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Research paper

The effect of enterococci on feline *Tritrichomonas foetus* infection *in vitro*

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ABSTRACT

Tritrichomonas foetus is a common cause of large bowel diarrhea in cats. Probiotics have been suggested to be effective for many intestinal pathogens; however, there are a lack of studies evaluating the effect of probiotics in *T. foetus* infection. *In vitro* studies were performed to evaluate the effect of a probiotic containing *Enterococcus faecium* (Efm) SF68 and a novel probiotic, *Enterococcus hirae,* on the inhibition of *T. foetus* growth, adhesion to, and cytotoxicity towards the intestinal epithelium. The effect of enterococci on *T. foetus* proliferation during co-culture was evaluated throughout log phase *T. foetus* growth. The previously validated *in vitro* co-culture model system using porcine intestinal epithelial cells (IPEC-J2) was used to evaluate the effect of enterococci on *T. foetus* adhesion and cytotoxicity towards intestinal epithelial cells. Cytotoxicity was assessed using fluorescent microscopy and spectrophotometry. Interactions of *T. foetus*, enterococci, and intestinal epithelial cells were assessed using scanning electron microscopy and immunofluorescence assays (IFA). *Enterococcus*-induced inhibition of *T. foetus* growth was demonstrated at concentrations as low as 10^4 enterococci colony forming units (CFU)/mL and was dependent, in part, on environmental pH and the presence of viable enterococci organisms. *T. foetus* adhesion, including with a ronidazole-resistant strain, was reduced with pretreatment of intestinal epithelial cells with enterococci but was not significantly affected when enterococci were introduced simultaneously or following *T. foetus* infection. Compared to Efm, *E. hirae* more effectively decreased *T. foetus* adhesion, suggesting its superior potential as a novel probiotic for *T. foetus* infection. There was no effect of enterococci treatment on *T. foetus*-induced intestinal epithelial cell cytotoxicity. Our results support further study into the investigation of a possible benefit of enterococci-containing probiotic treatment for prevention of *T. foetus* infection in at-risk uninfected cats.

1. Introduction

Tritrichomonas foetus a pathogenic intestinal protozoan, is a common cause of chronic diarrhea in domestic cats with a prevalence of up to 30%. Affected cats present with waxing and waning diarrhea and can remain subclinical carriers after resolution of clinical signs (Foster et al., 2004). Currently, ronidazole is the only effective treatment for feline *T. foetus* infection. However, a narrow margin of drug safety and identification of ronidazole-resistant strains of *T. foetus* support investigation of alternative therapies (Gookin et al., 2006, 2010).

Probiotics are live microorganisms that benefit the gastrointestinal tract and have emerged as potential adjunct therapies for other enteropathogens including *Giardia duodenalis*, another common intestinal protozoan in cats (Benyacoub et al., 2005; Carlin et al., 2006). Through colonization or interaction with the host's intestinal environment, probiotics may impart a multifactorial effect, such as creating an unfavorable environment for enteropathogens or modulating the host's immune system. Probiotics often contain lactic acid-producing bacteria (LAB) such as enterococci or lactobacilli, which are normal inhabitants of the colonic microbiota (Ritchie et al., 2008). The potential benefit of probiotics in the treatment of feline *T. foetus* infection is of particular interest for several reasons. Resident bacteria are thought to play an important role in the development and course of trichomonad infection. In a study of the human venereal trichomonad, *Trichomonas vaginalis, Lactobacilli* spp. inhibited adhesion of *T. vaginalis* to the vaginal epithelium (Phukan et al., 2013). Moreover, cats infected with *T. foetus*

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that are treated with antibiotics often have temporary improvement of diarrhea. Finally, changes in diet or other stressors can incite recurrence of diarrhea in chronically infected cats, which is thought to occur secondary to an alteration in the intestinal microbiota (Foster et al., 2004). Therefore, targeting the colonic microbiota through administration of LAB-containing probiotics may represent a novel, adjunctive treatment for feline trichomonosis. However, certain species of LAB may confer more benefit than others. The gram-positive enterococci are of particular interest as they are gastrointestinal commensals and appear to play a particularly important role in the health of cats. In a study evaluating the intestinal enterococci community of kittens, *Enterococcus hirae* was the most common species of ileum mucosa-associated enterococci in healthy kittens whereas non-*E. hirae* species were more commonly identified in kittens with terminal disease such as severe gastrointestinal illness (Ghosh et al., 2013). *Enterococcus faecium* (Efm) is also of interest as one strain of Efm is widely available as a commercial probiotic for cats and has been shown to be an effective therapeutic for shelter cats with diarrhea (Bybee et al., 2011). Moreover, in one unpublished *in vivo* study, Efm decreased the proportion of relapses of *T. foetus* infection in ronidazole-treated cats.[a]

Thus, the central objective of this study was to evaluate the effect of enterococci on growth, adhesion and cytotoxicity of feline *T. foetus* towards the intestinal epithelium *in vitro*. A second objective was to compare the efficacy of the commercial probiotic-containing *E. faecium* SF68, which has been previously suggested to be effective against feline trichomonosis, with a feline isolate of *E. hirae*, previously identified to be highly prevalent in the intestines of healthy kittens, in ameliorating *T. foetus* cytopathology towards the intestinal epithelium *in vitro*.

2. Materials and methods

2.1. IPEC-J2 cells

Non-transformed porcine intestinal epithelial cells (IPEC-J2) were cultivated as previously described (Tolbert et al., 2013). IPEC-J2 cells were seeded onto 24-well polystyrene plates (Corning Inc, Corning, NY) or chamber slides and cultured to confluence prior to use in assays with *T. foetus* and enterococci.

2.2. T. foetus isolates

Isolates of feline *T. foetus* (A, JT) were obtained from naturally infected cats including one isolate (JT) that was obtained from a cat who failed to clear the infection after two courses (30 mg/kg PO q24 h for 14 days each) of ronidazole therapy. *T. foetus* were cultivated as previously described (Tolbert et al., 2013; Tolbert et al., 2014). Assays were performed with *T. foetus* cultures passaged no more than 10 times since cryopreservation, at mid-log phase growth, and with > 95% viability based on motility.

2.3. Enterococcus spp. Isolates

Efm was cultivated from a commercially available probiotic (Purina Fortiflora©, Nestle Purina, St. Louis, MO) on Columbia 5% sheep blood agar plates (Becton Dickinson, Franklin Lakes, NJ). The identity of the isolated colonies was confirmed to genus level by growth on bile esculin agar and in 5% NaCl broth and to the species level using the commercial API20 strep test (Biomerieux-USA, Durham, NC). A single, well-characterized isolate of *E. hirae* was obtained by selective culture from the ileum mucosa of a healthy < 12-week-old kitten (Ghosh et al., 2013) and used for all studies. One pure culture of both Efm and *E. hirae* each were divided into aliquots and stored at −80 °C in SPGA cryopreservant (Bovarnick et al., 1950). Prior to each assay, one cryovial of either Efm or *E. hirae* was thawed and plated on Columbia agar with 5% sheep blood plates. The concentration of enterococci for each assay was determined by the optical density of Efm or *E. hirae* suspended in Dulbecco's Phosphate-Buffered Saline (DPBS) using the Densicheck Plus instrument (Biomerieux-USA, Durham, NC).

2.4. Growth curves

Antibiotic-free *T. foetus* growth media was inoculated with log-phase *T. foetus* at 37 °C. Enterococci were added at the time of *T. foetus* inoculation or after 18 h of mid-log growth of *T. foetus* at concentrations as low as 10^4 enterococci colony forming units (CFU)/mL. Co-culture was continued for up to 48 h. For growth curves using non-viable bacteria, heat-killed *E. hirae* were added to selected cultures after cultivation in antibiotic-free *T. foetus* media and heat-treatment at 100 °C for 60 min (Barman et al., 2013). Viability of heat-killed cultures was tested by plating *E. hirae* on Columbia agar with 5% sheep blood plates after heat treatment. The pH of the cultures was monitored at each time point using a pH meter (UltraBasic pH Benchtop Meter, Denver Instruments, Bohemia, NY). When necessary, pH of the co-culture media was titrated using 5 N $NaHCO_3$. Mean *T. foetus* concentrations were compared among groups.

2.5. Co-culture adhesion assays

To determine the effect of *Enterococcus* spp. on *T. foetus* adhesion to the intestinal epithelium, adhesion assays were performed as previously described (Tolbert et al., 2013) using monolayers infected with *T. foetus* alone or monolayers infected with *T. foetus* and treated with enterococci. Uninfected IPEC-J2 monolayers and monolayers treated with only enterococci were used as controls in all assays. Viable trichomonads were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies, Carlsbad, CA) as previously described prior to infection of IPEC-J2 (Tolbert et al., 2013). IPEC-J2 monolayers were grown to confluence in 24-well polystyrene plates prior to infection with *T. foetus* or enterococci. Immediately prior to adhesion studies, the IPEC-J2 culture media was replaced by co-culture media containing all constituents of IPEC-J2 media but devoid of serum and antibiotics, to prevent IPEC-J2 and trichomonad replication and to allow for enterococci survival, respectively. Increasing doses (10^5–10^8 CFU/well) of unlabeled, live enterococci suspended in antibiotic-free IPEC-J2 media were added to IPEC-J2 monolayers 6 h prior to or simultaneously with *T. foetus* infection. CFSE-labeled *T. foetus* were added to IPEC-J2 monolayers at 10×10^6 *T. foetus* per well and co-cultured for 6 h as previously described (Tolbert et al., 2013). Unbound trichomonads and enterococci were removed from monolayers by washing IPEC-J2 cells twice with DPBS. IPEC-J2 and *T. foetus* nuclei were counterstained with DAPI (Vectashield, Vector Laboratories, Burlingame, CA). Adherent trichomonads in individual wells were counted in six high power fields (HPFs) using an epifluorescence microscope (Nikon® Digital Sight DS, Melville, NY), with the average of six HPFs representative of one replicate. All assays were performed with a minimum of four replicate cultures per treatment group and repeated in triplicate experiments. Mean adherence was compared among groups.

2.6. Crystal violet cytotoxicity assays

To provide a semi-quantitative analysis of the effect of enterococci in decreasing *T. foetus*-induced epithelial cytotoxicity, crystal violet cytotoxicity assays were performed as previously described (Tolbert et.al 2014) using monolayers treated with enterococci or infected with *T. foetus* alone or monolayers infected with *T. foetus* and treated with enterococci. Uninfected IPEC-J2 monolayers and monolayers treated with only enterococci were used as controls in all assays. Unlabeled enterococci suspended in antibiotic-free IPEC-J2 media were added to IPEC-J2 monolayers at 10^6 CFU/well 6 h prior to *T. foetus* infection. At the end of 6 h, unlabeled *T. foetus* were added at 10×10^6 *T. foetus* per well and co-cultured for an additional 6 h. At the end of incubation, epithelial monolayers were gently washed with DPBS to remove

detached epithelial cells. They were then fixed with 2% paraformaldehyde in PBS for 15 min at RT, washed again with DPBS, and stained with 100 u L of 0.13% crystal violet solution dissolved in a 5:2 (vol/vol) ethanol-paraformaldehyde solution. After gentle washing with dH_20 the monolayers were air dried. Stained cells were solubilized in 100 u L 1% SDS in 50% ethanol and transferred to 96-well plates. The absorbance of the remaining crystal violet was measured at 570 nM with a reference wavelength of 650 nm to account for optical interference. All assays were performed with a minimum of four replicate cultures per treatment group and repeated in triplicate experiments. Mean or median optical density were compared among groups.

2.7. Indirect immunofluorescence

Immunofluorescence was used to qualitatively evaluate the interaction between IPEC-J2 cells, *T. foetus*, and enterococci. IPEC-J2 monolayers were grown in chamber slides and adhesion assays were performed as described above. After completion of the adhesion assay, wells were fixed with 80% acetone at 25 °C (RT) for 10 min. Slides were rinsed briefly in 1X PBS for 5 min and incubated in blocking buffer (1X PBS, 5% normal goat serum, and 2% BSA) overnight at 4° C in a humidified chamber on a rocker. Slides were then incubated with enterococcus polyclonal antibody diluted 1:100 in blocking buffer (Invitrogen, Carlsbad, CA) or rabbit IgG as a control for 4 h in a humidified chamber on a rocker at RT. Following incubation with primary antibody, slides were rinsed three times in 1X PBS for 5 min each followed by incubation with Cyanine3 (Cy3)-conjugated goat anti-rabbit IgG (H + L) (Invitrogen, Carlsbad, CA) diluted 1:750 in blocking buffer for 30 min in a humidified chamber at RT. Slides were once again rinsed three times with 1X PBS for 5 min each and the nuclear counterstain DAPI with mounting media (4',6-diamidino-2-phenylindole) (Vector Laboratores, Inc., Burlingame, CA) was applied for visualization of epithelial and *T. foetus* nuclei. Images were obtained using a fluorescence microscope (Leica DMi8, Buffalo Grove, IL).

2.8. Scanning electron microscopy

Scanning electron microscopy (SEM) was also used to qualitatively evaluate the interaction between *T. foetus* and enterococci and between IPEC-J2 cells, *T. foetus*, and enterococci in co-culture. For *T. foetus* and enterococci interactions, mid-log phase *T. foetus* cultures in antibiotic-free *T. foetus* media were inoculated with 10^4 enterococci CFU/mL and co-cultured for up to 8 h. Co-cultures were centrifuged $250 \times g$ for 5 min. The pellet was collected and preserved in Carson's fixative (Fisher Scientific, Hampton, NH). Additionally, IPEC-J2 cells were seeded on 12 mm circular coverslips. Adhesion assays were performed as previously described above. After washing with 1X PBS, the monolayers were preserved in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Samples were dehydrated with an ascending series of ethanol (15%, 30%, 50%, 70%, 95%) and two washes in 100% ethanol and then mounted on LEO microscope tapered end stubs (Electron Microscopy Sciences, Inc., Hatfield, PA) and sputter coated with gold using an SPI-Module sputter coater (SPI Supplies Structure Probe, Westchester, PA). Samples were viewed using a Carl Zeiss EVO 40 SEM for interactions between enterococci, *T. foetus* trophozoites, and IPEC-J2 monolayers.

2.9. Statistical analysis

Data were analyzed for normality (Kolmogorov–Smirnov) and variance (Levene median) using commercially available statistical software (SigmaStat, Jandel Scientific). Parametric data were analyzed using a Student's *t*-test or one-way ANOVA. Non-parametric data were analyzed using Mann–Whitney rank sum test or Kruskal–Wallis ANOVA on Ranks. Results are reported as mean ± standard deviation. For all analyses, $p \leq 0.05$ was deemed significant.

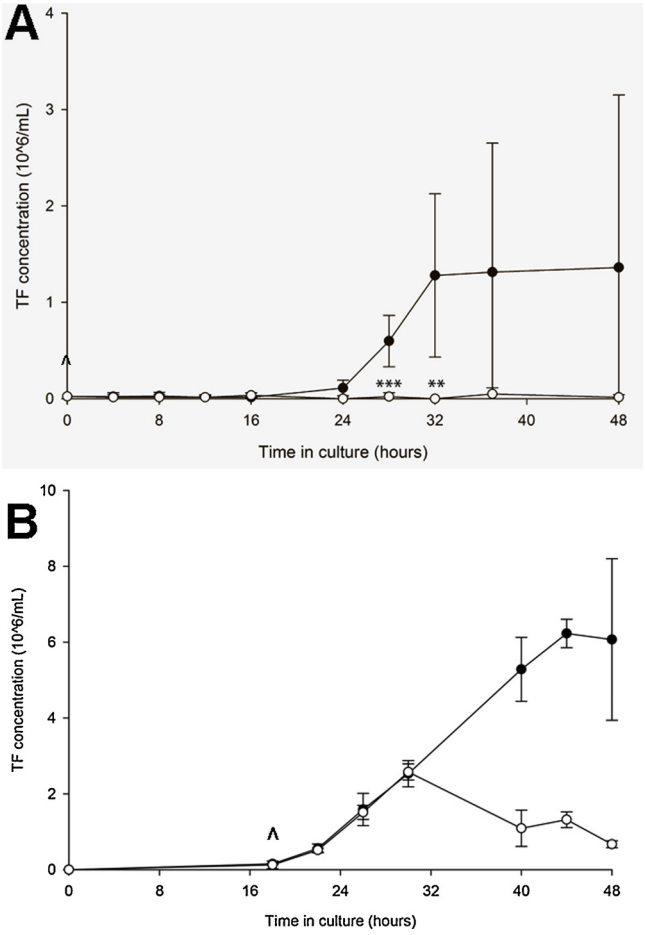

Fig. 1. The effect of enterococci on *Tritrichomonas foetus* growth (A) Growth curve of *T. foetus* (TF) inoculated at t = 0 with vehicle treatment (closed circles) *or Enterococcus faecium* (1Efm:10TF; open circles) and monitored for 48 h. ***p < 0.001, ** p = 0.002 (B) Growth curve of *T. foetus* inoculated at mid-log growth (18 h) with vehicle treatment (closed circles) or Efm (1Efm:10TF; open circles) and monitored for 48 h. ***p < 0.001, ** p = 0.002 Data points represent the means ± standard deviations from 4 replicate cultures. ˆ denotes addition of enterococci to *T. foetus* cultures. Above figures depict Efm; similar results were obtained using *Enterococcus hirae* (results not shown).

3. Results

3.1. Enterococci decrease growth of T. foetus

When enterococci and *T. foetus* were simultaneously added, enterococci completely inhibited the growth of *T. foetus* (Fig. 1A). Additionally, when enterococci were added after *T. foetus* had achieved early log-phase growth (18 h in culture), enterococci also significantly inhibited growth of the established *T. foetus* culture over time (p < 0.001 at 40, 44 h; p = 0.002 at 48 h) (Fig. 1B).

3.2. The inhibitory effect of enterococci on T. foetus growth requires viable bacteria

Co-culture with heat-killed *E. hirae* had no effect on *T. foetus* growth compared to live *E. hirae* as *T. foetus* continued to replicate through mid-log growth in the face of heat-killed *E. hirae* (p = 0.004 at 42 h; p < 0.001 at 46 and 48 h) (Fig. 2).

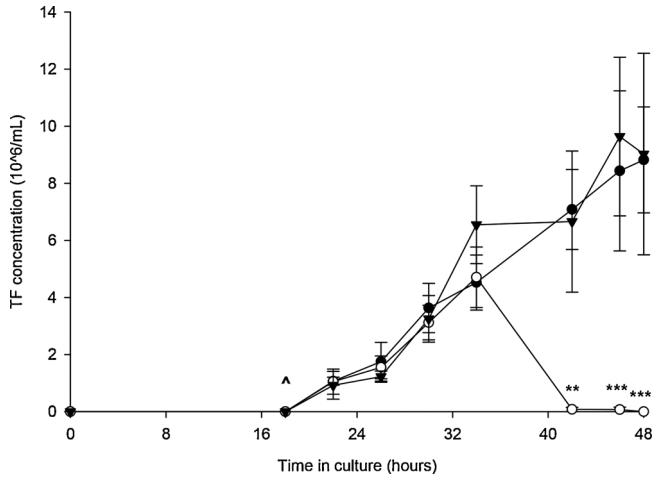

Fig. 2. Inhibitory effect of enterococci on *Tritrichomonas foetus* growth requires viable organisms. Growth curve of *T. foetus* (TF) inoculated mid-log growth (18 h) with either vehicle (closed circles), live *Enterococcus hirae* (open circles), or heat-killed *E. hirae* (upside down triangles) and monitored for 48 h. **p = 0.009, *** p < 0.001. Data points represent the means ± standard deviations from 4 replicate cultures. ^ denotes addition of enterococci to *T. foetus* cultures. Above figure depicts *E. hirae*; results not obtained using *Enterococcus faecium*.

3.3. Inhibition of T. foetus replication by enterococci is partially pH dependent

After approximately 12 h of co-culture with Efm, declining numbers of *T. foetus* was associated with a pH decrease > 1 unit that was not observed in *T. foetus* cultures not treated with Efm. When the pH of the Efm-treated co-culture was titrated to the same pH of the *T. foetus* only culture, Efm-treated *T. foetus* numbers reached a higher concentration than without pH adjustment; however, *T. foetus* growth remained below that of cultures without Efm (Fig. 3) suggesting that a change in environmental pH by enterococci is responsible for part but not all of its negative effect of *T. foetus* growth ($p < 0.001$ at 40, 44, and 48 h).

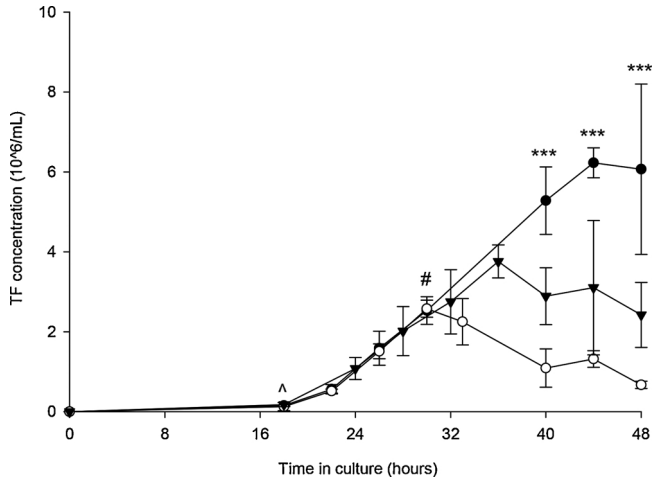

Fig. 3. The inhibitory effect of enterococci on *Tritrichomonas foetus* growth is partially pH dependent. Growth curve of *T. foetus* inoculated mid-log growth (18 h) with either vehicle (closed circles), live *Enterococcus faecium* (Efm) (open circles), or live Efm with pH adjustments (upside down triangles) and monitored for 48 h. Data points represent the means ± standard deviations from 4 replicate cultures. *denotes significant difference from all other groups. *** p < 0.001. ^ denotes addition of enterococci to *T. foetus* cultures. # indicates time at which a decrease in pH > 1 is noted. Above figure depicts Efm; results not obtained using *Enterococcus hirae*.

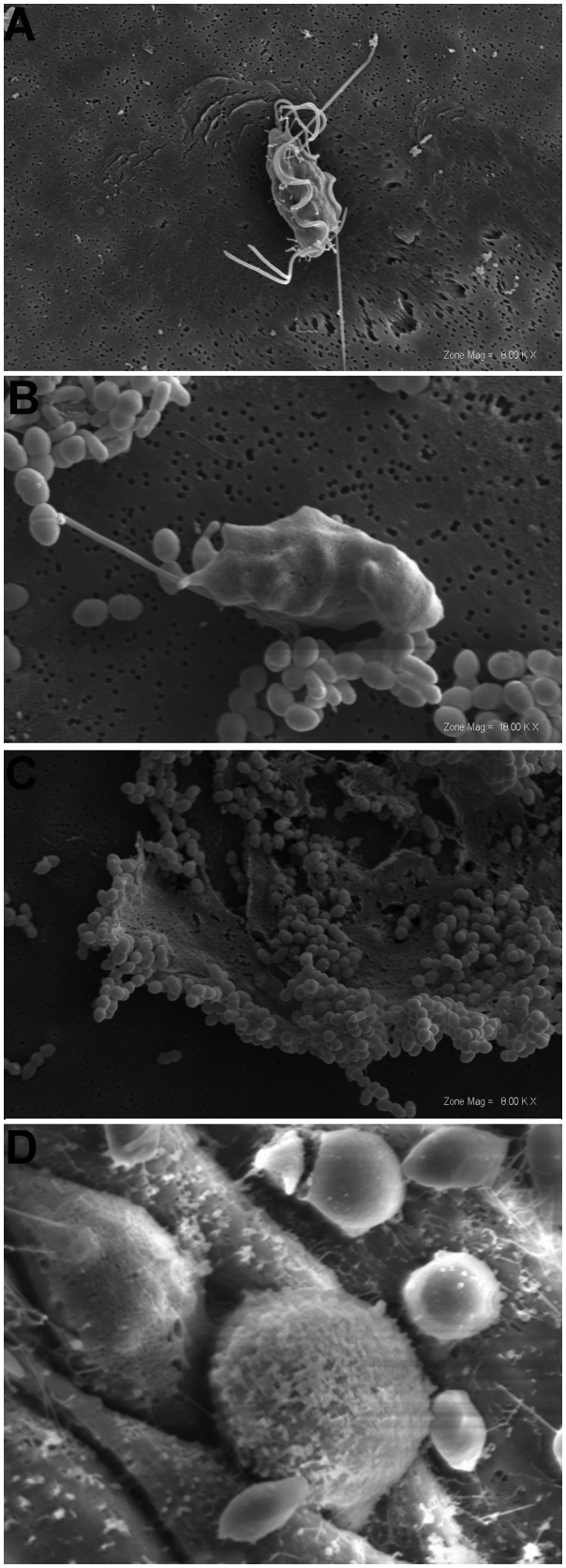

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Fig. 4. Enterococci directly interact with *Tritrichomonas foetus*. Scanning electron microscopy of feline *T. foetus* in co-culture with enterococci and IPEC-J2 monolayers. (A) A *T. foetus* trichomonad from an untreated culture. (B) A *T. foetus* trichomonad from a culture treated with *Enterococcus hirae*. Enterococci are seen adhering to the posterior and anterior flagella of the trichomonad after six hours of co-culture. (C) The *T. foetus* trichomonads are destroyed over time in the presence of *E. hirae*. (D) *Enterococcus faecium* and *T. foetus* directly interact with IPEC-J2 monolayers. Co-culture was performed using both *Enterococci* spp. and above images are representative, with specification of species for each image.

3.4. Enterococci directly interact with T. foetus and colonize the intestinal epithelium

Ultrastructural analysis of *T. foetus* (Fig. 4A) and enterococci co-culture using SEM revealed a close interaction of *T. foetus* and enterococci. Enterococci were noted to surround the surface of *T. foetus* and adhere to both the anterior and posterior flagella (Fig. 4B). Over time, *T. foetus* were destroyed when co-cultured with enterococci and no intact trichomonads were visible by SEM (Fig. 4C). Enterococci and *T. foetus* were also seen closely interacting with the IPEC-J2 monolayer (Fig. 4D), suggesting that similar direct interaction occurs between the protozoa and probiotic at the level of the intestinal monolayer. Both Efm and *E. hirae* demonstrated similar destruction of *T. foetus* and colonization of epithelial monolayers.

3.5. Inhibition of T. foetus adhesion by enterococci requires pretreatment of intestinal cells for enterococci colonization

Simultaneous infection with *T. foetus* and treatment with enterococci did not result in a significant decrease in *T. foetus* adhesion to intestinal epithelial cells compared to untreated groups ($p = 0.556$) (Fig. 5A). However, when IPEC-J2 monolayers were pretreated with enterococci 6 h prior to *T. foetus* infection, a dose-dependent significant decrease in the number of adhered trichomonads was observed ($p = 0.003$) (Fig. 5B). An immunofluorescence assay was used to qualitatively examine the interaction of *T. foetus* and enterococci with the epithelial monolayer. Imaging demonstrated colonization of the epithelium with enterococci as well as a subjective decrease in the density of adherent *T. foetus* (Fig.6 A–B). Additionally, *E. hirae* repeatedly decreased adhesion of *T. foetus* to IPEC-J2 monolayers more than Efm in side-by-side adhesions using the same *T. foetus* controls ($p = 0.018$) (Fig. 7).

3.6. Enterococci do not decrease T. foetus cytotoxicity to the intestinal epithelium

There was not a significant decrease in *T. foetus*-induced cytotoxicity towards IPEC-J2 monolayers after pretreatment with either *Enterococcus* spp. compared to untreated groups (Fig. 8).

4. Discussion

Probiotics have emerged as adjunct therapies for a broad range of infectious and inflammatory diseases ranging from *G. duodenalis* infection to inflammatory bowel disease. (Benyacoub et al., 2005; Rossi et al., 2014; Lippi et al., 2017). Probiotics containing enterococci are often used as adjunct therapy in cats with *T. foetus* infection; however, little research has been done to examine their efficacy. Thus, determination of the effect of enterococci on *T. foetus* infection using an established *in vitro* model of *T. foetus* pathogenicity was the central objective of this study.

Our first objective was to evaluate the effect of enterococci on the growth of *T. foetus*. The sum of these experiments demonstrated that enterococci actively inhibited *T. foetus* replication. When co-cultured with *T. foetus* simultaneously, enterococci completely inhibited the

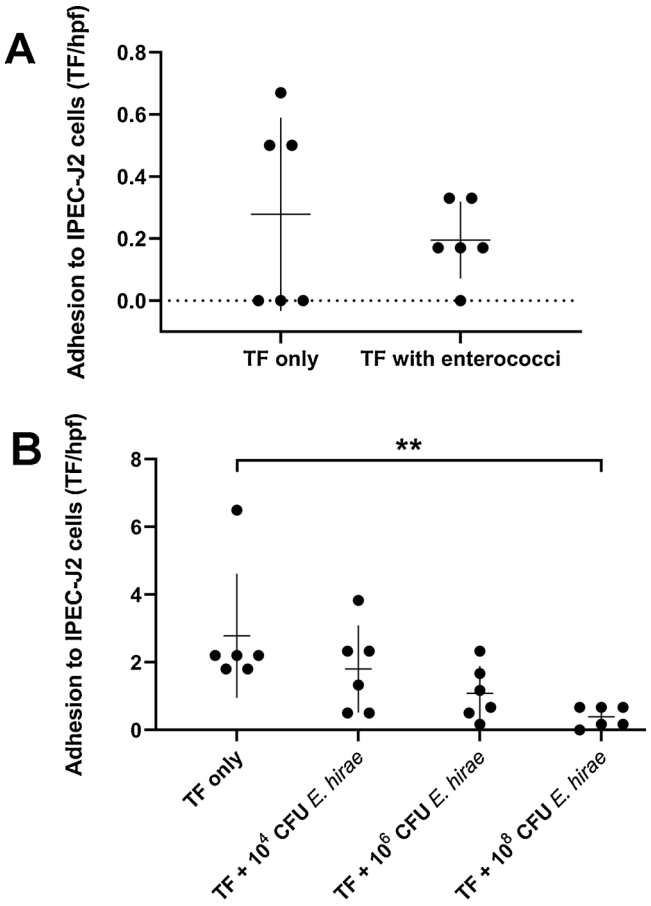

Fig. 5. Enterococci inhibition of *Tritrichomonas foetus* intestinal epithelial cell adhesion requires time. (A) Adhesion of *T. foetus* to intestinal epithelial monolayers (IPEC-J2) treated with 10^6 CFU *Enterococcus faecium* (Efm) at the time of *T. foetus infection*. (B) Adhesion of *T. foetus* to intestinal epithelial monolayers treated with increasing doses of *Enterococcus hirae* 6 h before *T. foetus* infection. **$p = 0.003$ among all groups. Each group represents 6 replicates of adhered *T. foetus* counted in 6 high power fields (hpf)/replicate. Similar results obtained using both *Enterococci* spp. (results not shown). Each group represents the mean ± standard deviations of 6 replicates of adhered *T. foetus* counted from 6 hpf/replicate.

growth of *T. foetus*. When added after *T. foetus* achieved early log-phase growth, enterococci decreased *T. foetus* growth over time. Using co-culture evaluation with heat-killed *E. hirae*, we demonstrated that this effect is an active process that requires viable bacteria. The host luminal pH has been demonstrated to be a critical factor in the ability of other trichomonads, including *T. vaginalis* and bovine *T. foetus*, to colonize and establish infection (Antonio et al., 1999; Morin-Adeline et al., 2015). Lactic-acid producing bacteria such as enterococci have the ability to actively acidify their local environment and could explain the negative effect on *T. foetus* growth over time. Thus, we sought to determine if enterococci were changing the pH of feline *T. foetus* cultures and if correction of that pH change would resolve the inhibitory effect on growth. Evaluation and alteration of the pH of culture media in the presence or absence of Efm demonstrated that Efm induced a change in environmental pH, which had an inhibitory effect on *T. foetus* growth. However, correction of the pH to match that of cultures containing *T. foetus* alone did not completely rescue the inhibitory effect of Efm on *T. foetus* growth. Thus, additional mechanisms, including competition for nutrients, direct interactions between the two organisms, or additional cytotoxic by products produced by the bacteria, are likely also involved. SEM imaging demonstrated that the enterococci surround and appear to adhere to *T. foetus* resulting in destruction of the trichomonads over

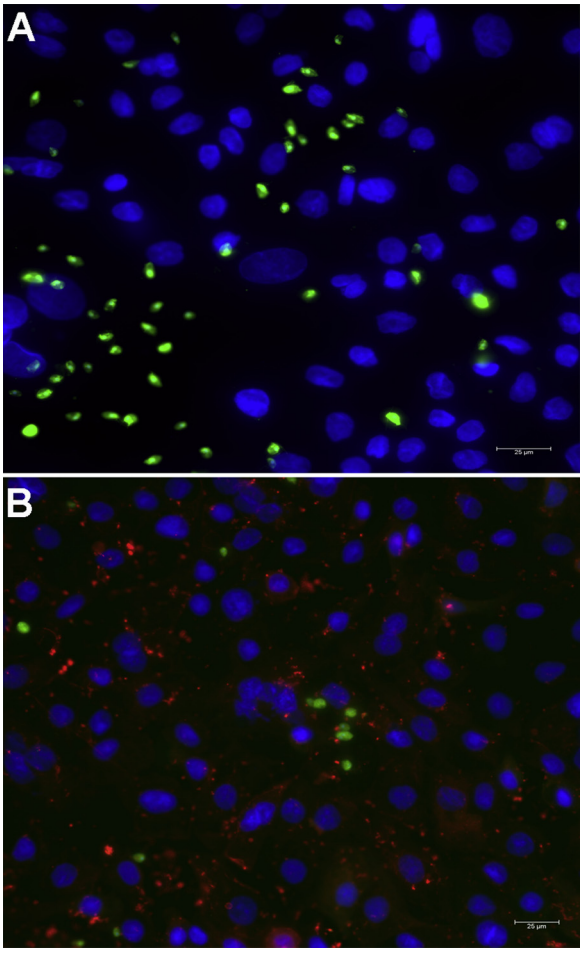

Fig. 6. Enterococci colonization of the intestinal epithelium reduces *Tritrichomonas foetus* adhesion. Indirect immunofluorescence demonstrates the colonization of IPEC-J2 monolayers with *T. foetus* only (A) or *T. foetus* and *Enterococcus hirae* (B). IPEC-2 are labeled with the nuclear stain DAPI (blue), *T. foetus* are labeled with the vital stain CFSE (green), and enterococci are labeled with Cy3-conjugated antibodies (red). 20x with 1.6x magnification. Above images depict *E. hirae*; images not obtained using *Enterococcus faecium* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

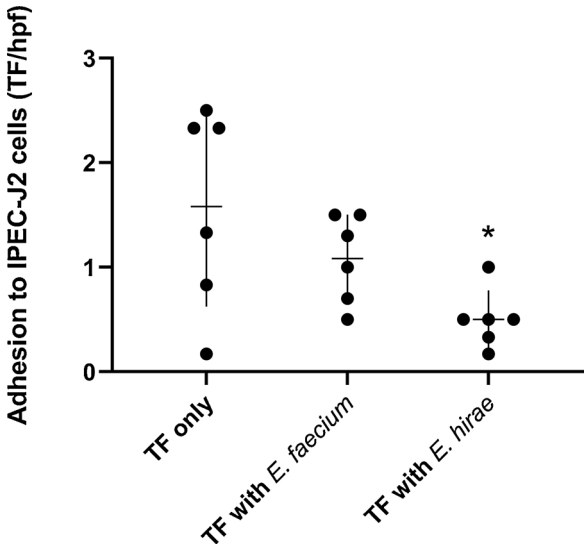

Fig. 7. Magnitude of enterococci inhibition of *Tritrichomonas foetus* adhesion to intestinal epithelium is species dependent. Comparison of side by side adhesions of *T. foetus* to intestinal epithelium monolayers (IPEC-J2) treated with either *Enterococcus faecium* (Efm) or *Enterococcus hirae* 6 h before *T. foetus* infection. *p = 0.018. Each group represents the mean ± standard deviations from 6 replicates of adhered *T. foetus* counted in 6 high power fields/replicate.

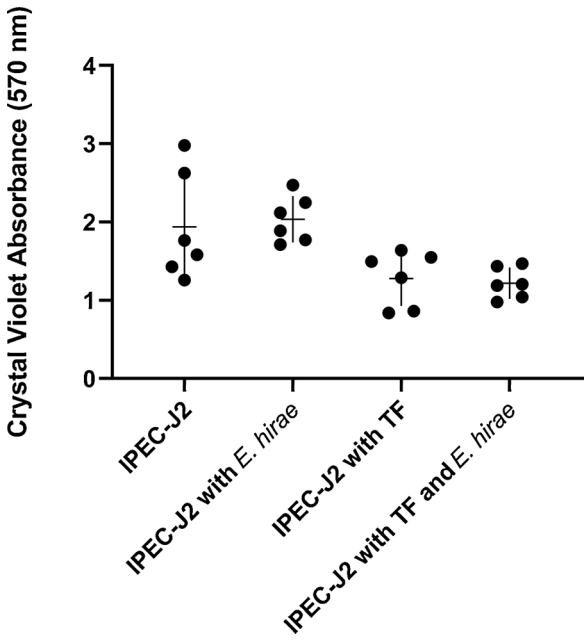

Fig. 8. Enterococci have no effect on *Tritrichomonas foetus*-induced intestinal epithelial cytotoxicity *in vitro*. Spectrophotometric analysis of crystal violet absorbance by IPEC-J2 monolayers following treatment with *Enterococcus hirae* 6 h prior to *T. foetus* and co-culture with *T. foetus*, enterococci, or both for 6 h. Data represent 6 cultures per treatment group and are reported as means ± SD. Above figures depict *E. hirae*; similar results were obtained using Efm (results not shown).

time. Bacterial aggregation, a process in which bacteria congregate around a pathogen and inhibit its access to the host epithelium, has been demonstrated with other bacteria contained in probiotics (Ekmekci et al., 2009). Aggregation prevents epithelial adhesion and promotes pathogen destruction by making the pathogen more susceptible to host immune defense mediators as well as bacterial secretions, such as lactic acid. Although more studies are needed, we hypothesize that, in the absence of a host, enterococci directly interact with and aggregate around *T. foetus* to promote trichomonad cell death.

Adhesion of *T. foetus* to the intestinal epithelium is an important first step in establishing infection and inducing cytotoxicity (Tolbert et al., 2013). We demonstrated a decrease in *T. foetus* adhesion when enterococci are added in advance of *T. foetus* infection. Based on immunofluorescent imaging, we suspect that enterococci colonize the intestinal epithelium resulting in occupation or blocking of receptors necessary for *T. foetus* to adhere. However, further studies would be needed to determine by what mechanism the bacteria are inhibiting *T. foetus* adhesion. No effect on adhesion was observed when enterococci were added at the same time as *T. foetus* to IPEC-J2 monolayers. Based on these findings, enterococci-containing probiotics might be most beneficial when administered to at-risk cats, such as those in shelters or catteries, prior to infection with *T. foetus*. Additionally, our findings were similar when using a *T. foetus* isolate harvested from a cat with ronidazole-resistant infection, suggesting that enterococci-containing probiotics could be of benefit when treatment with ronidazole is ineffective.

When considering bacterial species for use in probiotics, bacterial strain and host differences can dictate the behavior of the bacteria in the presence of infection or disease. For example, enterococci are generally considered intestinal commensals; however, their ability to

acquire antimicrobial resistance and their role in opportunistic infections can make some enterococci species serious pathogenic threats (Ghosh et al., 2012; Litster et al., 2009). A previous study identified *E. hirae* as the predominant commensal enterococci bacteria colonizing the ileum mucosa of healthy kittens. Isolates of *E. hirae* lack typical enterococci virulence factors and become displaced by more virulent species of enterococci such as *E. faecalis* in sick kittens (Ghosh et al., 2013). Thus, a second objective was to compare the efficacy of the commercial probiotic-containing Efm SF68, which has been previously suggested to be effective against feline trichomonosis (Lalor and Gunn-Moore, 2012), with a feline isolate of *E. hirae*. In our studies, *E. hirae* was repeatedly as effective as Efm at reducing *T. foetus* growth and more effective in inhibition of *T. foetus* adhesion to the intestinal epithelium. This finding suggests that further investigation into the benefit of *E. hirae* for treatment of feline trichomonosis is worthwhile.

Inhibition of feline *T. foetus* adhesion by enterococci was determined to be dose-dependent, perhaps because of the requirement for blockade of intestinal epithelial cell receptors to *T. foetus* adhesins. Based on our findings, successful adjunctive treatment of feline trichomonosis with orally administered probiotics would likely depend on the maintained viability of the enterococci and their successful transit to and colonization of the colonic epithelium. A study of a similar lactic-acid producing bacteria as a probiotic in dogs demonstrated that feeding 10^8 CFU of *Lactobacillus acidophilus* to dogs resulted in recovery of 10^4–10^6 CFU/g of feces, demonstrating survival of the probiotics through transit of the intestinal tract (Tang and Saris, 2014). Our studies showed that a dose as low as 10^6 CFU of enterococci per 2×10^5 IPEC-J2 cells (Tolbert et al., 2014) was able to decrease *T. foetus* adhesion *in vitro*. Similar studies in cats would support appropriate doses of *Enterococcus* spp. necessary for its colonization of the intestinal epithelium, and subsequent benefits.

Adhesion to the intestinal epithelium has been shown to be a vital first step in *T. foetus*-induced cytotoxicity (Tolbert et al., 2014), therefore the above findings would suggest that by decreasing adhesion, treatment with enterococci would also decrease *T. foetus*-induced cytotoxicity. However, in the current study, *T. foetus*-induced intestinal epithelial cytotoxicity was not significantly reduced by pretreatment of intestinal epithelial monolayers with either Efm or *E. hirae*. A similar pattern was seen in a prior study evaluating a *T. foetus* specific adhesin (Gould et al., 2017). This would suggest that both *T. foetus* adhesion and cytotoxicity are multifactorial processes. While enterococci may decrease adhesion of *T. foetus* to the intestinal epithelium by destruction of trophozoites or occupation of adhesion sites, once *T. foetus* adhesion has occurred, enterococci appear to be less effective at preventing intestinal epithelial cytotoxicity. Additionally, *T. foetus* might utilize virulence factors that are not dependent on adhesion. *T. foetus* are largely destroyed when co-cultured with enterococci alone, however the addition of intestinal epithelial cells appears to prevent complete destruction of *T. foetus* suggesting local support of the trophozoites by the epithelial cells. Our findings are in agreement with other studies in which the interactions and mechanisms of pathogenicity of *T. foetus* with and toward the intestinal epithelium have been demonstrated to be multifactorial and complex, which in turn may necessitate multimodal treatment to combat the infection.

5. Conclusion

In conclusion, this study was the first to demonstrate a beneficial effect of enterococci-containing probiotics in an *in vitro* model of feline *T. foetus* infection; however, *in vivo* studies in naturally infected cats are still needed. Based on our findings, we believe that enterococci-containing probiotics might be beneficial as a prophylactic therapy for cats at high-risk for *T. foetus* infection, particularly high-density population such as catteries and shelters, and as an adjunct but not sole therapy for cats with established *T. foetus* infection.

Declaration of Competing Interest

None.

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