WINN FELINE FOUNDATION GRANT PROPOSAL

INTESTINAL BIOFILM FORMATION BY ENTEROCOCCI IN KITTENS
– DETERMINING THE IDENTITY AND VIRULENCE DETERMINANTS OF THOSE ASSOCIATED WITH LIFE AND DEATH –

PRINCIPAL INVESTIGATOR	CO-PRINCIPAL INVESTIGATOR	CO-INVESTIGATOR
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AUTHORIZED ADMINISTRATIVE OFFICIAL

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THIS IS A 1-YEAR PROPOSAL WITH REQUESTED SUPPORT in the Amount of **$ 22,085.95**

FOR THE BUDGET PERIOD May 1, 2011 to April 30, 2012

SCIENTIFIC ABSTRACT

Diarrhea is frequently cited as a primary or contributing cause of death of foster-age kittens[1-4]. Among the suspect causes of diarrhea, bacterial culprits are perhaps the most problematic to identify. Gram-positive enterococci are generally considered to be gastrointestinal commensals, but also include significant pathogens such as *E.faecium* and *E.faecalis*. Virulence of enterococci is largely attributed to their ability to form resilient and antimicrobial-resistant biofilms[5-8]. In studies funded by the Winn Feline Foundation we uniquely discovered that formation of an extensive biofilm by *E.hirae* on the intestinal mucosa confers a significant survival advantage to kittens. Conversely, over-representation of the intestinal flora by *E.faecium* or *E.faecalis* was associated with mortality. Further, attachment of *E. coli* to intestinal epithelium was significantly associated with death and was not observed in kittens with biofilm formation by enterococci. This proposal will combine the expertise of KSU and NCSU investigators with the one-of-a-kind-resource of 476 feline *Enterococcus* spp. isolates obtained from our prior study. We will use these isolates to explore a Central Hypothesis that *E.hirae* is an indigenous probiotic of the feline intestine whose biofilm formation is able to out-compete a significant cause of kitten mortality – attaching and effacing *E. coli* infection. Conversely, that non-*E.hirae* enterococci such as *E. faecium* and *E. faecalis* are poorly able to out-compete epithelial colonization by attaching and effacing *E. coli* and possess virulence attributes that are directly pathogenic. These findings have important implications toward identifying species of probiotics that are likely to significantly impact the survival of foster-age kittens.

LAY ABSTRACT

Diarrhea is frequently a primary or contributing cause of death of foster-age kittens. Among the causes of diarrhea, bacterial culprits are perhaps the most problematic to identify. *Enterococcus* species are generally considered to be normal inhabitants of the intestine, but include members that are significant pathogens such as *E.faecium* and *E.faecalis*. Pathogenic enterococci are generally capable of forming extensive colonies called biofilm that adhere to host tissue and are very difficult to eradicate. In studies funded by the Winn Feline Foundation we discovered that a common enterococcus of the feline intestine called *E.hirae* can form an incredible biofilm along the lining of the intestine and those kittens with this biofilm have a survival advantage. Conversely, kittens with *E.faecium* or *E.faecalis* were more likely to have died or been euthanized. We also found a significant number of kittens that died with an *E.coli* rather than *E.hirae* biofilm lining the intestine. These observations led us to hypothesize that *E.hirae* is a natural probiotic of the kitten intestine that is able to form a biofilm that can protect against a significant cause of kitten death - attaching and effacing *E. coli* infection. Also, that *E. faecium* and *E. faecalis* are poorly able to out-compete *E. coli* and may be directly injurious. We will examine these mechanisms in laboratories at KSU and NCSU using 476 feline *Enterococcus* spp. isolates obtained from our prior study. Our findings may have an enormous impact on selection of probiotics that directly promote the survival of kittens.

Background

Diarrhea is frequently cited as a primary or contributing cause of death of foster-age kittens[1-4]. Among the suspect causes of diarrhea, bacterial culprits are perhaps the most problematic to identify. For example, the Gram-positive enterococci are generally considered to be gastrointestinal commensals, but also include medically-significant pathogens such as *E. faecium* and *E. faecalis*. Virulence of these enterococci is largely attributed to their ability to form resilient and antimicrobial-resistant biofilms[5-8]. Several case reports, including one of our own[9], suggest that common intestinal species such as *E. durans* and *E. hirae* are also able to form biofilms and do so by extensively colonizing the mucosal surface of the intestine in neonatal animals[9-17] where their presence is often associated with diarrhea. In studies funded by the Winn Feline Foundation (and detailed in our current progress report) we were surprised to find that colonization of the intestinal mucosa by *E. hirae* confers a survival advantage to kittens. On the other hand, over-representation of the intestinal flora by *E. faecium* or *E. faecalis* was associated with mortality. [redacted] We are seeking continued funding from the Winn Feline Foundation to determine the reason why an imbalance in colonization by beneficial biofilm-forming *E. hirae* and deleterious non-*E. hirae* enterococci is significantly associated with health versus mortality in the foster-age kitten population. Our specific observations so far are as follows:

- Attachment of ***E. hirae*** to the intestinal epithelium is significantly associated with **survival** and *E. hirae* is represented in greater numbers among *Enterococcus* spp. cultured from the intestine of surviving kittens. In surviving kittens, attachment of enteropathogenic *E. coli* was not observed.
- Conversely, attachment of **non-*E. hirae*** to the intestinal epithelium is significantly associated with **mortality** and *E. faecium* and *E. faecalis* are represented in greater numbers among *Enterococcus* spp. cultured from the intestine of dying kittens. Attachment of *E. coli* to intestinal epithelium was exclusively and significantly associated with death of foster kittens. Attaching and effacing *E. coli* infection was not observed in any cat in which enteroadherent *Enterococcus* spp. infection was observed.

The present proposal will use the existing one-of-a-kind-resource of 476 isolates of *Enterococcus* spp. obtained from the aforementioned cohort-controlled study of 50 healthy and 50 dying foster-age kittens. Using these isolates, we will pinpoint the attributes responsible for disease causation and/or protection in these kittens.

Our **Central Hypothesis** is that the intestinal tract of thriving foster-age kittens is colonized by beneficial biofilm-forming *E. hirae* whose attachment to the intestinal epithelium is able to out-compete pathogenic bacteria such as attaching and effacing *E. coli*. Conversely, foster-age kittens that fail to survive are colonized by non-*E. hirae* enterococci such as *E. faecium* and *E. faecalis* whose adherence may be directly pathogenic and is poorly able to compete with attaching and effacing *E. coli*.

Aim I will determine if *E. hirae* is better at forming a non-pathogenic, *E. coli*-competitive biofilm than *E. faecalis*, or *E. faecium*.

Aim II will determine if *Enterococcus* spp. cultured from the intestine of foster-age kittens that died have virulence or antimicrobial resistance phenotypes that are not present in those cultured from surviving kittens.

These studies will combine the efforts of KSU and NCSU investigators having expertise in the microbiology of enterococci (Zurek and Borst) and feline gastroenterology (Gookin).

The relevance of this study is its potential to identify *E. hirae* as not a new pathogen, but as an indigenous probiotic of the feline intestine that is able to out-compete a significant cause of kitten mortality – attaching and effacing *E. coli* infection. At the same time, the study will identify the virulence attributes responsible for why *E. faecalis* and *E. faecium* are more often associated with death. [redacted]

Research Design and Methods

Aim I Is *E.hirae* better at forming a non-pathogenic, *E. coli*-competitive biofilm than *E.faecalis*, or *E.faecium*?

a) We will first determine the **identity of the 164 non-*E. hirae* enterococci isolates** recovered from kittens in our prior study in order to determine if there are also significant differences in other species of enterococci between the healthy and dying populations. If such species are identified those isolates will also be included in all of the following studies. Identification of these isolates will be determined by PCR amplification and sequencing of the manganese-dependent superoxide dismutase gene (*sodA*) as previously described[18].

b) To establish if feline ***E. hirae* is better at forming biofilm** than *E. faecalis* or *E. faecium*, biofilm formation by each feline *Enterococcus* spp. isolate will be assayed.

Adherence to polystyrene is a time-honored way to quantify biofilm formation by enterococci and will be performed in the laboratory of Dr. Zurek as previously described[6]. Briefly, enterococci will be cultivated overnight at 37°C in M17 broth, diluted in replicates of 5 to a ratio of 1:100 and incubated without agitation for 24 h at 37°C in 96-well microtiter plates. Biofilm formation will be quantified by staining with crystal violet (**Fig. 1**) followed by spectrophotometry [19].

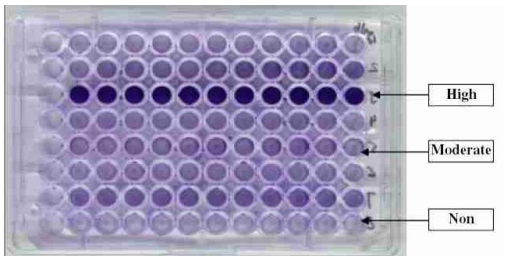

Fig. 1 Screening of biofilm producers. High, moderate and non-biofilm producers are differentiated by crystal violet staining in 96-well tissue culture plates

Adherence to intestinal epithelial cells will be performed in the laboratory of Dr. Gookin. Enterococci will be cultured overnight to log-phase, fluorescently labeled by incubation with CFSE and then used to infect 96-well microtiter plates that have been previously seeded with confluent cultures of non-transformed porcine small intestinal epithelial cells (IPECs). After 6, 12, and 24-hrs of incubation, unattached enterococci will be removed by washing and the remaining biofilm quantified by measurement of fluorescence emission using a fluorimeter. The number of adhering enterococci will be calculated using a standard curve of serially-diluted CSFE-labeled enterococci. For all biofilm experiments positive (*E. faecalis* V583) and negative controls (media, IPEC cells, unlabeled enterococci etc) will be included in each assay.

c) To investigate whether feline *E. hirae* are **able to outcompete attaching and effacing *E. coli*** infection during biofilm formation, feline *Enterococcus* spp. isolates will be pitted against a feline eae-positive (EPEC) strain of *E. coli* (obtained from the UPenn *E. coli* Laboratory). Briefly, equal numbers of enterococci and *E. coli* will be added in competition for biofilm formation as described in detail[20] using both polystyrene and IPEC cell substrates. The number of cfu of each species will be determined for both the inoculums (to confirm 1:1) and in the biofilm population by plating serial dilutions onto LB agar plates. While species differences in growth rate and biofilm formation between combatants have been shown not to influence competition assays of this nature[20], the influence of these factors and their contribution to our findings will also be assessed.

Aim II Do *Enterococcus* spp. from kittens that died have virulence or antimicrobial resistance phenotypes that differ from those found in surviving kittens?

d) Certain key genotypic and phenotypic attributes of the enterococci are thought to be responsible for their virulence[5-8]. Multiplex PCR will be performed to screen all of our feline *E. hirae*, *E. faecalis*, and *E. faecium* isolates (n=312) for four **virulence traits** including *gelE* (gelatinase); *cylA* (cytolysin); *asa1* (aggregation substance); and *esp* (enterococcal surface protein).[21] Isolates will be also phenotypicaly tested for gelatinase activity on Todd-Hewitt agar with 1.5% skim milk[6]. These studies will be performed in the laboratory of Dr. Zurek. The association of metabolic and antimicrobial resistance profiles with pathogenicity will be determined for selected isolates from healthy (17 isolates from 9 kittens) and dying (37 isolates from 16 kittens) kittens that were identified as having enteroadherent enterococci or *E. coli* infection using GenIII MicroPlates (Omnilog, Biolog) and the Sensititre Gram positive NARMS Plate Format (Trek Diagnostics). These assays will be performed in the laboratory of Dr. Borst.

e) To determine if feline *E. faecalis* and *E. faecium* are more deleterious to barrier function of the intestinal epithelium than *E. hirae*, respective isolates obtained from healthy and dying foster-age kittens will be used to dose-dependently infect IPEC cells at a multiplicity of infection ranging from 0 to 100 for 24-hrs at 37°C. Studies will be performed using polarized IPEC cells grown to confluence on transwell polycarbonate membranes that enable repeated recording of transepithelial electrical resistance (TER) over time as a measure of epithelial barrier function (**Fig. 2**). If loss of barrier function is observed, the cultures will be examined by light and/or transmission electron microscopy to ultrastructurally characterize the pathogenic effects. We will also examine whether *E. hirae* is able to abrogate the pathological effects of *E. coli* on epithelial barrier function when both species are added in competition for biofilm formation as previously described.

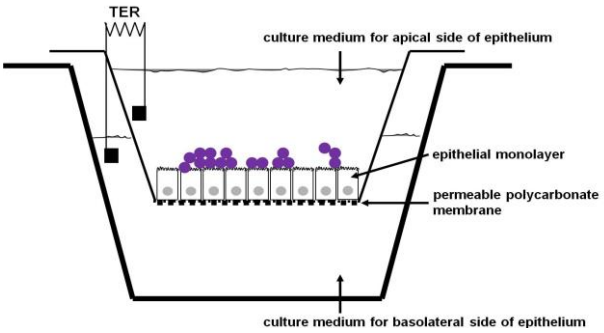

Fig. 2 The effect of feline enterococci on barrier function of intestinal epithelial cells will be quantified by measuring the loss of transepithelial electrical resistance (TER).

Expected Results We expect to demonstrate that feline *E. hirae* is a potent biofilm-former that lacks many of the genetic virulence traits that are characteristic of the pathogenic enterococci. Further, that *E. hirae* has no adverse effects on intestinal epithelial barrier function and can efficiently out-compete pathogenic *E. coli* for adhesion to the intestinal epithelium and abrogate *E. coli* deleterious effects. On the other hand, we expect to identify *E. faecium* and *E. faecalis* as poor biofilm-formers that promote loss of epithelial barrier function and possess many virulence traits characteristic of the pathogenic enterococci. *E. faecium* and *E. faecalis* will fail to compete with pathogenic *E. coli* for adhesion to the intestinal epithelium. These results will define the identity and mechanisms by which beneficial and deleterious enterococci are likely to promote survival versus death of foster-age kittens. [redacted]

Data Analyses For all experiments a minimum of 3-5 replicates will be performed. Positive and negative controls will be used in each assay, including our isolates of *E. faecium* cultured from the commercial probiotics. All data will be tested for normality and equal variance followed by parametric or non-parametric statistical comparison using a commercial software package (SigmaStat, Jandell Scientific). For most experiments we plan on testing all of the isolates we have. Given that the study from which the isolates were obtained was sufficiently powered to have shown statistically significant differences, we do not propose obtaining additional isolates for inclusion in the current study.

Pitfalls and Alternative approaches Most of the techniques we propose have been done in our laboratories already or are well described. Some of the more novel approaches, particularly those using intestinal epithelial cells will likely need some tweaking, including dose-response and time-course preliminary studies to establish our model system. It would be ideal to examine biofilm formation and pathogenic effects of the enterococci on feline intestinal epithelial cells (IEC) rather than porcine cells. The porcine cells are not an unreasonable alternative, as they are non-transformed and biofilm formation by enterococci in piglet gut has been well described (R). We are currently establishing methods to culture feline IEC in our laboratory and if we are able, such cells will be included in the planned experiments.

Timeline We anticipate completing the studies in 1 year. This is particularly do-able because of participation by 3 laboratories that will be capable of conducting their respective studies simultaneously on the same isolates.

Project Budget

Supplies	
Culture of *Enterococci* from frozen cryopreservates	
Blood agar plates (VWR #101319-486) 750 plates	$ 1,000.00
LB agar plates x 200	$ 400.00
Broth M17 (Fisher #OXCM0817B) 500 g x 2	$ 161.22
Misc culture supplies (loops, cryo-vials, DMSO etc)	$ 500.00
Identification of unknown *Enterococcus* spp. by *SodA* PCR	
DNA extractions	
164 extractions @ $5.77 each	$ 946.28
InstaGene Matrix (BioRad 732-6030) 2 @ $50.00 each	$ 100.00
Polymerase Chain Reaction - *SodA*	
SodA primers (100 nM – Bio-Synthesis Inc.)	$ 128.00
PCR assay reagents = 164 reactions @ $3.10 each	$ 508.00
Gel Electrophoresis = 200 PCR reactions/54 reactions per gel = 4 gels@ $6.24 each	$ 24.96
Purification of amplicons for DNA sequencing (Qiaquick PCR purification kit)	$ 464.00
Biofilm formation	
Polystyrene 96 well plates (Corning 3799) 476 isolates in 5 replicates each both with and without IPEC cells including positive and negative controls, and competition experiments = 200 plates	$ 330.00
Crystal violet stain	$ 100.00
CFSE stain (Invitrogen #C34554) 1 mg	$ 276.00
Gelatinase assays	
Todd-Hewett broth (gelatinase activity), skim milk and culture plates	$ 500.00
Multiplex PCR	
DNA extractions	
312 extractions @ $5.77 each	$ 1,800.24
InstaGene Matrix (BioRad 732-6030) 3 @ $50.00 each	$ 150.00
Polymerase Chain Reaction - gelE, cylA, asa1, and esp	
gelE, cylA, asa1, and esp primers @ $30.00 per pair	$ 120.00
PCR assay reagents = 1,248 reactions @ $3.10 each	$ 3,868.80
Gel Electrophoresis = 1,248 PCR reactions/54 reactions per gel = 23 gels@ $6.24 each	$ 144.21
Metabolic pathogenicity and antimicrobial susceptibility profiles	
BioLog GEN III Microplate @ $25.00 each x 54 isolates	$ 1,350.00
Sensititre Gram positive NARMS plate @ $6.00 x 54 isolates	$ 324.00
Barrier function	
Polycarbonate membranes (Fisher PIHP01250) – 24-well plate x 100	$ 192.24
IPEC cell culture media and supplies	$ 1,000.00
Services	
DNA Sequencing of *SodA* amplicons	
Estimate 164 bidirectional reactions @ $6.00 each	$1,968.00
Histopathology/Electron microscopy	$ 500.00
Technical Support	
Research Technician Salary + 25% fringe @ 10% effort	$ 5,000.00
TOTAL FUNDING REQUESTED	**$21,885.95**

Other Support

There are no current or pending sources of funding for this project. [redacted]

Key Personnel

Investigator	Role on Project	% Effort	Salary Requested	Responsibilities
Maria Stone	Technician	[redacted]	[redacted]	**Research Technician.** Bacterial cultures, IPEC cultures, DNA extractions, PCR, biofilm assays, competition experiments, data entry.
Jody Gookin	PI	[redacted]	[redacted]	**Gastroenterologist.** Assist with barrier function studies and biofilm studies. Compile, interpret and report results of study.
Ludek Zurek	Co-PI	[redacted]	[redacted]	**Microbiologist with expertise in *Enterococcus* spp. biofilm formation.** Biofilm formation on polystyrene, gelatinase assays, multiplex PCR virulence factor assays.
Luke Borst	Co-I	[redacted]	[redacted]	**Pathologist/Microbiologist.** Metabolic phenotyping and antimicrobial susceptibility testing.

[redacted]

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