tool for examining colobine primate GI health**. *Global Ecology and Conservation* 2016, **7**:225-237.
24. Avena CV, Parfrey LW, Leff JW, Archer HM, Frick WF, Langwig KE, Kilpatrick AM, Powers KE, Foster JT, McKenzie VJ: **Deconstructing the Bat Skin Microbiome: Influences of the Host and the Environment**. *Frontiers in Microbiology* 2016, **7**(1753).
25. Kohl KD, Weiss RB, Cox J, Dale C, Denise Dearing M: **Gut microbes of mammalian herbivores facilitate intake of plant toxins**. *Ecology Letters* 2014, **17**(10):1238-1246.
26. Stumpf RM, Gomez A, Amato KR, Yeoman CJ, Polk JD, Wilson BA, Nelson KE, White BA, Leigh SR: **Microbiomes, metagenomics, and primate conservation: New strategies, tools, and applications**. *Biological Conservation* 2016, **199**:56-66.
27. Clayton JB, Gomez A, Amato K, Knights D, Travis DA, Blekhman R, Knight R, Leigh S, Stumpf R, Wolf T *et al*: **The gut microbiome of nonhuman primates: Lessons in ecology and evolution**. *American Journal of Primatology* 2018, **80**(6):e22867.
28. West AG, Waite DW, Deines P, Bourne DG, Digby A, McKenzie VJ, Taylor MW: **The microbiome in threatened species conservation**. *Biological Conservation* 2019, **229**:85-98.
29. Keyburn AL, Yan X-X, Bannam TL, Van Immerseel F, Rood JI, Moore RJ: **Association between avian necrotic enteritis and Clostridium perfringens strains expressing NetB toxin**. *Veterinary research* 2010, **41**(2):21-21.
30. Shane S: **The significance of campylobacter jejuni infection in poultry: A review** *Avian Pathology* 1992, **21**(2):189-213.
31. Pisani J, Speer B, Howerth EW, Clubb SL: **Clostridial Infections in Psittacine Birds**. *Journal of Avian Medicine and Surgery* 1998, **12**(3):202-204.
32. O'Toole D, Mills K, Ellis R, Farr R, Davis M: **Clostridial Enteritis in Red Lories (Eos Bounea)**. *Journal of Veterinary Diagnostic Investigation* 1993, **5**(1):111-113.
33. Guimarães MB, Torres LN, Mesquita RG, Ampuero F, Cunha MPV, Ferreira TSP, Ferreira AJP, Catão-Dias JL, Moreno AM, Knöbl T: ***Clostridium perfringens* Type A Enteritis in Blue and Yellow Macaw (*Ara ararauna*)**. *Avian Diseases* 2014, **58**(4):650-653, 654.
34. Asaoka Y, Yanai T, Hirayama H, Une Y, Saito E, Sakai H, Goryo M, Fukushi H, Masegi T: **Fatal necrotic enteritis associated with Clostridium perfringens in wild crows (Corvus macrorhynchos)**. *Avian Pathology* 2004, **33**(1):19-24.

35. Buffie CG, Bucci V, Stein RR, McKenney PT, Ling L, Gobourne A, No D, Liu H, Kinnebrew M, Viale A *et al*: **Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile**. *Nature* 2015, **517**(7533):205-208.
36. Battaglioli EJ, Hale VL, Chen J, Jeraldo P, Ruiz-Mojica C, Schmidt BA, Rekdal VM, Till LM, Huq L, Smits SA *et al*: ***Clostridioides difficile* uses amino acids associated with gut microbial dysbiosis in a subset of patients with diarrhea**. *Science Translational Medicine* 2018, **10**(464).
37. Ferreira RBR, Gill N, Willing BP, Antunes LCM, Russell SL, Croxen MA, Finlay BB: **The intestinal microbiota plays a role in Salmonella-induced colitis independent of pathogen colonization**. *PloS one* 2011, **6**(5):e20338-e20338.
38. Gray KL: **After 14 lorikeets die, zoo hopes illness gone**. In: *Columbus Dispatch.* 2012.
39. Uzal FA, Vidal JE, McClane BA, Gurjar AA: **Clostridium Perfringens Toxins Involved in Mammalian Veterinary Diseases**. *Open Toxinology J* 2010, **2**:24-42.
40. Liu K: **Soybean Trypsin Inhibitor Assay: Further Improvement of the Standard Method Approved and Reapproved by American Oil Chemists' Society and American Association of Cereal Chemists International**. *Journal of the American Oil Chemists' Society* 2019, **96**(6):635-645.
41. Droual R, Farver TB, Bickford AA: **Relationship of Sex, Age, and Concurrent Intestinal Disease to Necrotic Enteritis in Turkeys**. *Avian Diseases* 1995, **39**(3):599-605.
42. Wobeser G, Rainnie DJ: **EPIZOOTIC NECROTIC ENTERITIS IN WILD GEESE**. *Journal of Wildlife Diseases* 1987, **23**(3):376-385.
43. Silva ROS, Lobato FCF: **Clostridium perfringens: A review of enteric diseases in dogs, cats and wild animals**. *Anaerobe* 2015, **33**:14-17.
44. Shivaprasad HL, Uzal F, Kokka R, Fisher DJ, McClane BA, Songer AG: **Ulcerative Enteritis-Like Disease Associated with Clostridium perfringens Type A in Bobwhite Quail (Colinus virginianus)**. *Avian Diseases* 2008, **52**(4):635-640.
45. McOrist S, Reece RL: **Clostridial enteritis in free-living lorikeets (Trichoglossus spp.)**. *Avian Pathology* 1992, **21**(3):503-507.
46. Rupiper DJ: **Hemorrhagic Enteritis in a Group of Great-Billed Parrots (Tanygnathus megalorynchos)**. *Journal of the Association of Avian Veterinarians* 1993, **7**(4):209-211.
47. Grau-Roma L, Navarro M, Blatter S, Wenker C, Kittl S, Uzal FA, Posthaus H: **Clostridium perfringens–Associated Necrotic Enteritis-Like Disease in Coconut Lorikeets (Trichoglossus haematodus)**. *Veterinary Pathology* 2021, **58**(2):423-427.
48. Ritchie BW, Harrison GJ, Harrison LR: **Avian Medicine: Principles and Application**. Lake Worth, FL: Wingers Publishing, Inc.; 1994.
49. Bildfell RJ, Eltzroth EK, Songer JG: **Enteritis as a Cause of Mortality in the Western Bluebird (Sialia mexicana)**. *Avian Diseases* 2001, **45**(3):760-763.
50. Góngora E, Elliott KH, Whyte L: **Gut microbiome is affected by inter-sexual and inter-seasonal variation in diet for thick-billed murres (Uria lomvia)**. *Scientific Reports* 2021, **11**(1):1200.
51. Drovetski SV, O'Mahoney MJV, Matterson KO, Schmidt BK, Graves GR: **Distinct microbiotas of anatomical gut regions display idiosyncratic seasonal variation in an avian folivore**. *Animal Microbiome* 2019, **1**(1):2.

52. Lewis WB, Moore FR, Wang S: **Characterization of the gut microbiota of migratory passerines during stopover along the northern coast of the Gulf of Mexico**. *Journal of Avian Biology* 2016, **47**(5):659-668.
53. Le Roy CI, Woodward MJ, Ellis RJ, La Ragione RM, Claus SP: **Antibiotic treatment triggers gut dysbiosis and modulates metabolism in a chicken model of gastro-intestinal infection**. *BMC Veterinary Research* 2019, **15**(1):37.
54. Langdon A, Crook N, Dantas G: **The effects of antibiotics on the microbiome throughout development and alternative approaches for therapeutic modulation**. *Genome medicine* 2016, **8**(1):39-39.
55. Dethlefsen L, Huse S, Sogin ML, Relman DA: **The Pervasive Effects of an Antibiotic on the Human Gut Microbiota, as Revealed by Deep 16S rRNA Sequencing**. *PLOS Biology* 2008, **6**(11):e280.
56. Dethlefsen L, Relman DA: **Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation**. *Proc Natl Acad Sci* 2011, **108** (1):4554-4561.
57. Modi SR, Collins JJ, Relman DA: **Antibiotics and the gut microbiota**. *J Clin Investig* 2014, **124**(10):4212-4218.
58. Lu J, Hofacre C, Smith F, Lee MD: **Effects of feed additives on the development on the ileal bacterial community of the broiler chicken**. *Animal* 2008, **2**(5):669-676.
59. Uzal FA, Navarro MA, Li J, Freedman JC, Shrestha A, McClane BA: **Comparative pathogenesis of enteric clostridial infections in humans and animals**. *Anaerobe* 2018, **53**:11-20.
60. Cooper KK, Songer JG, Uzal FA: **Diagnosing clostridial enteric disease in poultry**. *Journal of Veterinary Diagnostic Investigation* 2013, **25**(3):314-327.
61. Pattison M, McMullin P, Bradbury J, Alexander D (eds.): **Poultry Diseases (Chapter 18, Clostridia)**, 6th edn: Saunders Ltd.; 2007.
62. Altman RB, Clubb SL, Dorrestein GM, Quesenberry K (eds.): **Avian Medicine and Surgery**: Saunders; 1997.
63. Pizarro M, Höfle U, Rodríguez-Bertos A, González-Huecas M, Castaño M: **Ulcerative Enteritis (Quail Disease) in Lories**. *Avian Diseases* 2005, **49**(4):606-608.
64. Stanley D, Keyburn AL, Denman SE, Moore RJ: **Changes in the caecal microflora of chickens following Clostridium perfringens challenge to induce necrotic enteritis**. *Veterinary Microbiology* 2012, **159**(1):155-162.
65. Lin Y, Xu S, Zeng D, Ni X, Zhou M, Zeng Y, Wang H, Zhou Y, Zhu H, Pan K *et al*: **Disruption in the cecal microbiota of chickens challenged with Clostridium perfringens and other factors was alleviated by Bacillus licheniformis supplementation**. *PLOS ONE* 2017, **12**(8):e0182426.
66. Antonissen G, Eeckhaut V, Van Driessche K, Onrust L, Haesebrouck F, Ducatelle R, Moore RJ, Van Immerseel F: **Microbial shifts associated with necrotic enteritis**. *Avian Pathology* 2016, **45**(3):308-312.
67. Fasina YO, Newman MM, Stough JM, Liles MR: **Effect of Clostridium perfringens infection and antibiotic administration on microbiota in the small intestine of broiler chickens**. *Poultry Science* 2016, **95**(2):247-260.
68. Hadley TL: **Disorders of the Psittacine Gastrointestinal Tract**. *Veterinary Clinics of North America: Exotic Animal Practice* 2005, **8**(2):329-349.

69. Wilson GH, Ritchie BW, Greenacre CB, Fontenot D: **Clostridium: Passenger or pathogen?** In: *Annual Conference of the Association of Avian Veterinarians: 1999*. 251-253.
70. Boujon P, Henzi M, Penseyres JH, Belloy L: **Enterotoxaemia involving β2-toxigenic Clostridium perfringens in a white stork (Ciconia ciconia)**. *Veterinary Record* 2005, **156**(23):746-747.
71. Garmory HS, Chanter N, French NP, Bueschel D, Songer JG, Titball RW: **Occurrence of Clostridium perfringens beta2-toxin amongst animals, determined using genotyping and subtyping PCR assays**. *Epidemiol Infect* 2000, **124**(1):61-67.
72. Uzal FA, Songer JG, Prescott JF, Popoff MR (eds.): **Clostridial Diseases of Animals**: John Wiley & Sons, Inc.; 2016.
73. Silva ROS, Junior FCF, Marques MVR, Junior CAO, Martins NRD, Lobato FCF: **Genotyping and antimicrobial susceptibility of Clostridium perfringens isolated from Tinamidae, Cracidae and Ramphastidae species in Brazil**. *Ciência Rural* 2014, **44**(3):486-491.
74. Silva ROS, Ribeiro MG, Palhares MS, Borges AS, Maranhão RPA, Silva MX, Lucas TM, Olivo G, Lobato FCF: **Detection of A/B toxin and isolation of Clostridium difficile and Clostridium perfringens from foals**. *Equine Veterinary Journal* 2013, **45**(6):671-675.
75. Silva ROS, Santos RLR, Pires PS, Pereira LC, Pereira ST, Duarte MC, de Assis RA, Lobato FCF: **Detection of toxins A/B and isolation of Clostridium difficile and Clostridium perfringens from dogs in Minas Gerais, Brazil**. *Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]* 2013, **44**(1):133-137.
76. Schotte U, Truyen U, Neubauer H: **Significance of β2-Toxigenic Clostridium perfringens Infections in Animals and Their Predisposing Factors – A Review**. *Journal of Veterinary Medicine, Series B* 2004, **51**(10):423-426.
77. Crespo R, Fisher DJ, Shivaprasad HL, Fernández-Miyakawa ME, Uzal FA: **Toxinotypes of Clostridium Perfringens Isolated from Sick and Healthy Avian Species**. *Journal of Veterinary Diagnostic Investigation* 2007, **19**(3):329-333.
78. Berto G, Agnoletti F, Drigo I, Tonon E, Vascellari M, Fracas V, Bano L: **Clostridial co-infection episodes in commercial laying hens**. *Avian Pathology* 2015, **44**(3):200-203.
79. Forero AJ, Muñoz M, Camargo M, Soto-De León SC, Ríos-Chaparro DI, Birchenall C, Pinilla D, Pardo JM, Josa DF, Patarroyo MA *et al*: **High frequency of toxigenic Clostridium difficile and Clostridium perfringens coinfection among diarrheic patients at health care facility-onset (HCFO) and community-onset (CO) centers in Bogotá, Colombia**. *Gut Pathogens* 2019, **11**(1):27.
80. Uzal FA, Diab SS, Blanchard P, Moore J, Anthenill L, Shahriar F, Garcia JP, Songer JG: **Clostridium perfringens type C and Clostridium difficile co-infection in foals**. *Veterinary Microbiology* 2012, **156**(3):395-402.
81. Diniz AN, Silva ROS, Oliveira Junior CA, Pierezan F, Lobato FCF: **Clostridium perfringens type A netF and netE positive and Clostridium difficile co-infection in two adult dogs**. *Anaerobe* 2016, **38**:94-96.
82. Morrissey EM, Mau RL, Schwartz E, Caporaso JG, Dijkstra P, van Gestel N, Koch BJ, Liu CM, Hayer M, McHugh TA *et al*: **Phylogenetic organization of bacterial activity**. *The ISME Journal* 2016, **10**(9):2336-2340.

83. Hermans PG, Morgan KL: **Prevalence and associated risk factors of necrotic enteritis on broiler farms in the United Kingdom; a cross-sectional survey**. *Avian Pathology* 2007, **36**(1):43-51.
84. Kaldhusdal M, Skjerve E: **Association between cereal contents in the diet and incidence of necrotic enteritis in broiler chickens in Norway**. *Preventive Veterinary Medicine* 1996, **28**(1):1-16.
85. Fancher CA, Zhang L, Kiess AS, Adhikari PA, Dinh TTN, Sukumaran AT: **Avian Pathogenic Escherichia coli and Clostridium perfringens: Challenges in No Antibiotics Ever Broiler Production and Potential Solutions**. *Microorganisms* 2020, **8**(10):1533.
86. Gerlach H: **Bacteria**. In: *Avian Medicine: Principles and Applications.* Edited by Ritchie B, Harrison G, Harrison L. Lake Worth, FL: Wingers Publishing, Inc.; 1994: 949-983.
87. Gibert M, Jolivet-Renaud C, Popoff MR: **Beta2 toxin, a novel toxin produced by Clostridium perfringens**. *Gene* 1997, **203**(1):65-73.
88. Palliyeguru MWCD, Rose SP, Mackenzie AM: **Effect of trypsin inhibitor activity in soya bean on growth performance, protein digestibility and incidence of sub-clinical necrotic enteritis in broiler chicken flocks**. *British Poultry Science* 2011, **52**(3):359-367.
89. Bradbury JH, Hammer B, Tue N, Anders M, Millar JS: **Protein quantity and quality and trypsin inhibitor content of sweet potato cultivars from the highlands of Papua New Guinea**. *Journal of Agricultural and Food Chemistry* 1985, **33**(2):281-285.
90. Zhang Z, Corke H: **Trypsin inhibitor activity in vegetative tissue of sweet potato plants and its response to heat treatment**. *Journal of the Science of Food and Agriculture* 2001, **81**(14):1358-1363.
91. Senanayake SA, Ranaweera KKDS, Gunaratne A, Bamunuarachchi A: **Comparative analysis of nutritional quality of five different cultivars of sweet potatoes (Ipomea batatas (L) Lam) in Sri Lanka**. *Food Science & Nutrition* 2013, **1**(4):284-291.
92. Lacey JA, Stanley D, Keyburn AL, Ford M, Chen H, Johanesen P, Lyras D, Moore RJ: **Clostridium perfringens-mediated necrotic enteritis is not influenced by the pre-existing microbiota but is promoted by large changes in the post-challenge microbiota**. *Veterinary Microbiology* 2018, **227**:119-126.
93. Kiu R, Hall LJ: **An update on the human and animal enteric pathogen Clostridium perfringens**. *Emerg Microbes Infect* 2018, **7**(1):141-141.
94. Sorg JA, Sonenshein AL: **Bile salts and glycine as cogerminants for Clostridium difficile spores**. *Journal of bacteriology* 2008, **190**(7):2505-2512.
95. Austin MC, Hallstrand TS, Hoogestraat DR, Balmforth G, Stephens K, Butler-Wu S, Yeung CCS: **Rhodococcus fascians infection after haematopoietic cell transplantation: not just a plant pathogen?** *JMM Case Rep* 2016, **3**(2):e005025-e005025.
96. Glünder G, Siegmann O: **Iccurrence of aeromonas hydrophila in wild birds**. *Avian Pathology* 1989, **18**(4):685-695.
97. França M, Walker RL, Kokka R, Shivaprasad HL: **Aeromonas Species Associated with Necrotizing Enteritis and Septicemia in an Adult Male Ostrich (Struthio camelus)**. *Avian Diseases* 2009, **53**(2):310-316.
98. Brook I, Walker RI: **Pathogenicity of Clostridium species with other bacteria in mixed infections**. *Journal of Infection* 1986, **13**(3):245-253.

99. Keyburn AL, Sheedy SA, Ford ME, Williamson MM, Awad MM, Rood JI, Moore RJ: **Alpha-toxin of Clostridium perfringens is not an essential virulence factor in necrotic enteritis in chickens**. *Infection and immunity* 2006, **74**(11):6496-6500.
100. Brzęk P, Ciminari ME, Kohl KD, Lessner K, Karasov WH, Caviedes-Vidal E: **Effect of age and diet composition on activity of pancreatic enzymes in birds**. *J Comp Physiol B* 2013, **183**(5):685-697.
101. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F *et al*: **Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2**. *Nature Biotechnology* 2019, **37**(8):852-857.
102. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP: **DADA2: High-resolution sample inference from Illumina amplicon data**. *Nature methods* 2016, **13**(7):581-583.
103. AACC: **AACC Method 22-40: Measurement of trypsin inhibitor activity of soy products—spectrophotometric method**, 11th edn. St. Paul, MN: AACC International; 1999.
104. Preacher KJ: **Calculation for the chi-square test: An interactive calculation tool for chi-square tests of goodness of fit and independence** In.; 2001.
105. Mandal S, Van Treuren W, White RA, Eggesbø M, Knight R, Peddada SD: **Analysis of composition of microbiomes: a novel method for studying microbial composition**. *Microb Ecol Health Dis* 2015, **26**:27663-27663.
106. Breiman L: **Random Forests**. *Machine Learning* 2001, **45**(1):5-32.
107. Tibshirani R: **Regression Shrinkage and Selection via the Lasso**. *Journal of the Royal Statistical Society Series B (Methodological)* 1996, **58**(1):267-288.
108. Efron B, Hastie T, Johnstone I, Tibshirani R: **Least angle regression**. *The Annals of Statistics* 2004, **32**(2):407-499, 493.