Influence of the intestinal microbiota on disease susceptibility in kittens with experimentally-induced carriage of atypical enteropathogenic *Escherichia coli*

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A B S T R A C T

Typical enteropathogenic *E. coli* (tEPEC) carries the highest hazard of death in children with diarrhea and atypical EPEC (aEPEC) was recently identified as significantly associated with diarrheal mortality in kittens. In both children and kittens there is a significant association between aEPEC burden and diarrheal disease, however the infection can be found in individuals with and without diarrhea. It remains unclear to what extent, under what conditions, or by what mechanisms aEPEC serves as a primary pathogen in individuals with diarrhea. It seems likely that a combination of host and bacterial factors enable aEPEC to cause disease in some individuals and not in others. The purpose of this study was to determine the impact of aEPEC on intestinal function and diarrhea in kittens following experimentally-induced carriage and the influence of a disrupted intestinal microbiota on disease susceptibility. Results of this study identify aEPEC as a potential pathogen in kittens. In the absence of disruption to the intestinal microbiota, kittens are resistant to clinical signs of aEPEC carriage but demonstrate significant occult changes in intestinal absorption and permeability. Antibiotic-induced disruption of the intestinal microbiota prior to infection increases subsequent intestinal water loss as determined by % fecal wet weight. Enrichment of the intestinal microbiota with a commensal member of the feline mucosa-associated microbiota, *Enterococcus hirae*, ameliorated the effects of aEPEC experimental infection on intestinal function and water loss. These observations begin to unravel the mechanisms by which aEPEC infection may be able to exploit susceptible hosts.

1. Introduction

Diarrhea is responsible for the death of an estimated 500,000 children per year worldwide with the greatest mortality occurring in developing countries (Kirk et al., 2015; Kotloff et al., 2013; Liu et al., 2016). Diarrhea in most of these children is caused by a mere handful of infectious agents (Kirk et al., 2015; Kotloff et al., 2013). In particular, enteropathogenic *E. coli* (EPEC) is responsible for over 81 million cases of diarrhea per year of which 17 million cases are diagnosed in children (Kirk et al., 2015). Typical enteropathogenic *Escherichia coli* (tEPEC) is a

common bacterial cause of diarrhea in children and, compared to other common enteropathogens, is associated with the highest hazard of death (Kotloff et al., 2013). While tEPEC is largely undisputed as a cause of diarrhea, the pathogenicity of atypical EPEC (aEPEC), which lack the plasmid responsible for expression of bundle forming pili, is confounded by frequent observation in children with and without signs of diarrhea (Barletta et al., 2011; Enserink et al., 2014; Estrada-Garcia et al., 2009). A rising incidence of aEPEC infection in both developing and developed countries has renewed controversy over the pathogenicity of aEPEC in children (Hu and Torres, 2015).

Similar to children in developing countries, there exists a high mortality rate associated with diarrheal disease in young kittens residing in animal shelters across the United States and elsewhere (Ghosh et al., 2013; Murray et al., 2008). We recently identified that shelter kittens have a high prevalence of aEPEC carriage (Ghosh et al., 2013; Watson et al., 2017). The kitten carriage mirrors observations of aEPEC carriage in children by being observed in both sick and healthy individuals but with a significantly higher bacterial burden in individuals with diarrhea as compared to those with asymptomatic infection (Barletta et al., 2011; Watson et al., 2017). For both children and kittens it remains unclear as to what extent, and under what conditions, or by what mechanisms aEPEC serves as a primary cause of diarrhea. It seems likely that some combination of bacterial and host factors enable aEPEC to access the intestinal epithelium and thereupon promote pathogenicity in some hosts and not others. Directly pathogenic effects of EPEC are believed to commence when these bacteria attach to and efface the microvilli of intestinal epithelial cells and inject effector proteins into the epithelial cells resulting in disrupted regulation of paracellular permeability and ion transport mechanisms (Glotfelty et al., 2014; Hu and Torres, 2015; McNamara et al., 2001; Thanabalasuriar et al., 2010). While the cellular mechanisms have been well characterized *in vitro*, the pathogenesis of the infection *in vivo* is less well understood. Kittens with diarrhea-related death which test positive for aEPEC have more severe inflammation and epithelial injury within the small intestine and more often require parenteral fluid therapy compared to kittens with diarrhea-related death and negative test results for aEPEC (Watson et al., 2017).

We have previously demonstrated, in an autopsy study of kitten mortality, that aEPEC colonize the intestinal epithelium and this is associated with changes in the predominant species of the mucosa-associated enterococci (Ghosh et al., 2013). Specifically, prevalence of a major commensal species, *Enterococcus hirae*, was replaced by *Enterococcus faecalis*. Experimental animal models of EPEC infection similarly suggest a strong influence of the microbiome and specific members of the microbiome on disease outcome (Vong et al., 2015; Walsham et al., 2016; Wlodarska et al., 2011). Accordingly, the purpose of this study was to determine if aEPEC is sufficient to cause diarrhea or intestinal dysfunction in kittens with experimentally-induced infection and the influence of a disrupted intestinal microbiota on disease susceptibility. We additionally examined the effect of a probiotic formulation of feline *Enterococcus hirae* on the observed manifestations of aEPEC-induced disease. Our rationale was that identification of aEPEC as a primary cause of intestinal disease in kittens could provide a unique opportunity for development of life-saving treatment strategies for both kittens and possibly children with aEPEC infection. In particular, demonstration of a positive impact of commensal bacteria in treatment or prevention of aEPEC-associated disease would be of immediate benefit to improving the burden of diarrhea in infected kittens.

2. Materials and methods

2.1. Kitten acquisition and screening

Six-week-old kittens of either sex were obtained from a commercial vendor. A fecal sample was obtained from each kitten and sent to a commercial laboratory (IDEXX Laboratories Inc, Westbrook, Maine) for diagnostic qPCR testing to detect feline coronavirus, *Tritrichomonas foetus*, *Cryptosporidium spp*, feline panleukopenia virus, *Clostridium perfringens* alpha toxin and enterotoxin, *Giardia spp*, *Salmonella spp*, *Toxoplasma gondii*, *Campylobacter jejuni*, and *Campylobacteri coli*.

Individual kittens were housed in separate cages and fed a commercially available dry food formulated for cats (Hill's® Science Diet kitten healthy development; Topeka, Kansas) *ad libitum* throughout the study. Each kitten also received ¼ teaspoon of canned cat food (Hill's® Science Diet a/d) each morning. Water, which cultured negative for *E. coli* prior to kittens' arrival, was provided *ad libitum*. Kittens were
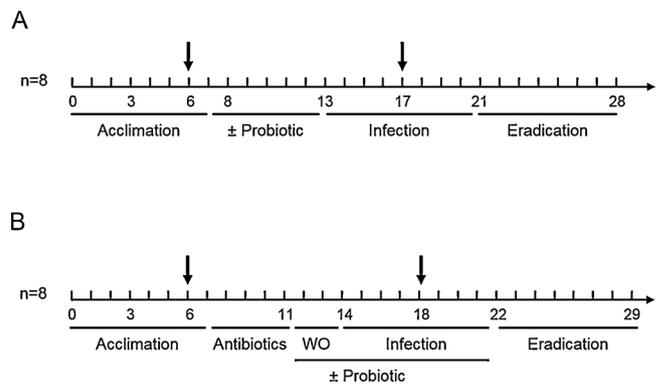

Fig. 1. Timeline (in days) for studies of experimental aEPEC infection in kittens. Panel A illustrates the timeline of kittens that did not receive antibiotics prior to aEPEC infection (Group A). Panel B illustrates the timeline of kittens that received antibiotics prior to aEPEC infection (Group B). Arrows denote specific days on which intestinal function testing was performed. WO = wash out period during which antibiotic administration was discontinued prior to aEPEC infection.

housed under conditions of controlled light cycle and ambient temperature and were handled in compliance with biosafety level 2 guidelines. Kittens were acclimated to this new environment for 5 days. Kittens were cared for in accordance with the Guide for the Care and Use of Laboratory Animals in AAALAC-accredited facilities at the North Carolina State University. The study was approved by the North Carolina State University Institutional Animal Care and Use Committee. No animals were euthanized for the purpose of the study.

2.2. Influence of antibiotic treatment

To determine the impact of an altered gut microbiota on susceptibility to aEPEC-associated intestinal disease, two separate groups of kittens were studied a shown in Fig. 1. Group A included eight kittens that did not receive antibiotics prior to experimental infection with aEPEC. After completion of the study in Group A kittens, a second group (Group B) of eight kittens were administered oral antibiotics prior to experimental infection with aEPEC. The antibiotics administered were amoxicillin/clavulanate (12.5 mg/kg twice daily) (Zoetis, Parsippany, New Jersey) and pradofloxacin (7.5 mg/kg once daily) (Bayer, Shawnee Mission, Kansas) given orally for 5 consecutive days followed by a washout period of 2 days prior to experimental infection. These antibiotics were selected to broadly disrupt the native gut microbiota by providing broad-spectrum activity against aerobic and anaerobic Gram-positive and Gram-negative bacteria.

2.3. Experimental aEPEC infection

As this study was an exploratory study on a bacterium of unknown pathogenicity in cats, we included 3 serologically and genetically distinct isolates of feline aEPEC in our experimental infection. Our rationale for selecting these 3 aEPEC was that they were each isolated from a kitten that died with diarrhea (Watson et al., 2017), selecting 3 should increase the likelihood of infection, and exposure to multiple isolates reflects a more natural setting of infection. Each isolate was confirmed to be *E. coli* by means of matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) (VITEK® MS; Biomérieux, Marcy l'Etoile, France) and identified as aEPEC by demonstrating the presence of *eae* and absence of genes encoding bundle-forming pilus and Shiga-toxins 1 and 2 (DebRoy and Maddox, 2001). Isolates were stored in 50% lysogeny broth (LB)-glycerol at −80 °C. Prior to use in experimental infection, each isolate was grown separately overnight on LB agar at 37 °C. A single colony from each plate was individually suspended in LB broth and grown overnight. The following morning, an

overnight culture of each isolate was individually sub-cultured at 1:25 concentration in LB and incubated on a shaker platform at 37 °C until an OD_{600} of 0.4 was obtained (~ 10^8 CFU/ml). To prepare the inoculum for each kitten, a one-milliliter aliquot of each isolate (3 ml total) was combined in a conical tube and centrifuged at 2000 × g for 15 min. The supernatant was removed and the pellet was reconstituted with 100 μl of sterile water. The 100 μl aliquot of aEPEC was then added to ¼ teaspoon of canned cat food which was offered to each kitten and promptly ingested. A tandem mixture of the isolates was simultaneously prepared for serial dilutions and plate counting to confirm the CFU/ml of the inoculum used for infection.

2.4. Characterization of aEPEC isolates used for experimental infection

Genomic DNA from each aEPEC isolate was examined by PCR for the presence of virulence-associated genes spanning the locus of enterocyte effacement (LEE). Included were genes that encode proteins of the T3SS, including an ATPase (*escN*) and a translocated protein (*escV*) and the LEE regulator (*ler*). Also selected for PCR was the non-LEE gene lymphocyte inhibitory factor (*lifA*) (otherwise known as enterohemorrhagic *E. coli* (EHEC) factor for adherence (*efa1*)). All conventional PCR assays were performed using previously published primer sequences and reaction conditions (Hazen et al., 2013; Kyaw et al., 2003; Narimatsu et al., 2010; Sharma and Zuerner, 2004). PCR products were separated by electrophoresis on 1.5% agarose gels stained with either ethidium bromide or commercially available DNA stain (GelRed, Biotium, Fremont, CA) and visualized using a UV imager (Bio-Rad, Hercules, CA or UVP LLC, Upland, CA).

Antimicrobial susceptibility testing was performed by the Clinical Microbiology and Molecular Diagnostics Laboratory at North Carolina State University. The minimum inhibitory concentration of each *E. coli* isolate was determined by microbroth dilution using a commercial platform (COMPAN2F plates; Trek Sensitre, ThermoFisher Scientific) and manufacturer-provided methodology. Clinical interpretations were made according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI VET01S).

2.5. Establishing a dose for experimental aEPEC infection

An inoculum of 10^8 cfu for each aEPEC used in experimental infections was chosen based on estimated burden of aEPEC in kittens with diarrhea, survival of aEPEC under simulated gastric conditions, and previously published inoculum sizes. We used qPCR data obtained from amplification of *eae* from the fecal DNA of naturally infected kittens and its application to a previously developed standard curve for the relationship between the cycle threshold (C*t*) of *eae* and CFU of aEPEC (Watson et al., 2017) to estimate the burden of aEPEC carriage in kittens with diarrhea. To determine the influence of the gastric environment on survival of aEPEC, growth of the aEPEC isolates was quantified under conditions designed to simulate the volume, acidity, ion composition, and pepsin concentration of feline gastric contents (Chandler et al., 1997). Briefly, a mixture of all 3 aEPEC isolates was inoculated into each of two 50 ml conical tubes containing 5 milliters of buffer. The buffer consisted of 2 solutions (0.2 M KCl and 0.2 M HCl) that were combined to achieve a target pH of 2 followed by the addition of 0.05 mg/ml pepsin. To replicate the vehicle intended for use in delivery of the experimental aEPEC inoculum, ¼ teaspoon of canned cat food (Hill's® Science Diet a/d) was included in each tube. The solution was incubated in a shaking water bath (30 RPM) at 37 °C for 2 h to simulate gastric temperature and emptying time (Chandler et al., 1997). Serial dilutions of the original inoculum and aliquots obtained after "infection" of the simulated gastric environments were plated and bacteria colonies counted to establish the CFU/ml of aEPEC prior to and after incubation in the gastric environment. Isolates obtained post-incubation were not identified by pulsotype or serotype.

2.6. Clinical disease assessment

Kittens were examined daily for clinical signs of illness including measurement of body weight, food consumption, and fecal consistency. Rectal temperature and heart rate were obtained every other day. Fecal consistency was scored using published criteria (Nestlé-Purina, St. Louis, Missouri) with scores ranging from 1 (very hard and dry) to 7 (watery with no texture). Fecal wet weight, a quantifiable measurement of fecal water content, was determined by obtaining an initial fecal weight followed by desiccation of feces at 60 °C for 24 h. Dried feces were weighed and the equation $\frac{Wi - Wd}{Wi} x\ 100$ ($i=$ initial fecal weight and $Wd=$ dried fecal weight) was used to calculate a % fecal wet weight.

2.7. Culture and molecular quantification of fecal aEPEC shedding

Each kitten had a fecal culture performed every other day for detection of aEPEC shedding. Fecal material was diluted in sterile PBS and plated on MacConkey agar. Eight morphologically representative *E. coli* colonies were subcultured onto LB agar plates and biochemical tests including indole, pyrrolidonyl arylamidase, and oxidase (Becton, Dickinson and Company, Franklin Lakes, NJ) were used to presumptively identify bacteria as *E. coli*. Each of the eight *E. coli* isolates was frozen in 50% LB-glycerol freezing media and stored at −80 °C. The DNA was evaluated for *eae* using qPCR with previously published primers and reaction conditions (Franck et al., 1998). Three EPEC isolates obtained from each kitten on day 3 post-infection were further characterized using pulsed-field gel electrophoresis. Molecular detection of aEPEC, by means of qPCR amplification of *eae* from fecal DNA, was performed every other day prior to infection and daily for 7 days post-infection. DNA extractions were performed using a commercial kit (Zymo Research Corp. Irvine, CA) and stored at −80 °C prior to use.

2.8. Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis was performed according to the PulseNet protocol. Briefly, overnight cultures of aEPEC were equilibrated in cell suspension buffer solution to an absorbency of 1.08–1.1 at a wavelength of 610 nm and mixed with 1% Seakem gold agarose (Thermo Fisher Scientific, Waltham, MA) for plug formation. The plugs were sectioned to an equal size with a width of 2-mm and then individually digested with restriction enzyme *XbaI* (New England Biolabs, Ipswich, MA). Plugs were embedded into an agarose gel and restriction fragments were separated by electrophoresis at 6 V/cm for 19 h. Standardization across gels was accomplished by including *Salmonella enterica* serotype Braenderup H9812 digested with *XbaI* in each gel. Gels were stained with a commercially available DNA stain (GelRed, Biotium, Fremont, CA) following the manufacturer's protocol and imaged with a UV imager (Bio-Rad, Hercules, CA). Gel images were subsequently inserted into and analyzed by BioNumerics software (Applied Maths, Inc., Austin, TX).

2.9. Intestinal function and permeability testing

Intestinal function testing was performed on each kitten on day 6 of acclimation and again on day 4 post-infection with aEPEC as shown in Fig. 1. At each time, kittens were lightly anesthetized by mask inhalation of isofluorane gas and then orogastrically intubated for administration of a mixture of iohexol (Klenner et al., 2009) and D-xylose (Eberts et al., 1979). Doses used for iohexol and D-xylose were extrapolated from previously published studies (Klenner et al., 2009; Nix et al., 1993). The final mixture contained 2 ml of a 350 mg/ml solution of iohexol (Omnipaque, GE Healthcare, Princeton NJ), 0.5 g of D-xylose (Sigma-Aldrich Corp., St. Louis, MO), and sufficient sterile water to achieve a total solution volume of 11 ml. At 1.5 h post-gavage, kittens were again lightly anesthetized by mask inhalation of isofluorane gas

and a 1 ml whole blood sample was collected by jugular venipuncture. Blood samples were allowed to clot for 30 min and then centrifuged at 2000 × g for 20 min to separate the serum from the red blood cells. Serum was stored at −20 °C prior to batch assay.

2.10. D-xylose absorption and iohexol permeability assays

Serum D-xylose concentrations were measured using a phloroglucinol assay as previously described (Eberts et al., 1979). The colorimetric reagent was composed of 0.25 g phloroglucinol (Sigma-Aldrich, Corp., St. Louis, MO), 50 ml of glacial acetic acid, and 5 ml HCl (Thermo Fisher Scientific, Waltham, MA). Twenty microliters of serum from each kitten was mixed with 2 ml of the color reagent and maintained at 100 °C for 4 min and then absorbency at 554 nm was measured using a spectrophotometer (BioTek Synergy 2, Biotek US, Winooski, VT). Standard dilutions of D-xylose in feline serum (Immunoreagents, Raleigh, NC) ranging from 3.125 mg/dl to 100 mg/dl were used to create a standard curve for each reaction. The direct effect of aEPEC bacteria on D-xylose concentration was examined by co-culture of aEPEC (10^8 CFU each) in fresh LB containing D-xylose (5 mg/ml) at 37 °C for 2 h in triplicate. D-xylose remaining after incubation with aEPEC was measured using the phloroglucinol assay. The effect of glucose on the D-xylose assay was determined by performing the assay on feline serum with standard dilutions of glucose. A commercially available ELISA (BioPhysics Assay Laboratory Inc. Worcester, MA) was used to determine serum iohexol concentrations following the manufacturer protocol (Frias et al., 2012; Klenner et al., 2009).

2.11. Eradication of aEPEC from experimentally infected kittens

After a 1 week duration of post-infection observation, kittens were treated with oral pradofloxacin (7.5 mg/kg) (Bayer, Shawnee Mission, Kansas) once a day for 7 days to eliminate aEPEC carriage. At the end of the antibiotic treatment, absence of shedding of aEPEC was established by negative results of fecal culture for aEPEC.

2.12. Impact of oral administration of commensal feline Enterococcus hirae

For each experimental group of 8 kittens, individuals were randomly and equally divided into two separate rooms containing 4 kittens each. Kittens in one room received a probiotic while those in the other received placebo/vehicle. The investigators were blinded as to which room of kittens received the vehicle versus the probiotic. The administered probiotic was manufactured from a single, well-characterized isolate of feline *Enterococcus hirae*. The isolate (1002-2) was cultured from the mucosa-associated flora of the ileum from a healthy kitten in which histological evidence of *E. hirae* attachment to the intestinal epithelium was documented. The isolate lacked all virulence characteristics, the results of which were previously reported (Ghosh et al., 2013).

The selected isolate of feline *E. hirae* was prepared for use as a probiotic by culturing in Biostat B-plus reactors fed with MB medium containing 20 g/L glucose using a variable speed pump (Bruno-Barcena et al., 1998). The dilution rate (D = 0.17 h^{-1}), temperature (37 °C), and pH (5.5) were maintained. The bacteria were collected from the reactor overflow and concentrated in PBS at pH = 7. Glucose consumption and accumulation of lactate and other fermentation products were monitored with high-performance liquid chromatography (Shimadzu Corporation, Kyoto, Japan) performed under isocratic conditions at 65 °C, a mobile phase of 5 mM sulfuric acid (H_2SO_4) at a 0.4 ml/min flow rate using an Alltech IOA-1000 organic acids column (300 mm x 7.8 mm, Alltech, IL, USA), and coupled to a refractive-index detector. Numbers of viable cells were confirmed by plating on M17 agar and incubating at 37 °C for 72 h. For each sample, three plates were prepared from each dilution and recorded as the average of three independent counts. Finalized probiotic and vehicle (bacteriological peptone) were stored at

-80 °C.

Aliquots containing 10–15 mg of probiotic powder (2.85–4.28 × 10^8 CFU of *E. hirae*) or vehicle were prepared under sterile conditions on a single day and stored at −80 °C until needed. One extra aliquot was prepared and plated to confirm CFU count. To confirm survival in a gastric environment, 2 additional aliquots were incubated in a simulated gastric environment as described for aEPEC. On each morning of administration, the aliquots were thawed, mixed with 100 μl of sterile water, and inoculated into ¼ teaspoon of canned cat food. The timing of administration of *E. hirae* or vehicle in relation to other interventions in the study of each experimental group is shown in Fig. 1.

2.13. Confirmation of E. hirae colonization

Fecal culture for isolation of enterococci was performed every other day over the duration of the study. Fecal material was mixed with sterile PBS and plated on m-Enterococcus (BD Difco™, Fisher Scientific, Waltham MA) plates. Five individual isolates of enterococci were subcultured and each isolate was frozen in 50% LB-glycerol and stored at −80 °C. DNA from each isolate was liberated from bacteria by heating at 100 °C for 30 min. Enterococci were identified as *E. hirae* by means of PCR using primers targeting the transglycosylase gene sequence specific to the species (*murG*) (Arias et al., 2006). Quantitative PCR for *murG* was performed to determine the amount of *E. hirae* DNA present in fecal DNA from each kitten. Amplifications were performed with an initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 2 min, and extension at 72 °C for 3 min.

2.14. Statistical analyses

Data were tested for normal distribution (Kolmogorov-Smirnov) and equal variance (Leven median test) and analyzed using parametric or nonparametric statistics as appropriate. Significant differences in proportion of observations between groups was analyzed using Chi-square and Fisher exact tests. Differences in the mean or median values of continuous data between groups of kittens were analyzed using Student's t-tests or Mann-Whitney rank sum test, respectively. Changes in absorption of D-xylose, permeability to iohexol, and % fecal wet weight between baseline and post-infection measurements were analyzed using Student's paired t-test. Analyses were conducted using commercially available software (Sigmaplot 12, Systat Software, Inc., San Jose, CA or Prism 7, GraphPad Software Inc., La Jolla, CA) and significance was assigned a p-value of < 0.05.

3. Results

3.1. Establishment of infectious dose and simulated gastric survival of aEPEC

Using previously reported qPCR cycle threshold values for amplification of *eae* from the feces of 18 kittens with aEPEC carriage (Watson et al., 2017), we estimated that naturally infected kittens shed a range of 3.5 × 10^3 CFU to 2.0 × 10^8 CFU (median of 1.8 × 10^6 CFU) of aEPEC per 100 mg feces. Based on these findings, an inoculation dose of 10^8 CFU per aEPEC isolate was chosen for use in experimental infections. A mixture containing all 3 experimental aEPEC isolates maintained bacterial quantity over a 2 h incubation time in the simulated gastric environment (average CFU pre-incubation = 3.3 10^7, post-incubation = 2.95 × ×10^7).

3.2. Pre-existing intestinal infections and aEPEC carriage in vendor-source kittens

During pre-experimental screening, 4/8 (50%) kittens in Group A

PCR Detection of virulence genes

Isolate ID	Serotype	Pulsotype	efa1/lifA	escN	escV	ler
5-1	O108:H21		-	+	+	+
53-1	O153:H21		-	+	+	+
33-2	O111:H8		-	+	+	+
K5-1	O88:H-		-	-	+	-
K15-1	O128:H2		-	-	+	-

Fig. 2. Designation, serotype, pulsed-field gel electrophoresis pulsotype, and results of PCR for virulence gene content of the three isolates of kitten aEPEC used for experimental infection (5-1, 53-1, and 33-2) and two isolates of aEPEC carried by kittens at the time of purchase from a commercial vendor (K5-1 and K15-1).

were identified with pre-existing (vendor-origin) aEPEC carriage based on results of fecal culture and in an additional 2 kittens based on PCR amplification of *eae* in fecal DNA. In effort to eliminate pre-existing aEPEC carriage in Group B kittens, queens and their litters were proactively screened by PCR to confirm absence of *eae* in fecal DNA prior to purchase from the vendor. Despite obtaining kittens from only *eae*-negative queens, 2/8 (25%) Group B kittens were confirmed to be culture positive for aEPEC on arrival (Supplementary Table 1).

Kittens in Group A shared a different pulsotype and serotype of vendor-origin aEPEC compared to kittens in Group B. Both of the "vendor-origin" aEPEC isolates differed in pulsotype and serotype from the isolates used for experimental infection (Fig. 2). Apart from identification of subclinical aEPEC carriage, all kittens were considered healthy and no abnormalities were observed on clinical examination. The median weight of kittens on arrival was 0.69 kg (range 0.5 to 0.83 kg). Fecal DNA from each kitten tested negative for feline coronavirus, *Tritrichomonas foetus*, *Cryptosporidium spp*, feline panleukopenia virus, *Clostridium perfringens* alpha toxin and enterotoxin, *Giardia spp*, *Salmonella spp*, *Toxoplasma gondii*, *Campylobacter jejuni*, and *Campylobacter coli*.

3.3. Experimentally infected kittens shed live aEPEC

Following experimental infection, all kittens shed aEPEC, as determined both by direct isolation of aEPEC colonies from feces and by qPCR amplification of *eae* from fecal DNA (Fig. 3). Using the cycle threshold values for *eae* to estimate CFU of aEPEC, Group A kittens shed significantly higher CFU of aEPEC in feces on day 3 post-infection (median, 1.25×10^7; range, $7.64 \times 10^5 - 6.31 \times 10^7$) compared to Group B kittens (median, 2.74×10^5; range, $6.64 \times 10^4 - 2.04 \times 10^6$) (P = 0.002; Mann-Whitney rank sum test).

3.4. Kittens shed only one pulsotype of aEPEC following experimental infection

Although three different pulsotypes of aEPEC were administered to each kitten for induction of experimental infection, only one of these pulsotypes (isolate 53-1, Fig. 2) was subsequently shed. Among the 6 kittens in Group A that also harbored a vendor-origin aEPEC (K5-1) prior to experimental infection, 2 kittens shed only the vendor-origin aEPEC pulsotype after the experimental infection, 2 kittens shed both the vendor-origin pulsotype as well as isolate 53-1, and 2 kittens shed only isolate 53-1. Despite the carriage of a vendor-origin aEPEC (K15-1) by 2 kittens in Group B at the time of purchase, all kittens in this group received antibiotics prior to experimental infection after which only the dominant experimental isolate 53-1 was shed.

3.5. Kittens with unaltered intestinal microbiota remain clinically asymptomatic after experimental aEPEC infection

Group A kittens did not receive antibiotics prior to aEPEC infection (Fig. 1A). Experimental infection of Group A kittens with aEPEC did not result in significant changes in food consumption, body weight gain, rectal temperature, or heart rate (Supplementary Fig. 1). No significant changes in fecal consistency score or percent fecal wet weight were observed in Group A kittens following experimental aEPEC infection (Fig. 4A).

3.6. Experimental infection of kittens with aEPEC results in significant occult changes in intestinal function

Despite an absence of changes in fecal consistency score or wet weight, experimental aEPEC carriage in Group A kittens was associated with a significant decrease in intestinal absorption of D-xylose and a significant decrease in intestinal permeability to iohexol (Fig. 5A and 5B). Importantly, direct culture of aEPEC with D-xylose (5 mg/ml) for 2 h *in vitro* did not result in a decrease in D-xylose concentration (post-
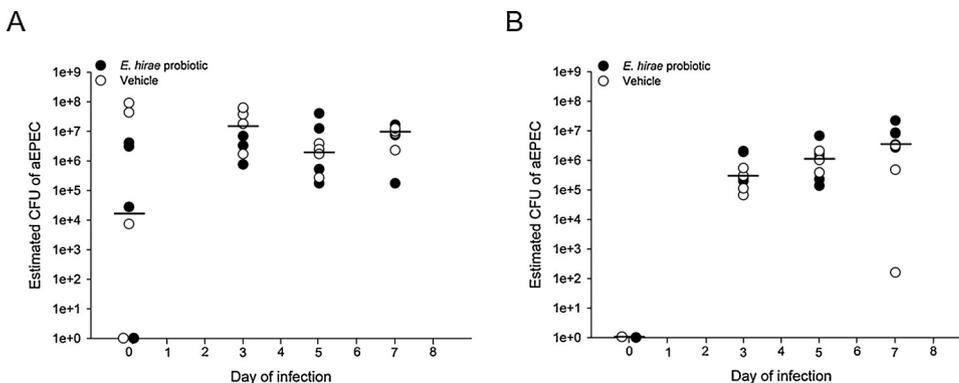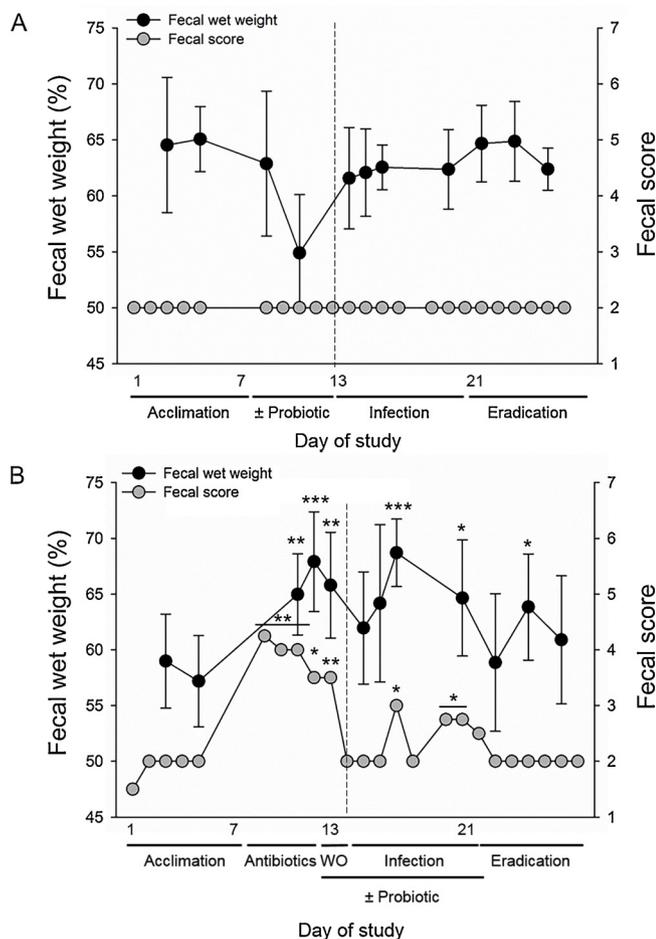
A

B

Fig. 3. Carriage of aEPEC in kittens following experimental infection. Quantification of aEPEC was performed using qPCR to amplify *eae* from fecal DNA. Results for *eae* were applied to a standard curve relating Ct to CFU of aEPEC to estimate infection load in each kitten at each time point (Watson et al., 2017). Panel A data represent kittens in Group A that did not receive treatment with antibiotics prior to experimental infection. Note on day 0 of infection, six kittens were identified as harboring a vendor-origin aEPEC. Panel B data represent kittens in Group B that received pre-treatment with antibiotics prior to experimental infection. Black circles = kittens that received probiotic; white circles = kittens that received vehicle only.

Fig. 4. Pretreatment with antibiotics to disrupt the intestinal microbiota impacts the development of clinical signs following experimental aEPEC infection. Panel A data represent kittens not treated with antibiotics prior to experimental aEPEC infection. Panel B data represent kittens pretreated with antibiotics to disrupt the intestinal microbiota prior to experimental aEPEC infection. Graphs represent mean ± standard deviation % fecal wet weight (black circles) and median fecal score (gray circles) of kittens over the defined periods of study shown on the x-axis. Dashed vertical line indicates the day of infection. Missing data-points reflect times of intestinal function assay. For panel B, all values were compared to values on day 5 of acclimation. Fecal scores were compared using Wilcoxon Rank Sum and percent wet weight of feces were compared using Student's paired t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

aEPEC incubation D-xylose, 4.7 and 4.8 mg/ml) and serum glucose concentration had no significant impact on D-xylose assay results (Supplementary Fig. 2).

3.7. Kittens pretreated with antibiotics to disrupt the intestinal microbiota prior to aEPEC infection become susceptible to diarrhea

To determine if perturbation of the resident intestinal microbiota increases susceptibility of kittens to aEPEC-associated clinical disease, Group B kittens were treated with antibiotics for 5 days followed by a 2-day washout period prior to experimental infection with aEPEC (Fig. 1B). Fecal aerobic cultures performed during antibiotic administration confirmed an absence of any Gram-negative bacteria and diminished numbers of enterococcus bacteria were observed when feces were cultured on m-Enterococcus agar. During treatment with antibiotics, significant increases in both fecal consistency score and percent wet weight of feces were observed. These measurements returned to normal over the 2-day antibiotic washout period prior to introduction of experimental aEPEC infection (Fig. 4B).

Similar to Group A kittens, infection of kittens in Group B with aEPEC had no significant effect on food consumption, body weight gain, rectal temperature, or heart rate (Supplementary Fig. 3). However, significant increases were observed in both fecal score and percent wet weight of feces after administration of aEPEC (Fig. 4B). Group B kittens also had decreases in absorption of D-xylose and permeability to iohexol after administration of aEPEC, however these changes were not statistically significant (Fig. 5C and D).

3.8. Oral administration of a probiotic formulation of feline E. hirae significantly increases culture and qPCR detection of E. hirae in feces

We have previously shown that *E. hirae* is the dominant member of the mucosa-associated enterococci in the small intestine of healthy kittens (Ghosh et al., 2013). To determine the specific influence of *E. hirae* on susceptibility of kittens to aEPEC-associated disease, both groups of kittens were randomized to receive either a probiotic formulation of *E. hirae* or vehicle. Prior to administration of the probiotic, each kitten's feces were determined to be culture negative for *E. hirae* but qPCR positive for the presence of the species-specific *murG* gene of *E. hirae*. In Group A kittens, probiotic or vehicle were administered orally once a day for 5 days prior to aEPEC infection (Fig. 1A). Kittens that received the probiotic shed cultivable *E. hirae* in feces and had a significantly greater quantity of *murG* in feces compared to kittens that received only the vehicle (Fig. 6). On day 5 of probiotic administration, 3 of the 4 kittens receiving the probiotic were fecal culture positive for *E. hirae* with an average of 4 (range 0–5) out of 5 enterococcus isolates confirmed as *E. hirae*. In comparison, none of the kittens receiving the vehicle were fecal culture positive for *E. hirae*. In Group B kittens, probiotic or vehicle were administered orally once a day for 9 days beginning immediately after antibiotic-induced disruption of the intestinal microbiota and continuing throughout the period of aEPEC carriage (Fig. 1B). Results of *murG* qPCR and fecal culture for *E. hirae* in these kittens was highly variable (Supplementary Fig. 4). On day 5 of probiotic administration, 3 of the 4 kittens receiving the probiotic were fecal culture positive for *E. hirae* with an average of 2 (range 0–4) out of 5 enterococcus isolates confirmed as *E. hirae*. In comparison, 2 of the kittens receiving the vehicle were culture positive for *E. hirae* with an average of 1 (range 0–3) out of 5 enterococcus isolates confirmed as *E. hirae*. No adverse health effects such as changes in food intake, body weight, rectal temperature, or heart rate were observed to differ between kittens administered *E. hirae* compared to those that received vehicle.

3.9. E. hirae promotes a decrease in intestinal permeability and ameliorates fecal water loss in kittens with aEPEC carriage

Kittens that were administered *E. hirae* had either a significantly greater decrease in intestinal permeability to iohexol (Group A; Fig. 7A) or lower permeability to iohexol at the post-infection time-point (Group B; Fig. 7B) compared to kittens that received only the vehicle. No differences were observed in the absorption of D-xylose after infection with aEPEC between kittens receiving *E. hirae* versus vehicle. In Group B kittens that were pretreated with antibiotics to disrupt the intestinal microbiota prior to aEPEC infection, those that received the *E. hirae* probiotic had a non-significantly lower fecal wet weight (mean ± SD = 67 ± 2%) on day 3 post-infection compared to kittens that received the vehicle (70 ± 3%) (P = 0.055; Students't-test) (Fig. 7C). The effects of *E. hirae* on intestinal permeability and fecal wet weight were not associated with any change in the apparent burden of aEPEC carriage based on estimated CFU in feces (Fig. 3).

3.10. Experimental aEPEC carriage is eradicated by treatment with pradofloxacin

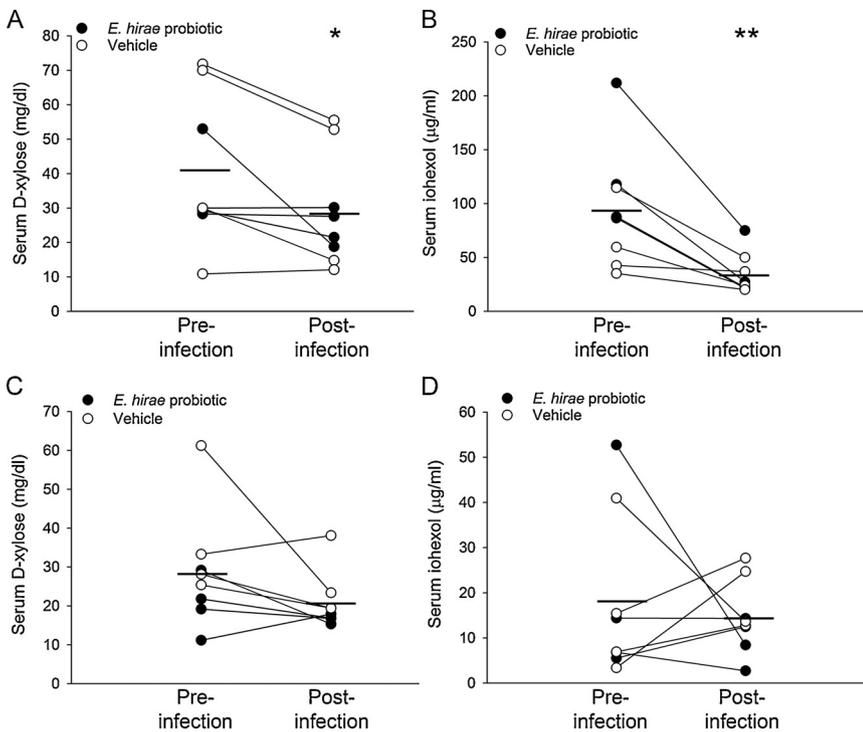On day 8 following aEPEC infection, all kittens were treated orally

Fig. 5. Effect of experimental aEPEC carriage on intestinal absorption of D-xylose and permeability to iohexol in kittens following oral administration at time points prior to infection (acclimation day 6) and on day 4 post infection. Panel A and B data represent the kittens with unaltered intestinal microbiota (group A). Panels C and D data represent the kittens that were pretreated with antibiotics to disrupt the intestinal microbiota prior to infection with aEPEC (group B). Data points represent individual kittens. Mean value of the data at each time point is represented by a horizontal bar. *P < 0.05, **P < 0.01, Student's paired t-test. Black circles represent kittens that received probiotic and white circles represent kittens that received vehicle only. The y-axis scale for iohexol permeability in group B kittens (panel D) reflects a lower baseline permeability in this group of kittens compared to kittens in Group A (panel B).
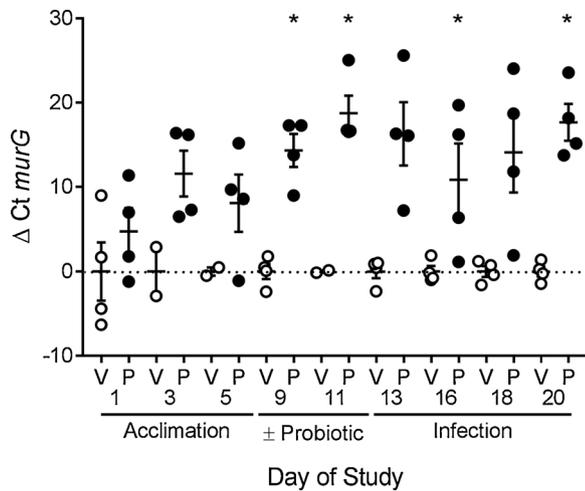

Fig. 6. Effect of probiotic administration on quantity of *E. hirae* in feces as determined by qPCR assay for presence of the *E. hirae* specific gene, *murG*, in fecal DNA. Each data point represents an individual kitten's Ct value compared to the average Ct value of kittens receiving vehicle at the same time point. Black circles represent kittens that received probiotic and white circles represent kittens that received vehicle (V = vehicle, P = probiotic). Experimental time-course is demonstrated along the x-axis. *P < 0.05; Mann-Whitney Rank Sum test.

once a day with the fluoroquinolone pradofloxacin (7.5 mg/kg, Bayer, Shawnee Mission, Kansas) for a duration of 7 days. Pradofloxacin, a fluoroquinolone, was selected based on antimicrobial susceptibility of all 3 aEPEC isolates to fluoroquinolones (i.e. enrofloxacin and marbofloxacin)(supplementary Table 2). On the 7th day of antibiotic treatment, fecal aerobic cultures were negative for presence of aEPEC. Likewise, no *eae* was amplified from the fecal DNA of any kitten at this time point.

4. Discussion

Results of this study have demonstrated that kittens experimentally-infected with aEPEC have sustained shedding of aEPEC and in quantities similar to that observed in naturally infected kittens (Watson et al., 2017) and children (Barletta et al., 2011) with diarrhea. A major objective of this study was to determine the pathogenicity of aEPEC in kittens. In the absence of an imposed disruption of the intestinal microbiota, healthy purpose-bred kittens (Group A) remained clinically asymptomatic after experimental aEPEC infection. In contrast, kittens pretreated with antibiotics to disrupt the intestinal microbiota (Group B) demonstrated significant increases in fecal scores and wet weights consequent to administration of aEPEC. These observations suggest an important role of the intestinal microbiota in influencing susceptibility to disease associated with aEPEC administration. These findings are similar to those observed in mice infected with the EPEC related
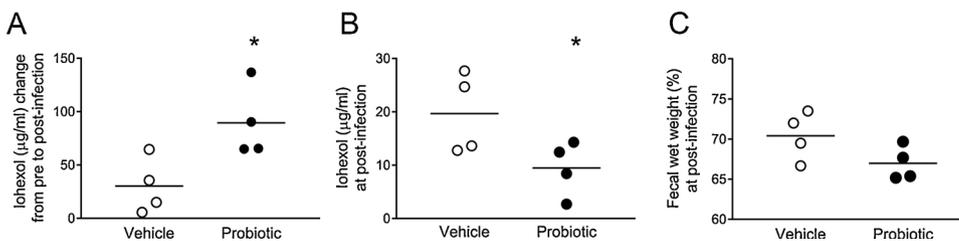

Fig. 7. Impact of *E. hirae* administration on intestinal function and % fecal wet weight of kittens experimentally infected with aEPEC. Panel A demonstrates the magnitude of decrease in intestinal permeability to iohexol in Group A kittens (undisrupted microbiota) that received vehicle versus *E. hirae* prior to infection with aEPEC (raw data shown in Fig. 5B). Panel B demonstrates intestinal permeability to iohexol at the post-infection time-point in Group B kittens (antibiotic-disrupted microbiota) (raw data shown in Fig. 5D). Panel C demonstrates fecal wet weights on day 3 of infection in Group B kittens treated with vehicle versus *E. hirae*. Black circles represent kittens that received probiotic and white circles represent kittens that received vehicle only. Bar represents the mean; *p < 0.05 compared to vehicle treated kittens (Student's unpaired t-test).

pathogen, *Citrobacter rodentium*, where pretreatment with antibiotics increases the severity of infection and this is associated with greater numbers of bacteria adhering to the epithelium (Wlodarska et al., 2011). We have previously shown that aEPEC adhere to the intestinal epithelium of kittens that have died from diarrhea (Ghosh et al., 2013; Nicklas et al., 2010; Watson et al., 2017). However, we did not examine adherence of aEPEC in the kittens in this study because they did not undergo euthanasia. Increases in intestinal water loss by these kittens could reflect a non-specific effect of *E. coli* administration, or alternatively the instigation of diarrheal mechanisms dependent on bacterial attachment. These mechanisms include inactivation of SGLT-1, inhibition of both NHE3 and the Cl^-/HCO_3^- exchanger SLC26A3, and internalization of aquaporins, resulting in impaired sodium, chloride, and water absorption by intestinal epithelial cells (Dean et al., 2006; Gill et al., 2007; Guttman et al., 2007; Hodges et al., 2008).

In addition to an impact of the intestinal microbiota in determining disease susceptibility, it is noteworthy that only one experimental isolate (53-1; O153:H21) established carriage in these kittens. Additionally, in a subset of kittens identified as harboring their own "vendor-origin" aEPEC, that isolate (K5-1; O88:H-) could outcompete the experimentally-introduced strains. In comparing the presence of conserved genes spanning the locus of enterocyte attachment and effacement (*escN*, *escV*, *ler*) or previously associated with diarrhea pathogenesis (*efa1/lifA*) (Cepeda-Molero et al., 2017; Slinger et al., 2017), we observed no unique differences between the experimental isolates involved in this study. The lack of molecular detection of *escN* and *ler* in the "vendor-origin" aEPEC may indicate that the T3SS is not functional in these isolates. Further genomic and phenotypic analysis of the experimental and vendor-origin isolates could provide further insight into virulence attributes (e.g. colicin production, T3SS functionality) capable of influencing carriage of some isolates and not others.

In an effort to gain further mechanistic insight into the pathogenesis of aEPEC carriage in kittens, but within the confines of a non-terminal study, we quantified intestinal absorptive and barrier function *in vivo* in kittens before and after experimental infection with aEPEC. These studies were conducted by oral administration of D-xylose and iohexol followed by measurement of their appearance in serum. The results demonstrated a significant effect of aEPEC administration on intestinal absorption and permeability. D-xylose is an exogenous monosaccharide whose oral absorption requires transport across the intestinal epithelium transcellularly via the sodium-glucose transporter SGLT-1 (Alvarado, 1966) or via passive diffusion (Ohkohchi et al., 1986). The concentration of D-xylose in serum following oral administration reflects the absorptive capacity of the intestinal epithelium. Therefore, our measurement of lower serum concentrations of D-xylose after, compared to before, aEPEC infection supports the onset of significant intestinal malabsorption. While we did not perform a histological examination of the intestines of kittens in this study, intestinal malabsorption could reflect a non-specific effect of *E. coli* administration, or alternatively the functional consequence of a number of cell-based mechanisms of EPEC pathogenesis including effacement of microvilli (Moon et al., 1983), villus blunting due to loss of epithelial cell numbers (Fagundes-Neto et al., 1997), or a specific inhibitory effect of EPEC effector proteins on SGLT-1 (Dean et al., 2006). It is also possible that aEPEC promoted changes in intestinal motility or in the host microbial community composition that negatively influenced the absorption of D-xylose. This study also demonstrated that kittens experimentally infected with aEPEC had significantly decreased intestinal permeability to iohexol. Iohexol is a nonionic, water-soluble, radiocontrast agent whose oral absorption requires passive diffusion through paracellar pathways between adjacent intestinal epithelial cells (Frias et al., 2012; Gerova et al., 2011). Our observation that intestinal permeability to iohexol decreased following aEPEC infection in kittens was somewhat unexpected as cell culture studies show that tEPEC increases tight junction permeability by injecting effector proteins (e.g. EspF, EspG1/G2, NleA and Map) into host cells *in vitro* (Glotfelty et al., 2014; McNamara et al.,

2001; Thanabalasuriar et al., 2010). It is possible that the decrease in intestinal permeability observed *in vivo* in these kittens reflects a non-specific effect of *E. coli*, or the presence of additional influences of aEPEC on epithelial surface area. For example, children with attaching and effacing *E. coli* often have villus blunting which reduces the overall surface area of paracellular pathway (Fagundes-Neto et al., 1997; Hyun et al., 1995). Recent studies of infants with environmental enteropathy demonstrate that total epithelial surface area has a greater influence on translocation of lipopolysaccharide (LPS) than does the presence of overt epithelial defects and tight junction leakage that were documented using confocal laser endomicroscopy (Kelly et al., 2016).

A final objective of this study was to explore the potential benefit of a member of the healthy intestinal microbiota on intestinal defense against the adverse effects of aEPEC administration in these kittens. We have previously established that *Enterococcus hirae* is the predominant mucosa-adherent enterococci species residing in the small intestine of healthy kittens (Ghosh et al., 2013; Nicklas et al., 2010). Moreover, in kittens with terminal illness, in which aEPEC colonization of the epithelium is prevalent, *E. hirae* is replaced by increases in mucosal colonization with genotypically diverse, virulent, and antimicrobial-resistant *E. faecalis* (Ghosh et al., 2013). This observation is strikingly similar to studies of *C. rodentium* in interleukin 22 receptor knockout (IL-22ra1$^{-/-}$) mice where opportunistic mucosal colonization by virulent *E. faecalis* promotes a greater burden of attachment of *C. rodentium* to the intestinal epithelium (Pham et al., 2014). Results of this study demonstrated that a probiotic formulation of *E. hirae* was highly effective in promoting intestinal colonization and fecal shedding of live *E. hirae* during administration. In kittens with experimental aEPEC carriage, *E. hirae* appeared to exert its most significant influence by promoting a decrease in intestinal permeability. This decrease in permeability may indicate an enhancement of the barrier function promoted by *E. hirae*. While the specific mechanism of this observation was not established by this study, others have shown that the lipoteichoic acid of *E. hirae* is able to protect against loss of epithelial tight junction function induced by TNF-α (Miyauchi et al., 2008). Additionally, *E. hirae* can limit the secretion of inflammatory mediators induced by LPS (Arokiyaraj et al., 2014; Miyauchi et al., 2008) and exerts bactericidal effects against numerous pathogens including *E. coli* and *E. faecalis* (Miyauchi et al., 2008).

A number of limitations of this study are worthy of discussion. Most importantly, we did not include a control group of kittens infected with non-pathogenic *E. coli* at a similar dose (i.e. 3×10^8 CFU). Including this control group would have allowed for differentiation between the specific influences of aEPEC and possible nonspecific effects of ingesting a relatively high dose of similar bacteria. Additional studies will be needed to answer this question. Our study utilized a relatively small sample size (n = 8) of kittens in each group. These samples sizes were further diminished by inclusion within each group of a subset of kittens receiving either an *E. hirae* probiotic or placebo. Despite these conservative numbers, statistically significant differences were disclosed for each of the major objectives of the study. Kittens pretreated with antibiotics had decreases in intestinal absorption and permeability of D-xylose and iohexol, respectively. However, these measurements were not significantly different due to a higher variability in the data collected from kittens that had received treatment with antibiotics. Differences in the baseline values between the kittens in Group A and Group B made it difficult to assess a comparison of severity of intestinal barrier dysfunction between kittens with a "normal" versus disrupted bacterial flora prior to aEPEC infection, respectively. Nonetheless, these studies support that a "normal" intestinal microbiota may be a strong contributing factor to maintaining an asymptomatic aEPEC carriage. To limit the number of animals used, we did not measure intestinal function in an uninfected group of kittens to control for any extraneous effect of time (11–12 days) between the pre- and post-infection measurements of intestinal absorption or permeability. Despite attempts to screen fecal samples from queens and kittens for aEPEC prior to their

purchase, a subset of kittens in each experimental group was determined to be carrying a "vendor-origin" aEPEC prior to inclusion in this study. No alternate source of kittens designated as specific pathogen free from aEPEC existed at the time of this study. The impact of preexisting aEPEC on the results of experimental aEPEC infection in these kittens was impossible to ascertain due to the low number of kittens falling into each combination of experimental variables. However, mice infected with *C. rodentium* can develop an IgG-dependent acquired immunity and decreased colonization upon re-infection (Maaser et al., 2004). Accordingly it is possible that pathogenic effects of aEPEC in kittens with prior infection was diminished in this study.

In summary, results of this study identify aEPEC as a potentially influential pathogen in kittens. An ability of aEPEC to promote intestinal malabsorption may explain the association of the infection with increased odds of mortality in kittens and children with diarrhea (Kotloff et al., 2013; Watson et al., 2017). The resident intestinal microbiota have an important impact on disease susceptibility, which may in part explain why some kittens and children with the infection remain asymptomatic while others are susceptible to diarrhea (Barletta et al., 2011; Watson et al., 2017). Fortification of the intestinal microbiota with commensal *E. hirae* has a potential to ameliorate the effect of aEPEC in kittens and is worthy of further study in susceptible populations.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.03.020.

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