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<tr><th colspan="6">Standard Operating Procedure</th></tr>
<tr><td colspan="2">Your Institution: The University of Arizona</td><td colspan="4">Short Title: C. diff control using probiotics</td></tr>
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<tr><td colspan="2">Title: Evaluating The Efficacy Of The Probiotic DiffBlok To Control Clostridium Difficile Using A Neonatal Pig Model</td><td colspan="4">Emergency Contact Information:
PI: Mikaylah Rivera, 915-321-9876
Attending Veterinarian: John Doe, 520-123-4569</td></tr>
<tr><td>Version</td><td>1</td><td colspan="2">Created on</td><td colspan="2">4/20/2026</td></tr>
<tr><td>Status</td><td>Waiting approval</td><td colspan="2">Biosafety Level</td><td colspan="2">BSL-2</td></tr>
<tr><td colspan="6">Purpose and field of application
The purpose of this standard operating procedure is to establish a standardized and reproducible method for using the neonatal pig as a translational model for biomedical research to evaluate the therapeutic potential and efficacy that our probiotic has against Clostridium Difficile infections.</td></tr>
<tr><td colspan="2">Author</td><td colspan="4">Mikaylah Rivera, student</td></tr>
<tr><td colspan="2">Approved by</td><td colspan="4">Name, title, signature</td></tr>
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(University of California, San Francisco, 2024; University of Southern California, 2024)

Rationale

In this study we aim to evaluate the probiotic DiffBlok as an enhancing intervention to support gut health and to lessen the severity of Clostridium difficile infections. Colostrum-deficient neonatal piglets are very vulnerable to severe health complications and face high mortality rates due to their lack of passive immunity and crucial nutrients initially received with colostrum intake. To justify the animal model choice, the neonatal pig was selected as the for this experiment because of its unique susceptibility C. difficile"s toxins; toxin A (TcdA), and toxin B (TcdB) (Nyblade et al., 2022). These toxins are important virulence factors that affect the intestinal epithelial cells directly and appears in clinical manifestations such as watery diarrhea and edema of the mesocolon, and lesions, closely resembling how the disease manifests in humans (Steele et al., 2010). To further rationalize, the pig's gastrointestinal tract, nutritional requirements, and immune development closely mimic that of a human infant (Lunney et al., 2021; Walters and Prather, 2013). These structural and pathological similarities ensure that findings regarding probiotic colonization and toxin neutralization can be trusted and are highly reliable, and can potentially be transferable for human clinical research and medicine.

Objective

The primary objective of this study is to evaluate the efficacy and potential of the daily administration of the probiotic DiffBlok to control infection by Clostridium difficile and severity in a colostrum-deprived neonatal pig model.

Methods

Subject Animals:

The models that will be used for the experiment will be conventional neonatal Yorkshire-cross *(Sus scrofa domesticus*) pigs that are colostrum-deprived. The total animals in the study is 40 piglets. The total number of piglets were determined using power analysis to increase statistical value and reduce for the 16 day long study, additionally because the piglets are colostrum deprived, the take rate is higher thus less piglets should be used. The piglets will be 0 days old when obtained, with an average weight around 1.2-1.8kg. These pigs will be obtained/caught at birth at the University of Arizona Swine Unit to minimize microbial exposure and ensure colostrum deprivation. These piglets will be moved to the Campbell Ave. farm. To justify, the neonatal pig was chosen as the model for this experiment because of its unique susceptibility to C. difficile toxin A (TcdA), and toxin B (TcdB) (Nyblade et al., 2022). These toxins are important virulence factors that affect the intestinal epithelial cells directly and results in clinical manifestations (e.g., watery diarrhea and edema of the mesocolon, and lesions) that closely resemble how the disease manifests in humans (Steele et al., 2010). Additionally, they are a better choice compared to rodent models that usually require extensive antibiotic pretreatment to overcome the resistance of C. difficile colonization and likely fails to replicate the characteristic of colon lesions found in humans (Steele et al., 2010). This makes them functional

animal models to investigate the effects that virulence attributes play, drug efficacy and evaluation of probiotic efficacy.

*C. Difficile*Inoculum Preparation and Genotype:

To simulate a highly relevant clinical challenge, the inoculum will consist of Clostridium difficile PCR Ribotype 078 (RT078 / Toxinotype V). This specific hypervirulent genotype is selected because it is the predominant strain responsible for up to 90% of naturally occurring C. difficile enteritis outbreaks in neonatal piglets globally. It also holds strong zoonotic significance from a One Health perspective due to its overlapping prevalence between swine operations and community-acquired infections in humans.

Housing:

The piglets will be housed at the Campbell Ave. farm individually in their own custom stainless steel metabolism cages, measuring 2.0 ft × 2.5 ft (providing 5.0 sq ft of floor space per animal). To prevent the cross-contamination of microbiota and ensure accurate, individual fecal sample collection, the housing allocation will strictly be 1 piglet per cage. Since these colostrum-deprived piglets lack the body fat required for autonomous thermoregulation, climate control will be strictly enforced by keeping the room temperature between 85°F and 95°F using overhead ceiling-mounted heat lamps. Cages will be sanitized and washed daily by laboratory personnel equipped with appropriate personal protective equipment (PPE).

Husbandry:

For feeding, piglets will be bottle-fed for the first 24 hours using a specialized milk replacement every hour and a half up to two hours, followed by a transition to feeding every 4 hours then to a regular feeding schedule through ad libitum for the remainder of the study. Due to the need for feeding and monitoring of the piglets, laboratory staff will be on-site 24 hours a day.

Experimental Procedure:

Groups:

To achieve statistically valid results while minimizing animal usage, a power analysis was conducted using G*Power software based on effect sizes from historical neonatal piglet C. difficile challenge data (Nyblade et al., 2022). Setting the power beta at 0.80, alpha at 0.05, and assuming a large anticipated effect size ($f = 0.40$) for clinical diarrhea scores and mucosal lesion severity, a minimum sample size of 8 piglets per treatment group is statistically required to detect significant therapeutic differences ($p < 0.05$) among the cohorts.

To control for sex-based physiological variations in early immune responses and weight gain, an equal distribution of males and females will be maintained across all treatment arms. Litters will be balanced across groups immediately upon arrival to eliminate confounding litter-matched maternal variables.

The animals (N = 24 total) will be randomly assigned to one of three specific treatment cohorts:

- **Group 1**: Negative Control (n = 8)
  Sex Distribution: 4 Males / 4 Females
  Protocol: Colostrum-deprived, sham-challenged with sterile saline, and fed standard milk replacer daily.

- **Group 2**: Positive Control (n = 8)
  Sex Distribution: 4 Males / 4 Females
  Protocol: Colostrum-deprived, challenged with Clostridium difficile spores on Day 2, and fed standard milk replacer daily without probiotic support.

- **Group 3**: Probiotic Treatment (n = 8)
  Sex Distribution: 4 Males / 4 Females
  Protocol: Colostrum-deprived, challenged with Clostridium difficile spores on Day 2, and administered the probiotic DiffBlok daily via the milk replacer.

- Rectal swabbing technique

Secure piglets in handler cradles
Use sterile PBS-moistened cotton swab
Insert cotton tip length inside rectum
Use a Gentle rolling motion during removal
Return swab to sterile cover for analysis

- Administration and dosage of DiffBlock

The probiotic DiffBlok will be administered continuously throughout the entire experimental period, rather than as a single dose, to ensure stable gastrointestinal colonization. Beginning on Day 1 (immediately following initial arrival and baseline checks) and continuing daily through Day 7, each piglet in Group 3 will receive the probiotic treatment.

The probiotic will be thoroughly dissolved and mixed into the milk replacer during the first morning feeding of each day. The precise dosing and volume parameters are structured as follows:

- **Probiotic Dosage**: Each treated piglet will receive a standardized daily dose of 1.0×10^9 Colony Forming Units (CFU) of DiffBlok.
- **Volumetric Preparation:** The daily 1.0×10^9 CFU dose will be completely suspended in 10 mL of sterile, lukewarm water.

- **Milk Integration**: This 10 mL probiotic suspension will be mixed directly into the piglet's first scheduled morning milk replacer allotment (the standard volume of milk per feeding is 50 mL on Day 1, scaling up to 120 mL by Day 7 based on body weight).
- **Administration Frequency:** Probiotic integration occurs once daily during the 06:00 feeding. The remaining feeding blocks throughout the day will consist of unsupplemented, standard milk replacer to ensure total volume intake is not disrupted.
- Verification: Personnel will visually confirm that the piglet completely consumes the probiotic-infused milk volume before leaving the housing unit.

Timeline and feeding protocol:

Table 1. Sample Collection Schedule

Study Day	Experimental Phase	Group 1: Negative Control (n=8)	Group 2: Positive Control (n=8)	Group 3: Probiotic Treatment (n=8)	Collection Matrix & Diagnostic Purpose
Day 1	Baseline Arrival	Baseline Screening	Baseline Screening	Baseline Screening	Rectal Swab: Baseline PCR to confirm absence of native C. diff or existing pathogens.
Day 2	Challenge Day	Sham Challenge (Saline)	C. diff RT078 Oral Inoculation	C. diff RT078 Oral Inoculation + Probiotic	None: Give piglets 12 hours post-challenge to stabilize before sampling.
Day 3	Acute Phase (24h PI*)	Clinical Observation	Clinical Observation	Clinical Observation	Fecal Score & Swab: Visual scoring (1–4); PCR / Culture to verify C. diff colonization.
Day 4	Acute Phase (48h PI)	Clinical Observation	Clinical Observation	Clinical Observation	Fecal Score & Swab: Visual scoring; ELISA testing for free Toxin A and Toxin B

					(\(TcdA/TcdB\)).
Day 5	Mid-Study Monitoring	Clinical Observation	Clinical Observation	Clinical Observation	Fecal Score Only: Visual monitoring for consistency changes or onset of watery diarrhea.
Day 6	Mid-Study Monitoring	Clinical Observation	Clinical Observation	Clinical Observation	Fecal Score & Swab: Visual scoring; Probiotic plate counts (Group 3) to verify gut colonization.
Day 7	Study Termination	Euthanasia & Necropsy	Euthanasia & Necropsy	Euthanasia & Necropsy	Tissue & Digesta: Cecal/colonic digesta for toxin load; Intestinal tissue for histopathology (mesocolonic edema/lesions).

PI = Post-Inoculation

Day-by-Day Experimental Tasks

Day 1: Baseline Arrival, Group Allocation, and Pre-Screening

- 06:00 – Feeding & Baseline Probiotic: Administer initial milk replacer feed (50 mL). For Group 3 piglets, mix the initial (1.0×10^9) CFU dose of DiffBlok probiotic (dissolved in 10 mL sterile water) directly into this morning milk volume. Visually confirm complete ingestion.
- 09:00 – Health Assessment: Conduct initial physical examinations, record baseline body weights, and apply unique ear tags for individual identification.

- 10:00 – Baseline Sampling: Collect individual rectal swabs from all piglets across Groups 1, 2, and 3. Submit samples for baseline PCR testing to confirm the total absence of native *C. difficile* or pre-existing enteric pathogens.
- 12:00, 18:00, 24:00 – Routine Husbandry: Provide standard, unsupplemented milk replacer feedings. Monitor room temperature to ensure it remains stable between 85°F and 95°F.

Day 2: Pathogen Challenge (Inoculation Day)

- 06:00 – Feeding & Routine Probiotic: Administer morning milk replacer. Group 3 receives its daily morning DiffBlok probiotic supplement mixed into the milk.
- 08:00 – Inoculum Preparation: Thaw and prepare the *C. difficile* PCR Ribotype 078 (RT078) master seed stock spores in sterile 0.9% physiological saline to a concentration of (1.0×10^6) spores/mL.
- 09:00 – Pathogen Administration: Administer a single 2.0 mL oral dose of the *C. difficile* RT078 spore suspension (2.0×10^6) total spores) to Group 2 (Positive Control) and Group 3 (Probiotic Treatment) via a syringe fitted with a flexible oral dosing catheter. Group 1 (Negative Control) receives a 2.0 mL sham dose of sterile 0.9% saline.
- 12:00, 18:00, 24:00 – Post-Challenge Care: Complete standard feeding rounds. Do not perform any invasive sampling or swabs for the next 12 hours to allow the piglets to stabilize post-inoculation.

Day 3: Acute Phase Monitoring (24 hours Post-Inoculation)

- 06:00 – Feeding & Routine Probiotic: Deliver morning milk feeds. Mix the daily DiffBlok probiotic dose into the Group 3 morning milk allocation.
- 08:00 – Clinical & Visual Inspection: Score the consistency of any fecal output using the visual 1–4 scale. Record systemic clinical parameters, including lethargy, hydration status, and responsiveness.
- 10:00 – Inoculation Verification Sampling: Collect rectal swabs from all animals. Submit samples for diagnostic PCR and anaerobic bacterial culture to verify successful *C. difficile* colonization in Groups 2 and 3, and to confirm Group 1 remains uninfected.
- 12:00, 18:00, 24:00 – Husbandry & Sanitation: Perform standard feeding rotations. Clean and sanitize all individual stainless steel metabolism cages to prevent environmental cross-contamination of shed spores.

Day 4: Peak Acute Phase & Toxin Assessment (48 hours Post-Inoculation)

- 06:00 – Feeding & Routine Probiotic: Administer morning milk replacer, including the daily DiffBlok probiotic dose for the Group 3 cohort.

- 08:00 – Clinical Assessment: Perform physical exams and record fecal scores. Watch closely for signs of severe clinical manifestations, such as acute watery diarrhea or abdominal distension.
- 10:00 – Toxin Bioassay Sampling: Collect fresh fecal samples via rectal swabs from all piglets. Submit samples directly to the laboratory for Enzyme-Linked Immunosorbent Assay (ELISA) testing to quantify the concentration of free Toxin A (\(TcdA\)) and Toxin B (\(TcdB\)).
- 12:00, 18:00, 24:00 – Routine Husbandry: Provide standard unsupplemented milk feedings. Monitor heat lamps continuously to maintain strict thermal support for sick animals.

Day 5: Mid-Study Clinical Monitoring

- 06:00 – Feeding & Routine Probiotic: Execute morning feeding routines, incorporating the daily DiffBlok probiotic treatment into the morning milk for Group 3.
- 08:00 – Visual Fecal Scoring: Inspect all cages and assign visual fecal scores (1–4) to track ongoing diarrhea trends, recovery indicators, or disease progression.
- 10:00 – Weight Check: Record mid-study individual body weights to track hydration status and nutritional maintenance.
- 12:00, 18:00, 24:00 – Husbandry & Spot Cleaning: Complete scheduled unsupplemented feeding blocks. Spot-clean cages immediately if diarrhea fouling occurs to keep the resting surfaces clean and dry.

Day 6: Colonization Dynamics Tracking

- 06:00 – Feeding & Routine Probiotic: Provide morning milk feeds. Supplement the Group 3 morning milk volume with its daily DiffBlok probiotic dose.
- 08:00 – Clinical Monitoring: Perform routine physical exams and document visual fecal scores across all experimental groups.
- 10:00 – Probiotic Colonization Sampling: Collect rectal swabs from all piglets. Submit Group 3 samples for selective bacterial plate counts to quantify and verify live probiotic colonization in the gut. Run parallel PCR screens on Groups 1 and 2 to monitor ongoing shed dynamics.
- 12:00, 18:00, 24:00 – Husbandry: Complete standard unsupplemented feeding blocks. Maintain high-level PPE protocols to avoid transferring pathogens between cages during feeding.

Day 7: Study Termination, Necropsy, and Tissue Collection

- 06:00 – Final Feeding & Weight Verification: Administer the final morning milk replacer feeding (including the final Group 3 probiotic dose). Record final endpoint body weights for all 24 piglets.
- 08:00 – Final Live Assessments: Document final clinical health scores and visual fecal scores for each animal.
- 09:00 – Humane Euthanasia: Euthanize piglets sequentially using an institutional-approved protocol (e.g., sodium pentobarbital overdose administered intravenously).
- 10:00 – Necropsy & Gross Pathology: Perform immediate post-mortem examinations. Inspect the intestinal tract to score gross pathological lesions and note any characteristic edema of the mesocolon.
- 11:00 – Core Matrix Sample Collection: Extract cecal and colonic digesta samples to analyze final luminal toxin loads. Harvest formal tissue sections from the ileum, cecum, and colon, and place them immediately into 10% neutral buffered formalin for histopathological evaluation (measuring mucosal lesions and crypt depths).

<u>Recording Results</u>

- Rectal swabbing for pathogen shedding

Conduct method between day 7 to day 16 for initial screening and post challenge duration. Sterile swabs are inserted rectally to collect fecal matter then samples are immediately diluted in PBS and plated in triplicate on CEFEX selective agar. For data analysis, the results are recorded as log10 CFU/gram of feces. A One-way ANOVA will be used to compare the mean shedding levels between the treatment and positive Control groups at each of the time points.

- ELISA testing for C. diff toxin quantification

Conducted on day 16 during necropsy where the luminal contents from the colon and cecum are harvested and samples are snap- frozen at 80°C. For data analysis samples are thawed out and processed with an ELISA kit to quantify TcdA and TcdB toxins. These toxin concentrations using units ng/ml, will be compared across all groups that were challenged using ANOVA in order to determine the extent of the neutralizing effects of DiffBlok on the toxin production in vivo. Success is defined as a statistically significant reduction in toxin levels of concentration in the probiotic group versus the positive control group.

- Necropsy and tissue examination for GI tract lesions

  On day 16 during the terminal necropsy the PI will conduct the gross examination and score the severity of edema and lesions on a scale grade 0-3. The microscopic damage will be evaluated by first harvesting 1cm sections of the colon and cecum that is fixed in 10% neutral buffered formalin for analysis. Tissues will be stained with hematoxylin and eosin and evaluated blindly by our lab pathologist to measure the mucosal hyperplasia,

determined by measuring intestinal crypt depth in micrometers (Steele et al., 2010). The statistical significance will result from comparing the mean crypt depth in micrometers across all groups on a one-way ANOVA followed by a HSD test to identify the specific significant differences between said groups post ANOVA.

- Score grading for lesions and ANOVA guide for groups:

G0 - No lesions and no visible edema, thin and transparent mesocolon
G 1 -+ mild lesions, slight swelling or clear fluid that has accumulated within colon coils
G2 -++ moderate lesions, spread out swelling, noticeably thick, fluid-filled mesocolon
G3-+++ severe lesions, extensive edema and expansion of mesocolon

Monitoring and sampling:

Milk consumption will be checked every four hours after the first 24 hour period and weight will be recorded in the mornings before feeding daily. Clinical health check-ins will also be conducted at a frequency of every four hours post-challenged. Symptom tracking for clinical manifestation of *C. difficile.* Symptom tracking will be performed by daily weight recording to ensure weight gain and intake. For dehydration which will be measured through performing a skin tugger test where the nape is pinched. Visual assessments and scoring of stools will be performed to check for watery or bloody diarrhea.

- Clinical/health Scoring scale 1-4:

1. Normal: Active, normal stool.

2. Mild: Soft stool, slightly reduced activity.

3. Moderate: Watery diarrhea, skin turgor >2s, lethargy.

4. (Severe/Humane Endpoint) yellow watery diarrheal, inability to stand, >10% weight loss, or non-responsive to stimulus, foggy eyes.

For humane Endpoints animals reaching a score of 4 will be immediately euthanized by CO2 inhalation.

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