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Probiotic studies in neonatal mice using gavage

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Cover Letter

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September 7, 18

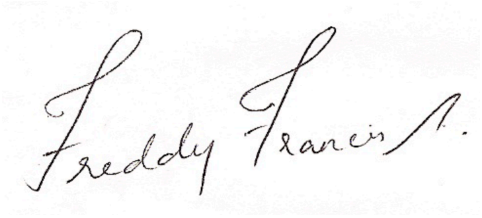
To:
Dr. Jaydev Upponi
Science Editor | Immunology and Infection
Editorial Department, JoVE
1 Alewife Center | Suite 200 | Cambridge | MA 02140 | USA

We would like to submit to you for publication in *JoVE Immunology and Infection* our invited technical methods entitled **'Experimental considerations for probiotic studies in neonatal mice using gavage.'** by Freddy Francis, Natallia Varankovich, Byron Brook, Nelly Amenyogbe, Rym Ben-Othman, Bing Cai, Danny Harbeson, Aaron C. Liu, Ben Dai, Shelly McErlane , Kris Andrews, Tobias R. Kollmann and Pinaki Panigrahi. This work presents the intricacies of developing an experiment using probiotic in neonatal mice. The use of probiotics in early life has shown promising results and the proper establishment of a mouse model can assist in unpacking the details behind the protective effects.

This manuscript has not been published previously and is being submitted only to *JoVE Immunology and Infection*. It will not be submitted elsewhere while under consideration and, should it be published in *JoVE Immunology and Infection*, it will not be published elsewhere – either in similar form or verbatim – without permission of the editors. All authors are responsible for reported analysis and have participated in the concept and design, analysis and interpretation, drafting or revising, and have approved this manuscript as submitted.

If there is anything else we can do to help with this manuscript submission, please do let us know.

Yours sincerely,



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TITLE:

Probiotic Studies in Neonatal Mice Using Gavage

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KEYWORDS:

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SUMMARY:

This study details the process of gavaging precise amounts of probiotics to neonatal mice. The experimental set-up was optimized to include but is not limited to probiotic dosing, methods of administration, and quantification of bacteria in the intestines.

ABSTRACT:

Adult mouse models have been widely used to understand the mechanism behind disease progression in humans. The applicability of studies done in adult mouse models to neonatal diseases is limited. To better understand disease progression, host responses and long-term impact of interventions in neonates, a neonatal mouse model likely is a better fit. The sparse use of neonatal mouse models can in part be attributed to the technical difficulties of working with these small animals. A neonatal mouse model was developed to determine the effects of probiotic administration in early life and to specifically assess the ability to establish colonization in the newborn mouse intestinal tract. Specifically, to assess probiotic colonization in the neonatal mouse, *Lactobacillus plantarum* (LP) was delivered directly into the neonatal mouse gastrointestinal tract. To this end, LP was administered to mice by feeding through intra-esophageal (IE) gavage. A highly reproducible method was developed to standardize the process of IE gavage that allows an accurate administration of probiotic dosages while minimizing trauma, an aspect particularly important given the fragility of newborn mice. Limitations of this process include possibilities of esophageal irritation or damage and aspiration if gavaged incorrectly. This approach represents an improvement on current practices because IE gavage into the distal esophagus reduces the chances of aspiration. Following gavage, the colonization profile of the probiotic was traced using quantitative polymerase chain reaction (qPCR) of the extracted intestinal DNA with LP specific primers. Different litter settings and cage management techniques were used to assess the potential for colonization-spread. The protocol details the intricacies of IE neonatal mouse gavage and subsequent colonization quantification with LP.

INTRODUCTION:

In infants, early probiotic exposure has been associated with immunomodulatory effects leading to reduced incidence of diseases like necrotizing enterocolitis, atopic dermatitis and sepsis[1–5]. However, the mechanism behind this immunomodulatory response is challenging to explore given the limitation to sampling in newborn human trials (i.e., sequential blood draws and biopsies). Neonatal mouse models can help study the mechanism of action involved in neonatal immune regulation associated with probiotic use and changes in the intestinal microbiota. Unfortunately, most mouse models for probiotics have largely focused on adult mice; however, the impact of probiotics is likely to be highest early in life, suggesting models specific for this age group will be useful[3,6]. In addition, neonatal mouse models are better suited to study diseases and interventions intended for application in early life of human infants as they are expected to more closely mimic a developing immune and microbial system[7,8,9,10]. The aim was to study the extent and patterns of probiotic colonization of neonatal mice with a focus on the mechanistic interaction between the host and its microbiome. Suitable descriptions of newborn models were not found in the literature, and thus a need for the development of robust and standardized method was addressed.

Established methods of oral administration of various compounds to newborn mice include maternal transfer of desired compounds through milk by treating the water source for pregnant dams[11] or using feeding needles to facilitate administration of desired compounds into the oropharynx[12]. These methods are useful for experiments that do not have precise dosage requirements and where the treatment is readily ingested by the recipient mouse. Probiotics

are often administered in conjunction with a prebiotic such as galactooligosaccharide and fructooligosaccharide (FOS) that serve as a source of nutrition for probiotic bacteria; these additive compounds make the solution viscous and challenging to administer via the above-mentioned methodologies. Devising a method to administer precise amounts of probiotics and prebiotics to newborn mice starting as early as the first day of life (DOL) was necessary. In the process of developing the gavage technique, the possibility of colonization-spread (as observed in other probiotic studies between the treatment and the control arms[13–16]) was tested and the relative abundance of colonized *Lactobacillus plantarum* (LP) in the intestines of pups with different gavage schedules was assessed. The probiotic preparation used in the experiments consisted of 10^9 colony-forming units (CFU) per gavage of LP (ATCC-202195 strain), mixed with FOS (prebiotic) and maltodextrin (excipient) as described in the recent human trial[3]. The probiotic delivery was accomplished using IE gavage and the process is detailed in the protocol below. The colonization profile of the probiotic was evaluated using real time amplification of DNA extracted from whole intestines using LP specific primers.

PROTOCOL:

All procedures were carried out pertaining to the guidelines established by the support staff at the Animal Care Facility at the University of British Columbia and all procedures were approved by the UBC Animal Care Committee.

1. Quantification of probiotics administered

NOTE: This step is recommended to determine the exact amount of probiotic CFU that can be administered in a single dose. The quantity of probiotics and vehicle (FOS and maltodextrin) determine the saturation conditions of the solution. From experience, no more than 30 µL (~20 mL per kg) of fluid can be administered to mice on DOL 2 as any greater volume increases risk of aspiration.

1.1 Prepare six 1.5 mL microcentrifuge tubes for serial dilutions with each tube containing 180 µL of sterile 5% dextrose saline.

1.2 Weigh a 0.2 g aliquot of a probiotic-prebiotic mixture and dissolve it in 1 mL of 5% dextrose saline in a sterile manner.

1.3 Vortex for 30 seconds and pipette to break clumps. Repeat until no visible clumps are observed.

NOTE: Maltodextrin makes the solution viscous and contribute to solution saturation.

1.4 Perform a serial dilution using tubes prepared in step 1.1. Vortex to mix.

1.5 Plate 40 µL of every dilution onto a labelled quadrant of the MRS agar plate. Plate each dilution in duplicate.

1.6 Incubate under anaerobic (or microaerophilic) conditions at 37 °C for 48 h in a vacuum jar using a gas pack.

1.7 Count each plate within a range of 20-70 colonies per quadrant. Average plate counts with the same dilution and calculate back to the desired units.

2. Preparation of probiotics and prebiotics for gavage

NOTE: The proper dissolution of probiotic and prebiotic is necessary to ensure the smooth injection of liquid through the feeding needle during gavage.

2.1 Combine the required amount of lyophilized probiotic organism with the desired amounts of prebiotics and vehicle in a sterile microcentrifuge tube.

2.2 Add appropriate amounts of solvent (5% dextrose saline) to dissolve the probiotic-prebiotic mixture.

NOTE: The capacity of dissolution is limited by the prebiotic and vehicle used. From experience, the synbiotic combination (with FOS and maltodextrin) reached saturation at approximately 0.3 g/mL while dissolving in a 2 mL microcentrifuge tube using 1 mL of solvent.

2.3 Vortex to mix until all solids are dissolved. Use a pipet to break up globules of solid particles in the solvent by pipetting up and down.

2.4 Incubate the solution in a 37 °C water bath for 20 min.

NOTE: This step can be skipped if the probiotic-prebiotic solution is created from a live culture.

2.5 Plate a five series 10-fold dilution on MRS agar plates before gavage to accurately quantify the probiotic administered to the pup. This step can be skipped if the accurate CFU count is not needed.

3. Preparation of the biosafety cabinet

3.1 Use a biosafety cabinet when working with probiotics to maintain aseptic technique. Set the cage with the dams and pups on a heating blanket (set to approximately 38 °C) on one half of the blanket. Place a clean, empty animal cage on the other half of the heating blanket.

3.2 Place a disinfected or sterile, absorbent pad on the heating blanket to tend to the mouse during gavage.

3.3 Collect nesting material for the pups from the top of the existing, dam-created nest and create a new conical nesting cup using gloved hands, disinfected using 70% ethanol and dried.

Place this new nest in the clean, empty holding cage. This facilitates the transfer of the scent of the nest to the gloved hands and thus minimizes the introduction of other scents on the pup while handling them for the procedure, reducing the risk of cannibalization.

3.4 Move the pups into the conical nest holding cage and remove the cage with the dam from the cabinet. This decreases the stress for the dam by preventing it from hearing the pups during the procedure.

NOTE: If the probiotic is a known colonizer of the murine intestines, the treatment conditions must be separated by cages or even different biosafety cabinets to avoid the possibility of cross colonization.

4. Intra-esophageal gavage of neonatal mouse

4.1 Open the syringe packaging for easy access. Open the packing of the needle in a sterile manner and attach it to the head of the syringe. Wash the needle with 70% ethanol and autoclave prior to the procedure. Use different sets of needles for the treatment and the control group to avoid contamination.

4.2 Draw a little more than the desired amount of probiotic-dye solution into the syringe. Hold the syringe facing up. Then pull down further and flick with finger to dislodge bubbles and push on the plunger to expel bubbles and the extra liquid volume until the desired volume is reached. This ensures that there is no air space in the needle. For DOL 2 mouse, the volume of gavage must not exceed 30 µL.

4.3 Place the pup onto the sterile absorbent pad on top of the heating pad. Use the feeding needle (24 Gauge, 1" needle length, 1.25 mm ball diameter) externally to measure the length of the esophagus by placing the ball of the needle just below the xiphoid process (bottom end of the sternum). Mark the needle at the level of the snout to note the limit of insertion of the needle (**Figure 1**). Observe the pup for health signs, which include regular breathing and pink coloration of the skin.

4.4 Dip the tip of the needle in the dipping solvent (5% dextrose saline or - the medium used to dissolve the probiotic and pre-biotic) to lubricate the external surfaces of the feeding needle. This facilitates the smooth entry of the needle into the esophagus of the mouse.

4.5 Lift the pup by the scruff or by gently holding the head and body between the thumb and index finger. Ensure the head, neck and body are held in a straight position. Do not hold the pup by the scruff for longer than 60 s as there is a risk of obstruction of the trachea leading to suffocation. Ensure the pup can breathe. Signs of scruffing too hard can include inability to breathe, significant gasping and the tongue extended out the mouth. Monitor the pup's color and breathing during the entire procedure.

4.6 Insert the bulb of the needle into the center of the mouth of the pup at a 45° angle to the plane of the torso until it reaches the back of the throat.

4.7 Gently change the angle of the needle by pivoting on the bulb of the needle and moving the syringe away from the gavaging person (towards the dorsal side of the pup) until it is parallel to the plane of the pup's vertebral column. Scruffing the pup helps keep the needle in place in the back of the throat, and also prevents the mouse from squirming. Make sure the ball of the needle does not advance or exert any pressure against the back of the throat during the angle change.

4.8 If the mouse attempts to swallow the needle, allow it to naturally slide downward and arrest the movement when the marking on the needle aligns with the snout. The syringe and needle are usually heavy enough to slide down the due to gravity. Support the weight of the needle at all times so the needle slides easily down the esophagus with no downward pressure from the person carrying out the gavage.

4.7.1 If the needle meets resistance in the back of the throat, withdraw the gavage needle slightly to dislodge the ball of the needle and re-angle the needle inside the mouth towards left of the mouse (handler's right) slowly in small, 1 mm increments. The needle should start to slide easily down the esophagus.

4.7.2 If the needle stops before the marking on needle reaches the mouth, do not inject the solution.

4.7.3 Do not keep the needle inserted for more than 20 s. If this occurs, retract the needle slowly while keeping the syringe parallel to the torso and let the pup rest on the paper towel for 30 s to 1 min. Try gavaging again after lubricating the external surface of the needle with the solvent.

NOTE: Anaesthesia is not used for the procedure as the mouse's response is necessary to gauge the success of gavage.

4.9 When the marking on the feeding needle is above the snout and aligned with the tip of the snout, do not let the needle move or advance any further. Slowly inject the desired volume of liquid. If the liquid is aspirated or observed to bubble through the nose, stop the injection immediately and slowly retract the needle.

4.9.1 Place the pup upright on the paper towel on the heating pad to aid in its recovery. Monitor closely for continued breathing problems or change in color of the pup which indicates aspiration. Euthanize pups that have aspirated immediately.

4.10 Once the gavage is complete, gently withdraw the feeding needle at the same angle it was inserted. Place the pup on the paper towel on the warmed heating pad. Wait 10 s for the pup to regain normal activity and breathing pattern. A healthy pink hue should appear over the

pup's body and the dye should only be visible in the stomach compartment. Move it back to the cage with the other pups.

NOTE: Gavaging blue food coloring is an excellent way to practice the procedure outlined above. If the gavage is successful, the stomach of the mouse will be visible as a blue hue. If the blue dye is found outside the stomach of the pup (neck, chest or axillary region), the animal should be humanely euthanized (in accordance with the animal care rules), as this indicates a rupture of the esophagus or aspiration.

5. Collection of intestional samples for colonization analysis

5.1 During subsequent monitoring or gavaging, collect fecal microbiome samples from the pups.

NOTE: The pup frequently urinates and defecates when gavaged and this time can be used as an opportunity to collect the fecal samples for microbiome analysis.

5.2 For termination of experiments, collect the intestines from duodenum to rectum after euthanasia of the pups. Pin the pup to a surgical board and disinfect the skin with 70% ethanol. Cut away the skin into four quadrants without damaging the peritoneal layer using tools sterilized with 70% ethanol and a hot bead sterilization at 250 °C.

5.3 Use a different set of sterile tools to cut the peritoneum into four quadrants and move it away from the center in a way that the visceral organs are exposed.

5.4 Locate the stomach and use a clamp to pinch below the pyloric sphincter and at the end of rectum. Run the length of the intestine using a blunt tool or forceps to streamline the intestine and free it from the connective tissue and mesenteric tissue. Once the entire length of the intestine has been freed of connective tissue, cut at the clamped ends.

5.5 Mark the aluminum foil with the orientation of the intestine, wrap in a secure manner and freeze at -80 °C.

NOTE: The DNA extraction procedure can be carried out at this point without freezing. The blue dye was also seen to pass through the intestine over 24 h and collection of samples for colonization analysis is best when the intestines are collected at least 24 hours post the last gavage. Signals might be amplified before that timepoint by the non-adhered bacteria transiently passing through the gavage mixture.

6. DNA extraction from intestines for colonization analysis

NOTE: The DNA extraction is done using a commercial kit with optimizing modifications made to the protocol for the intestine DNA extraction. Ensure the heating apparatus is set to the

desired temperature and the solutions that need alterations or pre-warming are prepared appropriately.

6.1 Prepare the Enzymatic Lysis Buffer (ELB) as follows: Make a solution with 20 mM Tris-Cl, 2 mM sodium EDTA and 1.2% Triton X-100. Adjust the pH to 8.0. Immediately before using ELB, add lysozyme to a final concentration of 20 mg/mL.

6.2 Pre-weigh the garnet bead tubes on analytical balance with the caps removed.

NOTE: This is done so that if the required weight is overshot, it is easier to remove the intestinal contents.

6.3 Cut the intestines into small segments using a sterile disposable scalpel and scoop the desired segments into the pre-weighed garnet bead tubes.

NOTE: Make sure to change scalpels between every sample as DNA is ubiquitous and can affect PCR results.

6.4 Add 1 mL of ELB with lysozyme (from step 6.1) to each tube, place on the vortexing bead beater and run at maximum setting (14) for 5 minutes.

6.5 Once the tissue is disrupted, transfer the tubes to the 37 °C water bath and incubate for 30 minutes.

NOTE: This step is done to activate lysozyme and induce the breakdown of cell wall peptidoglycan of gram-positive bacteria.

6.6 Prepare tubes with 20 µL of Proteinase K for every sample at a concentration of 600 mAU per mL.

6.7 Centrifuge the tubes at 400 x *g* for 10 minutes. The lysate should look clear with some tissue residue on top of the beads.

6.8 Transfer 180 µL of supernatant (the upper phase) into a tube containing Proteinase K and then add 200 µL of AL buffer to the tube. Vortex for 15 s to mix.

6.9 Place tubes on the heating block at 56 °C for 10 minutes.

6.10 Add 200 µL of 100% ethanol to the tube and mix by vortexing for 15 s.

6.11 Add approximately 600 µL of lysate to the spin column (from kit).

6.12 Centrifuge for 1 minute at 8,000 x *g*. Discard flow through.

6.13 Repeat step 6.12 until all the lysate has been drawn through the column.

6.14 Place the column in a new collection tube. Add 500 µL of AW1 buffer and centrifuge at 8,000 x *g* for 1 minute.

6.15 Discard the flow through. Add 500 µL of AW2 buffer and centrifuge at 8,000 x *g* for 3 min.

6.16 Discard the flow through and centrifuge the column in an empty collection tube at 8,000 x *g* for 3 min.

6.17 Transfer the column to DNA elution tube. Add 60 µL of PCR grade ultrapure water directly onto the membrane and incubate for 2 minutes at room temperature.

6.18 Use the pre-warmed elution water at 37 °C to elute. The elution can be done twice by using half the final elution volume and repeating step 6.15 twice to increase yield.

6.19 Centrifuge for 1 minute at 8,000 x *g* to elute DNA.

6.20 Measure the concentration of the DNA eluted using the desired quantification method. The extraction process yields are in the range of 10-40 ng/µL DNA.

6.21 Store the eluted DNA at -20 °C.

7. qPCR setup

7.1 PCR Conditions

7.1.1 Turn on the machine and load the program in **Table 1** into a real time qPCR machine.

7.1.2 Loop steps 3 to 5 in **Table 1** for 40 cycles and hold the sample at 4 °C at the end of the reaction.

7.2 PCR experimental setup

7.2.1 Use the primers and temperature found in **Table 2**. Use the concentrations and reaction conditions found in **Table 3.** Set up each reaction in triplicate to control for procedural variation.

7.3 Place the PCR reaction tubes/plate in the qPCR system and the run the program loaded into the system from step 7.1.

7.4 Remove the tube at the end of the run, place it on 4 °C and prepare for gel loading.

8. Quantification of LP colonization

8.1 Prepare qPCR mixes for 10 µL or 20 µL reactions according to **Table 3**.

8.2 LP genomic DNA standard curve 10^7 to 10^1 copies/µL

NOTE: Since 4 µL of each dilution will be plated, 10^7 copies in 4 µL or 2.5 x 10^6 copies per µL is required in the starting stock. Use the same principle for the rest of the curve.

8.2.1 Prepare a 1:4 dilution: 10^7 copies per µL in 50 µL.
3.147 µL of LPDNA + 46.85 µL of dH_2O = 2.5 x 10^6 copies per µL

8.2.2 Serially dilute 10-fold: add 5 µL to 45 µL dH_2O for 1.25 x 10^5 copies/µL.

8.2.3 Plate 4 µL per dilution per well.

8.3 Visualization of LP amplicons

8.3.1 Use a 2% agarose gel to reach a clear separation of the ~ 197 bp LP amplified fragment.

8.3.2 Load 9 µL of each PCR product in the gel.

8.3.3 Run the gel for 30 minutes at 120 V.

REPRESENTATIVE RESULTS:

The uniqueness of this method rests in its adaptation of the gavaging technique to the size and frailty of a neonatal mouse. The previous section described the important steps in carrying out a successful gavage procedure on a DOL 2 mouse. To establish a good quantification scale, a standard curve was generated using pure LP DNA with three technical replicates (**Figure 2**). The standard curve provided a dynamic range of detection of the LP DNA using the primers. The dynamic range was between 7 and 28 cycles where a range of 10^1 to 10^7 copies of LP DNA was detected. The steady slope of the standard curve represented the efficiency and scalability of the reaction.

The procedure of IE gavage has been used in adult mice with relative ease. However, the upper gastrointestinal tract of a neonatal mouse is fragile and required calibrated movements of the gavage needle during the procedure. Repeated gavages could increase the chances of intra-esophageal irritation, injury and failure or rejection by the dam due to the handling. Thus, two different gavaging schedules were tested and the intestinal colonization was quantified using DNA from whole intestine homogenates. Mice were gavaged from DOL 2 through DOL 8 with probiotic administered every day or every two days (**Figure 3**). Each sample contained one technical replicate and every condition had at least two biological replicates. The pups gavaged every day with 7 doses had around 10^3 copies of LP whereas the pups gavaged every two days with 4 doses had around 10^5 copies. The consistency of results between the replicates add

credit to the precision of technique. There was more LP detected in intestines of pups gavaged every two days in comparison with pups that were gavaged every day. Given this, subsequent experiments were set up with a gavage schedule of every other day as it also reduces the stress for the pups.

It is important to avoid intra-litter probiotic cross contamination when working with probiotics. The microbiome of littermates was expected to be similar as they share the same mother and nesting environment. This proves a problem for probiotic studies if the treatment and control conditions were present within the same litter as the probiotic organism has the potential to become a part of the microbiota ("colonization spread'). To determine if a probiotic will contaminate and colonize untreated littermates, half of a litter was gavaged as above and the intestines were collected for qPCR. Intestinal qPCR analysis of DOL 10 mice showed expected amplification of LP DNA in the gavaged mice but also, to a lesser degree in the non-gavaged littermates (**Figure 4**). The intestines of the same DOL mice from an untreated cage showed no amplification or minimal amplification at cycles greater than 32. This provided evidence for the communal sharing of the microbiome within a litter in a cage. Thus, for experiments with probiotics the treatment groups should be separated by cages to control for variability through cross contamination. The use of foster dams can be considered if an experiment is to be set up within a litter setting, but confounding effects like diminished care from the foster dam and rejection should be evaluated and optimized for. When mice gavaged until DOL 8 were left untreated for six days and the intestinal DNA was analysed at DOL 14, approximately 10 copies of LP were found (**Figure 5**). Thus, the colonization of LP was found to be transient and the detectable population diminished over time.

FIGURE LEGENDS:

Figure 1. Measuring the length between the xiphoid process (lower end of the sternum) and the snout to make maximum insertion marking for the needle.

Figure 2. Standard curve established using LP primers and ATCC LP DNA. A serial dilution of the ATCC LP DNA was made to establish the dynamic detectable range for the primers used in the study.

Figure 3. LP amplification of intestinal DNA from DOL 10 pups treated between DOL 2 and DOL 8 in scheduled gavages every day (7 doses) and every other day (4 doses). Gavaging every other day showed higher intestinal LP in comparison with gavaging every day.

Figure 4. LP amplification of intestinal DNA from DOL 10 pups with 2 treated and 2 untreated in a litter of 4 pups. The gavage was between DOL 2 and DOL 8 in scheduled gavages every day (7 doses). The two probiotic treated pups show the expected amplification profile. The untreated pups show variable amplification of LP indicating communal sharing of the probiotic organism within a litter.

Figure 5. LP amplification of intestinal DNA from DOL 14 pups treated between DOL 2 and DOL 8 in scheduled gavages every day (7 doses) and every other day (4 doses). The LP load drops below cycle 28 indicating clearance of LP over the course of 6 days post last probiotic gavage.

TABLE LEGENDS:

Table 1. qPCR amplification conditions. The temperature and number of cycle conditions for the PCR reaction.

Table 2. Details of components of the qPCR reaction. The details on the primers, their annealing temperature and expected fragment size in the PCR reaction.

Table 3. Per reaction volumes and concentrations. The concentration of reagents and volumes for reactions.

DISCUSSION:

The procedure of IE gavage was developed to safely administer a specific dose of a probiotic to neonatal mice. Small amounts of liquid are delivered to the upper gastrointestinal tract using a feeding needle to prevent aspiration while ensuring the delivery of the dosage in confidence. The intestines of the mice were collected for colonization analysis two and six days post gavage. The procedure for DNA extraction was modified to ensure high yield of the probiotic Gram-positive organism. The qPCR analysis of the DNA extracted two days post last gavage showed relatively higher colonization of LP in mice gavaged every two days in comparison to mice gavaged every day between DOL 2-8. There was also a decrease in the amount of LP over six days, showing this probiotic to be a transient organism in the intestines of the mouse. The results of these experiments establish the conditions to conduct research with high rigor in this age group.

To observe the long-term effects of probiotics in neonatal mice, it was administered to neonatal mice on DOL 2; a similar starting time point to the human trial. Oropharyngeal feeding of neonatal mice is previously described in literature and has been carried out only after DOL 5-8[12,17] when the risk of aspiration is lower due to a well-developed swallowing mechanics. However, oropharyngeal feeding is not well suited for DOL 2 mice as higher rates of aspiration were observed in the pilot study (data not shown). The viscous nature of the probiotic and prebiotic solution added to the risk of aspiration. Following the IE gavaging procedure minimized the risk of aspiration in DOL 2 mice while delivering the desired volume directly to the upper gastrointestinal tract. The success of the procedure was first validated using food coloring infused probiotic gavage. The food coloring acts as a marker that is visible through the skin of the pup. No negative effects were observed in mice gavaged with food coloring, and it is recommended to validate the gavaging procedure in this manner prior to commencing large-scale experiments. The rapid resolution of the gasping reflex seen post gavage can also be used as an additional indicator for a successful gavage. Once the mouse is placed on the heating blanket post-gavage, the gasping reflex will subside and an increase in the breathing frequency

will be observed within 20 seconds. The continuation of the gasping reflex for longer than 30 seconds indicates a failed gavage. Successful gavage also depends on appropriate insertion of the feeding needle with the bulb sitting right above the opening of the cardiac sphincter of the stomach. This can be facilitated by ensuring that the marking on the needle measuring the length between the xiphoid process and the tip of the snout, does not go past the snout of the mouse during gavage. This minimizes the chance of injury to the mouse. The frequency of gavage can have a significant impact on the experimental results. Frequent gavaging also can create more stress for the pups and the mother due to constant perturbation of the cage and the nest. The most optimal gavage schedule is when the gavages are the least frequent and over a shorter duration of time without losing the expected effect in the system. To ensure the safety and sterility of the procedure the gavage needle must be sterilized by washing and autoclaving in-between use. Washing rigorously on the outside using a scrub and the inside by forcing water through the needle using a syringe before autoclaving is necessary as any leftover particles can encrust on the needle during autoclaving and can interfere with the gavaging procedure.

Higher LP colonization was observed in pups that were gavaged every other day when compared to pups gavaged every day. This can be due to the reduced stress on pups gavaged every other day and potentially the probiotic getting more nutrients through the relatively more milk ingested by these pups. The dose dependency of probiotic treatment has been previously studied in mouse models[18,19] and thus the administration of correct dosage is important. The probiotic solution prepared is plated before every gavage to get an accurate count of CFU administered. If the probiotic organism is anaerobic, it is important to see if there is difference in CFU when cultured aerobically or anaerobically. Since LP is a facultative anaerobe, it was cultured using both methods and no difference in CFU was observed.

The post gavage intestinal LP load analysis was done using qPCR and high-quality DNA samples. To minimize LP DNA contamination between the treatment and the control groups, different feeding needles, biosafety cabinets and surgical equipment were used to ensure highest quality samples. The accurate measurement of the probiotic in the intestine required an optimized DNA extraction method. Most efficient methods for the extraction of DNA from stool involves multiple bead beating steps[20–22]. This method was adopted for the extraction of intestinal bacteria using bead beating and observed diminished representation ($<10^2$ copies recovered) of LP in the whole intestine DNA extraction. As LP is a Gram positive organism with a substantial amount of peptidoglycan in the cell wall, the protocol was optimized with a peptidoglycan dissolution step using lysozyme[23,24] added to the enzymatic lysis buffer. This increased the representation of LP in the same intestinal sample by greater than two-fold. The lysozyme treatment ensures the dissolution of the outer layer while the bead beating step facilitates the lysis of the organism. Optimization of amount of tissue, the type of garnet bead and the duration of disruption using the beads is necessary for obtaining optimal DNA products to conduct the PCR analysis.

The positive impact of probiotics administered as prophylaxis or treatment in the pre-term and term neonates is evidenced in recent studies[25–28]. The establishment of a proper neonatal

mouse model for probiotics is warranted to unpack the protective effect of probiotics. This protocol outlined here represents a guide for researchers unfamiliar with neonatal mouse work using probiotics. Notwithstanding the issues with rodent microbiota while studying human health and disease, this method can be extended to research focused on understanding the changes of the microbiome due to probiotics. This model also provides a platform to study host-microbe interaction and immune responses over the course of different developmental stages.

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No conflict of interests to disclose.

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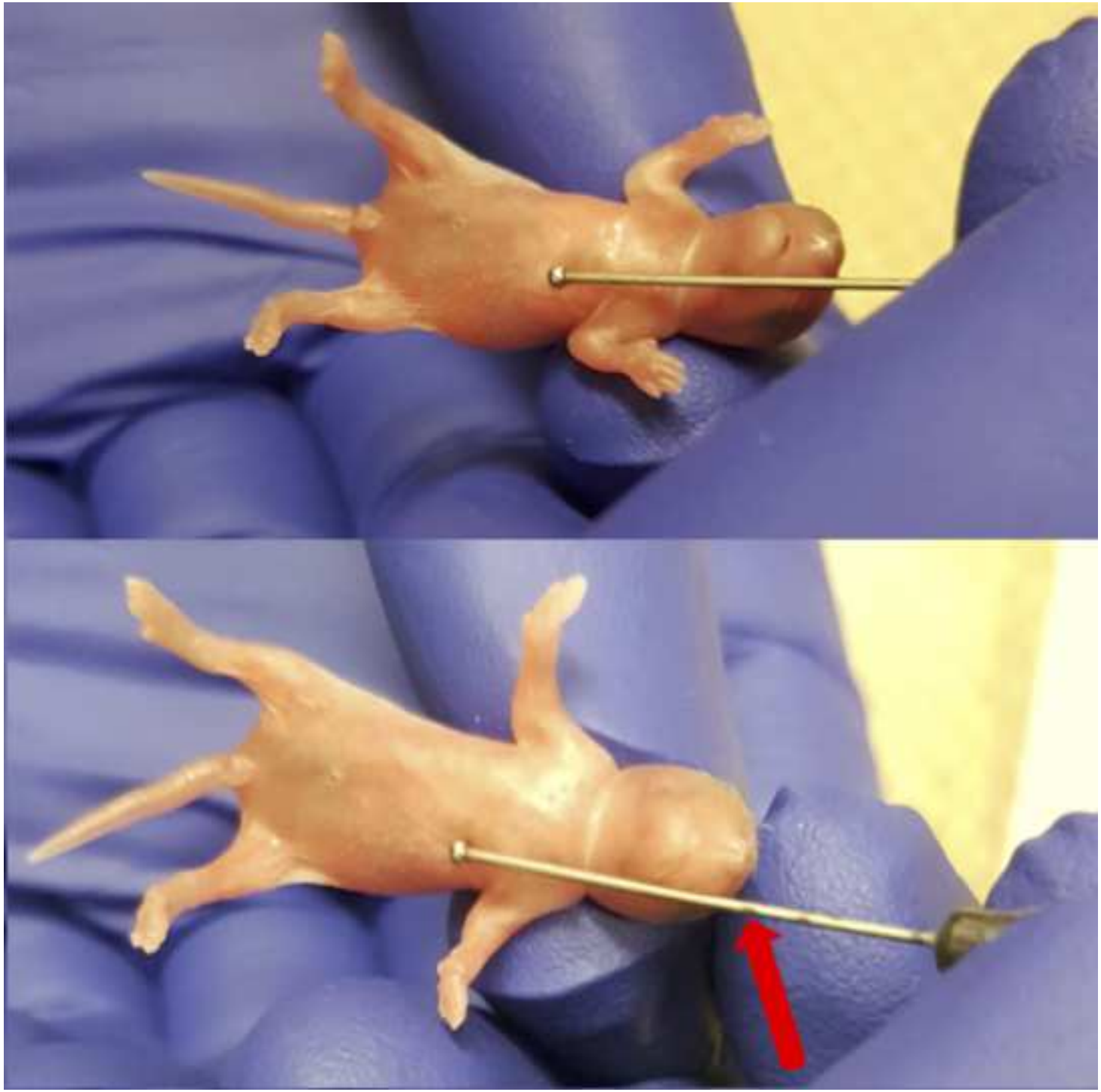

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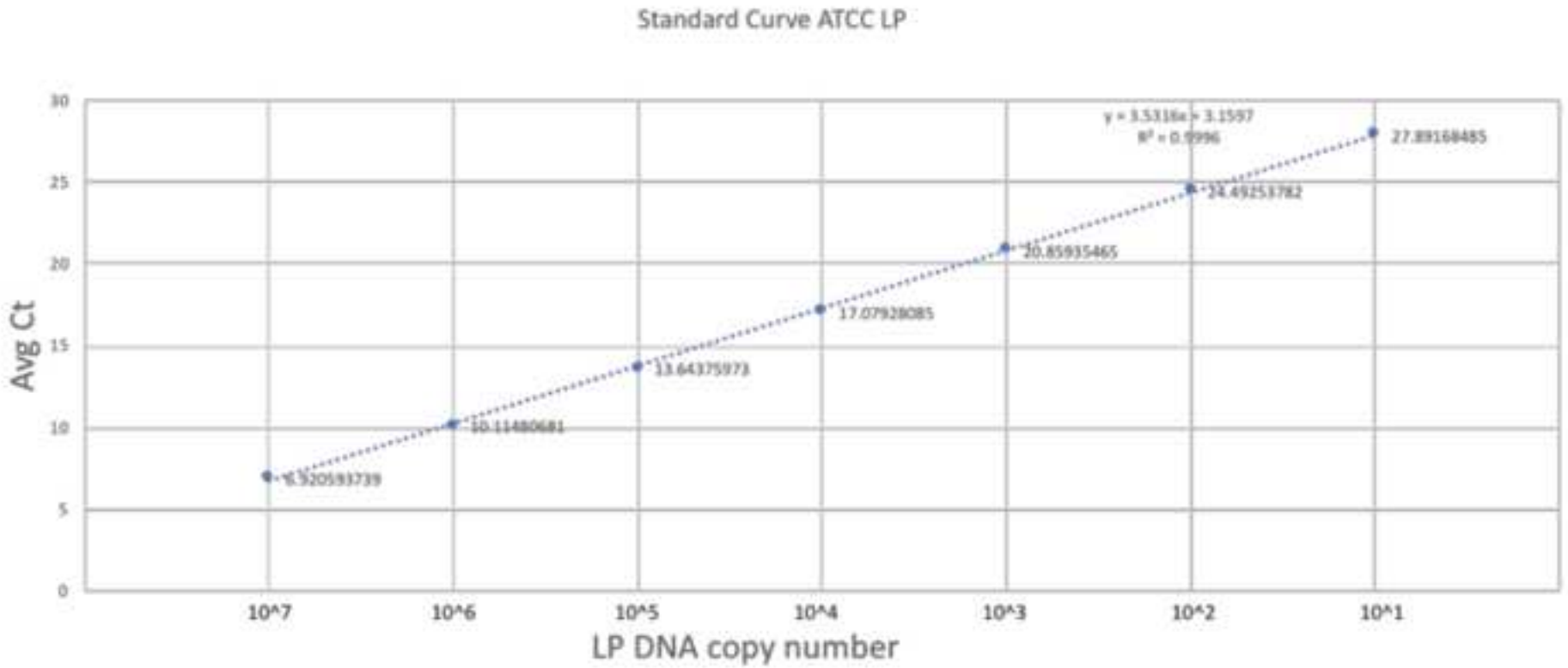

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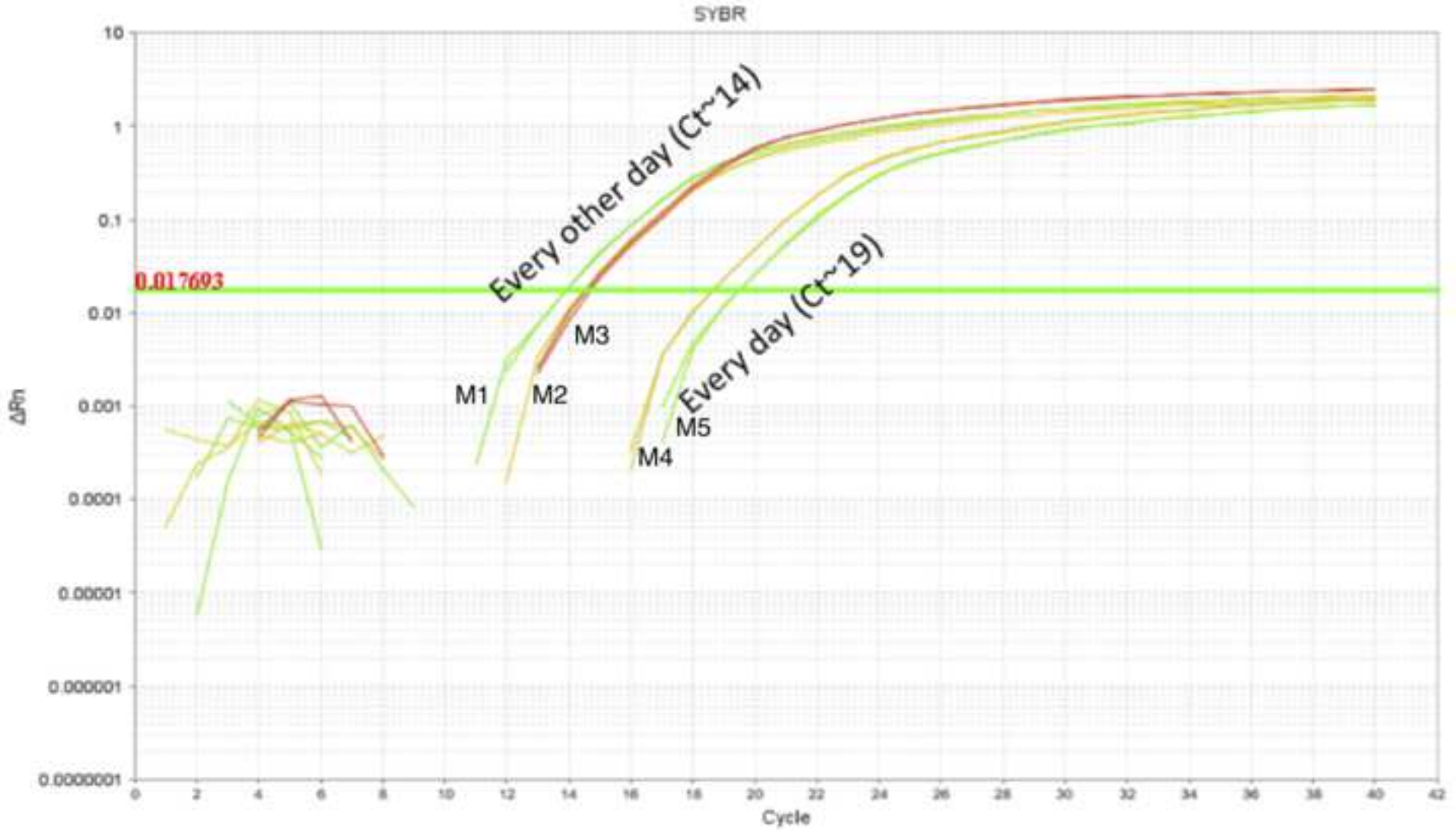

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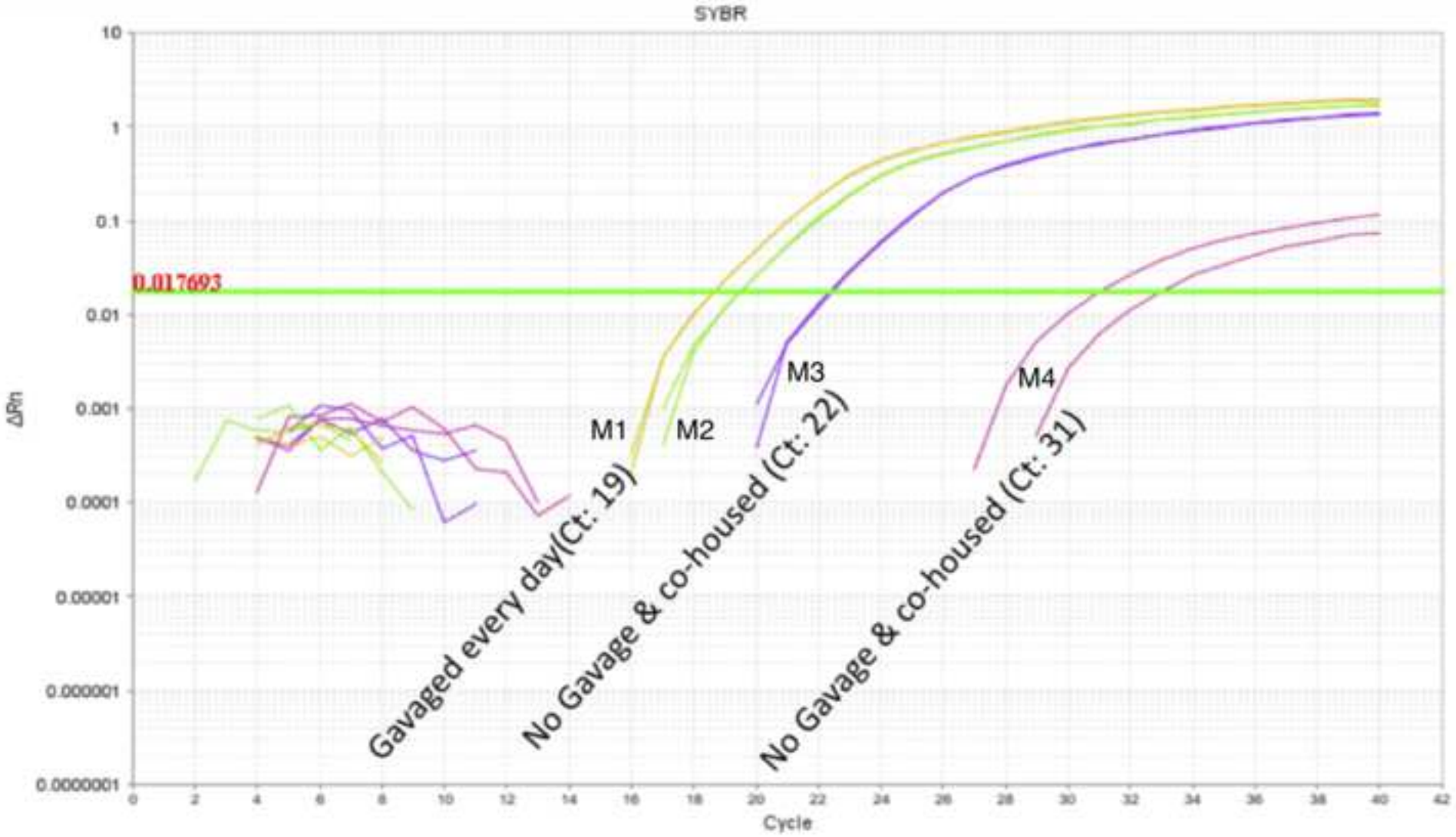

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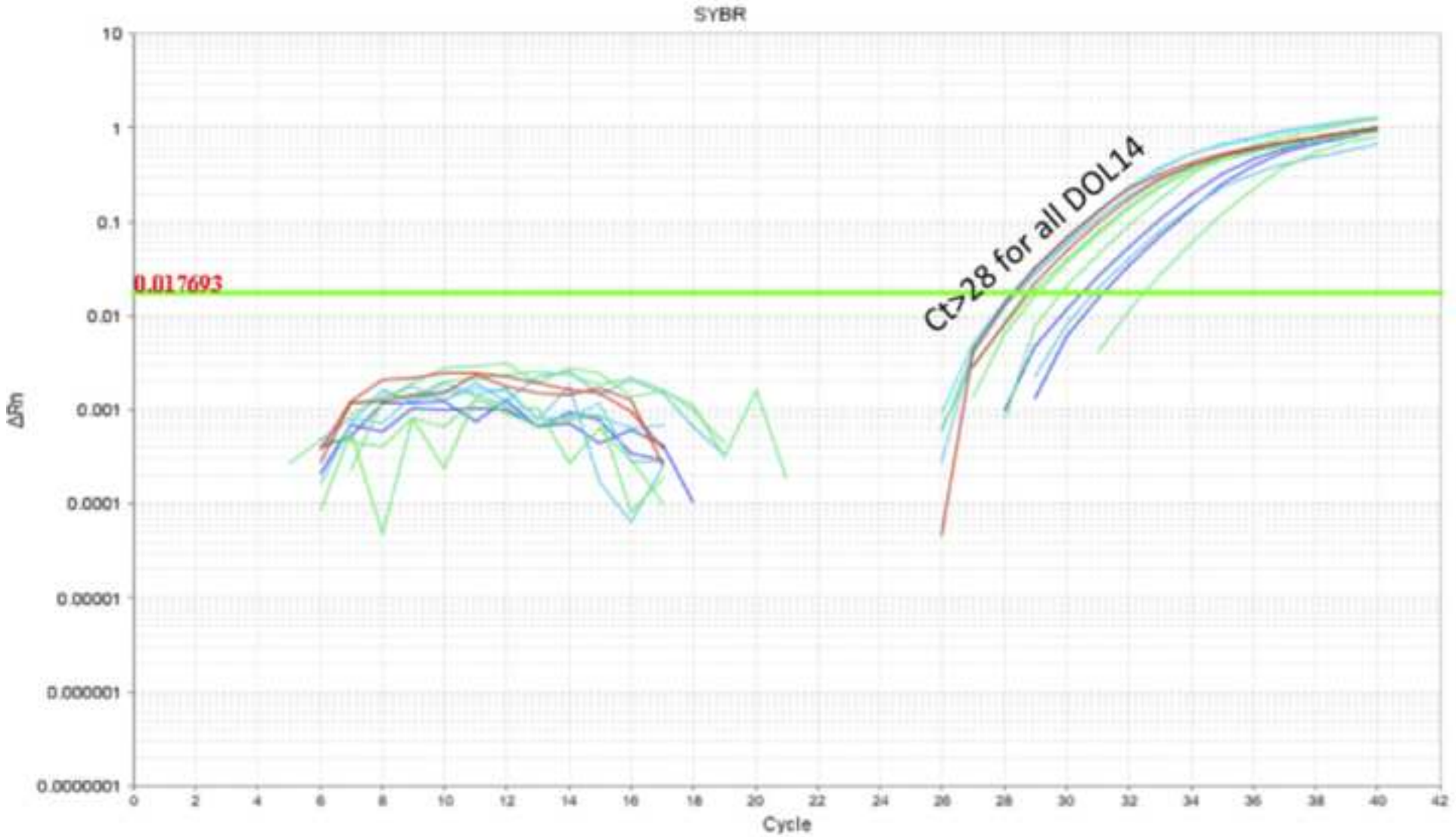

Table 1

Step	Temperature	Time
1	50 °C	2 minutes
2	95 °C	3 minutes
3	95 °C	30 seconds
4	58 °C	30 seconds
5	72 °C	30 seconds

Table 2

Target	16S-23S intergenic spacer region
Expected fragment size	144 bp
Primer Tm	58˚C
Forward primer (FP)	Lpn-1: TGG ATC ACC TCC TTT CTA AGG AAT
Reverse primer (RP)	Lpn-2: TGT TCT CGG TTT CAT TAT GAA AAA ATA

Table 3

	Concentration	10 µL reaction	20 µL reaction
Template DNA	200 pg/µL	1 µL	1 µL
SYBR Master Mix	-	5 µL	10 µL
FP	10 µM	0.3 µL	0.6 µL
RP	10 µM	0.3 µL	0.6 µL
dH_2O	-	3.4 µL	8.8 µL

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
1 mL tuberculin syringe with slip tip	BD	309659
1.2% Triton X-100	Millipore-Sigma	X100-100ML
2 mM sodium EDTA	Thermo Fisher Scientific	15575020
20 mM Tris·Cl	Thermo Fisher Scientific	15568025
5% dextrose and 0.9% NaCl injection solution	Baxter Corp.	JB1064
Alphaimager	Alpha Innotech	N/A
Anaerobic jar	Millipore-Sigma	28029-1EA-F
BD GasPak EZ anaerobe container system sachets	BD	260678
BD Difco Lactobacilli MRS Broth	BD	288130
Disruptor Genie	Scientific Industries Inc.	SI-D236
Feeding/oral gavage needles for newborn mice and rats	Cadence Science Inc.	01-290-1
Fructooligosaccharides	Millipore-Sigma	F8052
Garnet bead tubes 0.70 mm	Qiagen	13123-50
iTaq Universal SYBR Green Supermix	BioRad	172-5120
Lactobacillus plantarum (Orla-Jensen) Bergey et al.	ATCC	BAA-793
Lyophilized probiotic bacteria	N/A	N/A
Lysozyme	Thermo Fisher Scientific	89833
Maltodextrin	Millipore-Sigma	419672
Mini-Sub Cell GT Cell	BioRad	1704406
Nanodrop 1000	Thermo Fisher Scientific	N/A
QIAamp Blood and Tissue kit	Qiagen	51504
StepOnePlus Real-Time PCR System	Thermo Fisher Scientific	4376600
UltraPure Agarose	Invitrogen	16500-500
Ultrapure dH_2O	Invitrogen	10977023

	Comments/Description
Gel imaging system	
2.5 L	
24 Gauge, 1” needle length, 1.25 mm ball diameter	
from chicory	
for qPCR standard curve	
dextrose equivalent 4.0-7.0	
Gel chamber	

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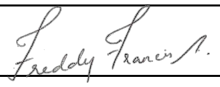
Signature:	
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The original editor's comments are in *greyed italics* with our responses in normal type face.
To the Editor:

We thank the editor for their comments and feedback on our manuscript and video and our response for each point mentioned is addressed below.

Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript has been proof read for spelling and grammatical errors.

2. Please shorten the title: Remove "Experimental considerations for"

The title has been shortened.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

The commercial language has been removed from the manuscript.

4. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

The commercial language has been removed from the table of materials and methods.

5. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

All personal pronouns have been removed from the text.

6. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Thank you for this comment. We want to equip the end-user of this protocol with the detailed steps of the protocol. Some of the points that might be considered discussion of the protocol by the editor might be points of caution specific to that step of the protocol. The protocol section of the manuscript has been revised to exclude any extraneous discussion and certain points have been re-worded to sound like caution statements for that specific step of the protocol.
7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

The protocol has been revised to include more steps about the how the procedure is done (for examples – Step 5)

8. Is anesthesia used? How is euthanasia performed?

The information regarding if anesthesia is used is added to protocol step 4.7.4. The information regarding euthanasia is added to protocol step 4.8 and 4.9.

9. Please avoid generalized directions. Please specify all volumes and concentrations used throughout. What are the desired amounts? We need specific values.

The manuscript has been proof-read and the specific details have been added wherever necessary and possible.

10. 5.2: How are the intestines collected?

More details on how the intestines are collected have been now added to step 5 (5.2-5.4).

11. 6.12/6.19: Again at 400 x g?

The steps with centrifugation have been now been fixed to explicitly state the speed at which they are to be centrifuged.

12. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

All tables have been removed and the respective titles and descriptions have be added to the manuscript after the results section.

Changes to be made by the Author(s) regarding the video:
1. Please include an introduction in the video.

An introduction has been added to the video. With an introduction title card and some explanations about the protocol.

2. Please increase the homogeneity between the video narration and the written protocol. There are more details in the video narration than in the written protocol text.

The written protocol has been edited to increase its homogeneity with the video.

3. Please have a card that says Representative Results.

The card that says representative results has been added.

4. Please include a Conclusion title card in lieu of the summary card.

The summary has been edited to be represented as conclusion with a title card and an explanation card.

5. Please include an introduction other than the initial title card.

This concern has been addressed with the edits made in point 1 of the editor's comments regarding the video.

6. Text/formatting issues
• 0:01 - There are numbers and symbols next to the authors' names, but they don't refer to anything. The affiliations should be added to this card. The animal use disclaimer can be a separate card after the title card.

Affiliations have been added to the title card now.

7. Frame size/proportions issues
• 8:50-9:03, 9:10-9:27 - The white background of these figures should be extended to eliminate the black borders.

The figure aspect ratio issues have now been fixed to make the figure fit the full frame.

We thank the editor again for the thoughtful remarks and their assistance in making the revisions successful.

The original reviewer's comments are in *greyed italics* with our responses in normal type face.

To Reviewer #1:

In general this is a well-written paper that provides details of the steps needed to administer probiotics to neonatal mice, various QC steps on the probiotic/synbiotic itself, and how to determine colonisation. The authors correctly suggest that the most interesting aspect of the study is the actual description of the gavage procedure, and I felt that the details of the qPCR etc. were of less general interest. Throughout, the mixture of highly specific instructions (e.g. PCR reactions), with much broader suggestions (e.g. the attempts to provide a generalised framework for assessing CFU for probiotics) can be off-putting, I would prefer the authors just to be absolutely specific about what they did with their synbiotic, other investigators will be able to adapt.

Unfortunately the 'results' are rather poorly presented - comprising a series of assertions that are not supported by details of the numbers of biological/technical replicates, interpretable raw or summarised data, or significance testing. The qPCR plots on their own are not useful, the authors should provide properly annotated and interpreted results or omit the whole section (I feel that a lot of this would be better suited to a standalone paper and is superfluous to a video description of/protocol for neonatal gavage). The authors talk about 'colonization' but provide no information on whether they are talking about in stool, in small intestine etc. From reading the introduction I was hoping for a presentation of longitudinal colonisation data across the length of the small/large intestine, which would have been very interesting, and proper high-quality data on risk of cross colonisation to littermates, but these do not materialise. The comparison of two gavage schedules appears superficial (though hard to judge with inadequate data), and the proposed mechanisms for the effect seen do not make sense to this reader. I think that the minimum 'results' data that are required for this submission are comparisons of safety and colonisation at ages where this is feasible with oropharyngeal gavage, and the issues of colonisation spread might be best dealt with in a submission where they can receive the proper treatment (with much more discussion about how to get around this where littermate controls are crucial and transfer to a foster mother might have impacts on health in and of itself).

We thank the reviewer for their thoughtful and constructive comments on our submission. They were very helpful in re-evaluating the presentation of the manuscript. The suggestions on reorganizing the results were well taken and more information about the technical and biological replicates have been now added to the representative results section and the figures have been annotated to show biological replicates. We hope this will better guide the audience in understanding the results. The reviewer's comments about the results being represented in a stand-alone paper is well taken and we are in the works of further investigating, validating and adding more layers to this data-driven story. However, the results presented in this manuscript are meant to be representative, providing an example of an outcome that can be obtained following the protocol we detail. We understand that some of the information about the qPCR reactions can be adapted by the end-user of the protocol, but we wanted to include it to present a complete picture of our workflow and provide a starting point/ framework for the experimental set up. The unclear representation of colonization commented on by the reviewer has been

addressed and clarified now stating that the whole intestine homogenate was used for the colonization analysis (see introduction (line 117), results (line 440) and discussion (line 567). The reviewer's comments on the gavage schedule were very helpful – our observation of consistent qPCR results between the three biological replicates for both schedules in two technical replicates per sample provided confidence in choosing one schedule over the other. The comments of the reviewer on safety and colonization at different ages were crucial. Specifically, the oropharyngeal gavage though arguably safer, did not provide confidence in the delivery of a calibrated amount of substance. Our protocol can really only provide a starting guide for the setting up the specific colonization experiments, to which the end-user has to then add their specific question of interest, such a colonization over time by potentially using longitudinal stool microbiome tracking.

The most important of the 'specific comments is that the authors provide no detail on how reliably one can administer 30 ul of any solution using a gavage needle and syringe whilst holding a delicate pup, I would have thought this would be the most challenging aspect of the procedure.
I was disappointed not to see a video of the gavage procedure (though perhaps this is provided after review in JoVE?)

It is unfortunate that the reviewer did not get a chance to view the video attached to the manuscript and we hope that the video is received without fail in this iteration of the review submission. The video addresses the reviewer's concern about the details of gavaging a young pup. More elaborate information has now also been added to the manuscript to better portray the action of gavaging in words (please refer to protocol step 4). This video also best pictures the usefulness of the blue dye used in the gavage, as requested by the reviewer.

Please provide details of the contributions of each of the 13 authors

Freddy Francis designed and conducted the experiments. Natallia Varankovich assisted with the probiotic quantification, gavage work and filiming of the video. Byron Brook, Nelly Amenyogbe, Rym Ben-Othman Bing cai, Danny Harbeson and Aaron Liu all assisted with monitoring the mice pre and post gavage. They also assisted in proof reading and editing the protocol document and video as it was undergoing development. Ben Dai assisted with establishing breeding schedules. Shelly McErlane and Kris Andrews are animal care facility staff who trained and assisted us with the development of this novel protocol. Tobias R. Kollmann and Pinaki Panigrahi are guides who have assisted us with ideation, development and implementation of the developed protocol and video production.

Response to the specific comments:
-*82 - not certain 'immunomodulatory effects' is the right way to describe, there may be other important mechanisms, the authors immediately go on to state that the mechanisms are not fully understood*

- Regarding the use of the term immunomodulatory, it was used in the introduction to present information from the literature. The connection of probiotics to the immune system has already been made in the published literature(Amenyogbe, Kollmann, &

Ben-Othman, 2017). The statement about the transfer of compounds has been re-written to provide a better explanation.

-98 - probiotics are live bacteria, not 'compounds', and in reference 11 the mothers microbiota is modulated by antibiotic exposure but I'm not sure milk-transfer of antibiotic is a major mechanistic issue

-102 - remove 'furthermore', 106 remove 'thus'

- Grammatical errors pointed by the reviewer have been addressed.

-110 - spell out LP first time it's used in the main body of the manuscript

- LP is spelled out in the body of the manuscript.

-111 - description of the synbiotic would be better suited to the methods

- The explanation of synbiotic is now addressed in procedure step 2.

-110 - I would suggest that LP colonisation was your main readout of the efficacy of the strategy, and colonisation spread was a secondary outcome? If so be clear about this

- Regarding the readout of this procedure – our primary aim was to safely administer the liquid compound to a newborn mice with confidence in the amount delivered. The secondary readouts of degree of colonization and spread were to give some examples for the end-user to acquire data using our procedure.

-120 - 'carried out pertaining to the guidelines established by the support staff at the Animal Care Facility at the University of British Columbia' I'm not clear what is meant by this, I think you could remove and just say this investigative work was approved by ACC

- The protocol was contributed to and approved by two different animal care centers at the university and at the research institution and thus both names are provided to give appropriate credit.

-Quantification - you are referring to 'saturation', but make no reference to actually determining whether the solution is saturated. It seems that you are just taking a commercial dried (lyophilized?) synbiotic, making a serial dilution and plating out to determine CFU count. How does this compare to the stated CFU count for this preparation? Presumably you spread the dilutions on the plate though no mention is made of this. Also, are you counting up to 250 colonies on a quadrant of a plate? That seems incredibly ambitious - I actually think it would be more practical to spread on the whole plate and measure?
-Preparation - you now seem to be adding the probiotic and prebiotic separately is that the case? In which case the preceding quantification step was superfluous since you could surely administer more-or-less as much as you want if you were just resuspending bacteria in some solute? Did you compare lyophilized bacteria to provision of growing bacteria e.g. at log phase from broth culture? 'FOS and malodextric reach saturation at 0.3 g/mL' - has this been determined empirically? Is a second probiotic CFU quantification essential every time?

- The reviewer's comments regarding the word usage 'saturation' has been addressed and has been elaborated to better explain the empirically determined conditions. The plate count for the CFU was done in different dilutions to find the active concentration of bacteria and plates containing between 25 and 250 colonies counted; there typically are considered ranges when only one dilution would be plated on one plate. This information was poorly and ambiguously worded in the original manuscript and the

colony count ranges have been edited to portray expected colonies per quadrant as the protocol set up for the plate is in the quadrant format. A second plate count every time before gavage is done to have an accurate representation of the CFUs administered and this step also serves as a quality control step for the consistency of CFUs administered.

-203 - measuring from the mouth/incisors? - no, I see later should be the nose, please state.

- The representation of this information was ambiguous and has been now clarified in the manuscript. (Protocol steps 4.1, 4.7 and 4.8; Line 542)

-207 - how do you accurately administer 30 ul? What kind of syringe do you use? How do you deal with dead space?

- The syringe details are now better highlighted in protocol step number 4.1 and the more information on the companies and sizes of the needles and syringes can be found on the addendum (materials and methods document).

-282 - you seem to freeze the whole intestine for later evaluation of colonisation longitudinally through the intestine (after cutting into 'small segments' line 303) - is there a benefit of this compared to just sequencing the intestinal contents? Or intestine with the contents squeezed out? (I can imagine there might be and those data might be provided)
-For the quantification, you provide absolute numbers but what are the units? Per cm intestine?

- We appreciate the reviewer's ideas on getting the whole intestine DNA sequenced. This was however not the focus of our current protocol. The quantification number for the copy counts provided were per 'whole intestine' and can be represented in either per cm intestine (if doing sectional analysis of microbiome) or per gram intestine (if exploring relative abundance). We clarified this in the revised manuscript in the introduction, results and discussion.

-The authors assert that their method is more reliable than oropharyngeal administration but provide no evidence to support this - in fact the fact that more colonisation is achieved with fewer administrations is concerning for the reliability of the technique - is this a real biological effect or stochastic? Has it been replicated by the authors?

- Our primary goal for this procedure is to have no regurgitation of the administered solution, commonly observed with oropharyngeal gavage. The primary success of our result was the elimination of regurgitation of gavage and the consistent copy number representation of the probiotic in our qPCR biological replicates as an added confirmation of the accuracy of this technique. While these two methods complement each other, they were not set up as a validation, i.e. not meant to provide statistical significance. This was now clarified in the manuscript (line 444).

-446 - treatment groups should be separated by cage - but should litter mates still be used? Therefore would the separated neonates need to be 'fostered' by another dam able to provide milk? It depends on how much of an effect/spread is meant by 'a lesser degree' (this is particularly inadequately reported)

- The reviewer's comments on the possibility of using foster mothers to solve cross-colonization of the probiotic is interesting. This was a very important discussion missing form our manuscript and now has been added (line 462).

-508 - 'above the opening of the cardiac sphincter' are the authors trying to gavage into the stomach or the distal oesophagus? I would have thought that they are trying to gavage into the stomach if measuring below the xiphisternum?

- The postulation to describe the higher copy number in pups gavaged every two days has been edited to better portray logic. The gavage needle bulb is not forced past the cardiac sphincter to avoid any damage to the crucial contractile muscle. We have empirically found that the external measurement of needle to the lower end of the xyphoid process has given us the best anticipated results, and clearly state this now in the manuscript.

-536 'observed diminished representation…' - please put all results in the result section and provide data to support assertions

- The results section has been revised to best represent the data presented in accordance with the reviewers concerns.

-549 - avoid 'roadmap', this is a 'protocol'.
-Figures 1-4 are not helpful, data presented needs to be presented properly (keeping a representative PCR plot for example if wished)
-Figure 5 please label all structures. Do you have a picture with the blue food dye?

- The phrasing suggested has been fixed and the graphs are labelled with biological replicates to better present the data.

The results section has been edited to address the overarching concerns of the reviewer. The figures have been revamped to better represent the data. We thank the reviewer again for the thoughtful and detail feedback and hope that we have adequately made edits to address the reviewer's concerns and have improved the quality of our manuscript with their assistance.

The original reviewer's comments are in *greyed italics* with our responses in normal type face.

To Reviewer #2,

Manuscript Summary:

Francis et al described the protocol of oral administration pf probiotics to neonatal mice using gavage. This method is of great interest in current field of probiotics and microbiome research, and will benefit several scientific groups. The paper is written well to follow, however this reviewer find few minor comments, that may help to increase clarity for readers.

Minor Concerns:

1. Is there any recommendation for optimizing probiotics content? The maximum number of bacteria to be given for neonates.

We thank the reviewer for the thoughtful and encouraging comments on the manuscript. We are excited to see that the reviewer finds our work as a significant contribution to the field. The suggestions have been taken into consideration in the revision of our manuscript. Specifically, the optimization of the probiotic usually is built off its previously known effectiveness. A dose titration can be done to determine the safe zone for the administration of the probiotics. If the probiotics are administered in combination with the prebiotics (a synbiotic formula), then the viscosity of solution creates a challenge for gavage and dosing. In this case the limits on the doses are due to the physical properties of the gavaged liquid.

2. Page 4, line 160, what is the suggested solvent in dissolving the mixture? I believe it 5% dextrose solution, if so mentioning here in bracket may help for readers.

The solvent for dissolution has now been named as suggested (page 4). We agree that it is best to be explicit in a protocol, so it is presented as a ready-to-use format for the end-user.

3. Page 6, line 222, does the description "by moving it away from you" mean "by moving it away from your hand"?, making more specific may help to readers.

The suggestion for page 6 regarding the calibrated movement of the syringe while holding the pup has also been addressed and elaborated in a way to create better imagery of the small movements needed do undertake this procedure.

4. For the fecal samples collection on page 7, was there any optimization was suggested, how long it will take for the gavaging mixture to go through the intestine?

The comment regarding how long the gavage mixture will take to pass through the intestine is addressed in protocol step 5.5.

5. Since different doses of LP copies were used in the two gavaging schedules, were the values/data were comparable?

We apologise for the unclear representation of the dosage; in both gavage schedules, the dosage was the same (10^6 CFU) per gavage but the frequency of the procedure was different – once every 24 hours versus once every 48 hours. This setup made the values and data comparable.

We thank the reviewer again for the sincere and thoughtful comments and we hope we have adequately addressed the concerns and have improved the quality of the manuscript.