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Standardized Preparation of Fecal Microbiota Transplantation Products for Companion Animals

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Protocol status: Working

Step-by-step protocols for standardized fecal microbiota transplantation (FMT) preparation for dogs and cats. These protocols are utilized by the Companion Animal Fecal Bank at the Ohio State University College of Veterinary Medicine.

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Abstract

Step-by-step protocols for standardized fecal microbiota transplantation (FMT) preparation for dogs and cats, including:

Protocol 1: Standardized FMT Preparation for Double Centrifuged Fecal Slurry

Protocol 2: Encapsulation of Fecal Slurry for Oral Capsular FMT Administration

Protocol 3: Standardized FMT Preparation for Lyophilized FMT Products

Protocol 4: Fecal Lyophilization and Encapsulation of Lyophilized Fecal Slurry for Oral Capsular FMT

Protocol 5: Standardized FMT Preparation for Fecal Slurry without Centrifugation

Protocol 1: Standardized FMT Preparation for Double Centrifuged Fecal Slurry

1 Standardized FMT Preparation for Double Centrifuged Fecal Slurry

- 1.1 Personal protective equipment (PPE) must be worn at all times while handling feces. Minimum PPE includes a lab coat, goggles, and gloves. A face mask is also recommended to protect the nose and mouth from any possible splash of biohazardous materials while processing feces. 
- 1.2 For dogs, the owner collects feces immediately following defecation using a clean plastic bag. Feline owners are instructed to collect naturally voided feces from the litter box within 12 hours of defecation using a clean plastic bag.
- 1.3 The owner labels the plastic bag and stores feces in a standard refrigerator (4°C) within 15 minutes of defecation (for dogs) or immediately upon collection (for cats). Owners store feces at 4°C for no more than 24 hours prior to delivery to a BSL-2 laboratory. 
- 1.4 Owners should deliver refrigerated feces on ice to a BSL-2 laboratory for processing. Feces should be weighed (grams) and processing should be started immediately upon arrival to laboratory (within 24 hours of original defecation). 

Note: If processing feline feces, use a metal spatula to remove litter material prior to weighing feces.

- 1.5 Combine all feces from a single donor into a sterile round bottom Stomacher™ 400 Classic Standard bag™ (Seward™, round bottom Stomacher™ bag for Model 400, Fisher Scientific, catalog # 14-285-284). Seal the bag using a standard Stomacher™ bag clip and place the sealed bag on ice. Gently hand-knead the bag until feces is homogenized.

Note: If additional testing (i.e. pathogen testing, 16S rRNA amplicon sequencing, Dysbiosis Index) of raw feces is desired, use a sterile spatula to transfer 1-gram aliquots of homogenized pooled feces into sterile cryovials. Store filled cryovials at -80°C until future use.

- 1.6 Add sterile 0.9% saline to the Stomacher™ bag with homogenized feces at a ratio of 1:4 feces to sterile saline (4x weight of feces in grams = mL of saline to add to feces).

Example Calculation: If processing 100 g feces
 $100 \times 4 = 400$ mL saline added to feces



- 1.7 Carefully hand-knead the saline and pooled feces mixture for 3-5 minutes or until completely homogenized.
- 1.8 Aliquot 45 mLs of the fecal saline mixture into sterile 50 mL conical tubes until all of the mixture has been dispensed. 
- 1.9 Within the conical tubes, centrifuge the fecal saline mixture at 400g (or RCF) for 30 mins at 4°C (Centrifuge 5810 R, 15 amp version, Eppendorf, Enfield CT, USA). Balance the centrifuge by loading the centrifuge rotor symmetrically and ensuring opposite tubes are the same weight. Store filled conical tubes awaiting centrifugation at 4°C.  
- 1.10 After centrifugation, using a transfer or serological pipette, remove supernatant to a 1:0.75 ratio of fecal sediment to supernatant. Avoid disturbing the sediment layer while decanting. 

Example:
45 mL fecal saline mixture with 10 mL of sediment, remove 17.5 mL of supernatant to leave 17.5 mL of solution with sediment in the tube.
- 1.11 Vortex the remaining supernatant with the fecal sediment until the mixture is completely homogenized. Pour the resulting fecal solution into the integrated strainer element of a sterile Stomacher™ Circulator Strainer bag (Seward™ Strainer bag for Stomacher™ 400, Fisher Scientific, Catalog # 14-285-22).
- 1.12 Using a fresh clean glove, gently detach the integrated strainer element from the plastic component of the Stomacher™ bag. Clip or hold the end of the strainer element to ensure no spillage of fecal material. Gently squeeze the strainer element until liquid is no longer produced and the retained material feels chalky and dry.
- 1.13 Aliquot 45 mL of the filtered fecal suspension into sterile 50 mL conical tubes and centrifuge the fecal material at 400g for 15 mins at 4°C.   
- 1.14 After centrifugation, using a transfer or serological pipette, remove supernatant to a 1:0.75 ratio of sediment to supernatant. Avoid disturbing the sediment layer while decanting. 
- 1.15 Vortex the fecal sediment with the remaining supernatant until homogenized.
- 1.16 If planning to freeze FMT slurry (at -80°C), add sterile glycerol (Millipore Sigma, Sigma-Aldrich, Catalog # G9012) to the fecal slurry, to a final concentration of 10% glycerol. 

Example Calculation: If fecal slurry volume = 40 mL



40 mL x 0.11 = 4.4 mL glycerol

Add 4.4 mL glycerol to 40 mL fecal slurry to reach a final concentration of 10% glycerol

- 1.17 Vortex the final FMT mixture thoroughly.



Note: This final fecal slurry can be: (1) administered immediately via enema, endoscopy, or nasogastric tube; (2) encapsulated and frozen at -80°C for later use as frozen FMT capsules (see Protocol 2); or (3) stored at -80°C for up to 6 months later use as a fecal slurry.

- 1.18 If stored at -80°C as fecal slurry, rapidly thaw FMT slurry in a 37°C water bath for 30-45 minutes and keep warm until administration. Once thawed, FMT product should be utilized within 12 hours.



Protocol 2: Encapsulation of Fecal Slurry for Oral Capsular FMT Administration

2 Encapsulation of Fecal Slurry for Oral Capsular FMT Administration

- 2.1 Maintain capsules over dry ice prior to encapsulation to ensure the fecal slurry is kept cold.



- 2.2 Gently separate the two parts of the capsule, then use a repeater micropipette to dispense the fecal slurry into the open capsule.



For dogs: Size 0 gelatin or delayed-release capsules are recommended.

For cats: Size 4 gelatin or delayed-release capsules are recommended.

- 2.3 Over encapsulate the gelatin or delayed-release capsules with a slightly larger commercially available gelatin capsule (i.e. Size 00 for dogs; Size 3 for cats). Continue to maintain the filled capsules on dry ice until ready for long term storage.



Note: This step is important to prevent erosion and degradation of the interior capsule and ensure the integrity of the capsule for oral administration.

- 2.4 Place the filled fecal slurry capsules in a sealed airtight container and store at -80°C until ready for use.



- 2.5 For oral administration, remove from -80°C and place in -18°C to -20°C freezer. Frozen capsules can be easily administered in canned food or manually *per os* while still frozen. Capsules that reach room temperature will quickly disintegrate, thus compromising capsule integrity.



Protocol 3: Standardized FMT Preparation for Lyophilized FMT Products

3 Standardized FMT Preparation for Lyophilized FMT Products

- 3.1 For dogs, the owner collects feces immediately following defecation using a clean plastic bag. Feline owners are instructed to collect naturally voided feces from the litter box within 12 hours of defecation using a clean plastic bag. 
- 3.2 Personal protective equipment (PPE) must be worn at all times while handling feces. Minimum PPE includes a lab coat, goggles, and gloves. A face mask is also recommended to protect the nose and mouth from any possible splash of biohazardous materials while processing.
- 3.3 The owner labels the plastic bag and stores feces in a standard refrigerator (4°C) within 15 minutes of defecation (for dogs) or immediately upon collection (for cats). Owners store feces at 4°C for no more than 24 hours prior to delivery to a BSL-2 laboratory. 
- 3.4 Owners should deliver refrigerated feces on ice to a BSL-2 laboratory for processing. Feces should be weighed (grams) and processing should be started immediately upon arrival to laboratory (within 24 hours of defecation). 

Note: If processing feline feces, use a metal spatula to remove litter material prior to weighing feces.

- 3.5 Maltodextrin (Millipore Sigma, Sigma-Aldrich, Catalog # 419680) and D-(+)-trehalose dihydrate (Millipore Sigma, Sigma Aldrich, Catalog #90210) are dissolved in 0.9% sterile saline at 40°C to respective final concentrations of 18.75% and 6.25%. The solution is cooled on the benchtop to room temperature and filter-sterilized using a 0.22 µm PES membrane (Titan3™ PES Syringe Filters, Fisher Scientific, catalog # 42225-PS). 

Example: If the desired final volume of solution is 100 mL

Add 18.75 g maltodextrin and 6.25 g trehalose into a sterile glass beaker. Then add sterile saline to achieve a total volume of 100 mL.

- 3.6 Combine all feces from a single donor into a sterile round bottom Stomacher™ 400 Classic Standard bag™ (Seward™, round bottom Stomacher™ bag for Model 400, Fisher Scientific, catalog # 14-285-284). Seal the bag using a standard Stomacher™ bag clip and place the sealed bag on ice. Gently hand-knead the bag until feces is homogenized. 

Note: If additional testing (i.e. pathogen testing, 16S rRNA amplicon sequencing, Dysbiosis Index) of raw feces is desired, use a sterile spatula to transfer 1-gram aliquots of homogenized pooled feces into sterile cryovials. Store filled cryovials at -80°C until future use.



- 3.7 Add the maltodextrin and trehalose (M:D) solution to homogenized feces at a ratio of 1:4 feces to M:D.

Example: If processing 25 g feces
 $25 \times 4 = 100$ mL M:D solution added to feces

- 3.8 Carefully hand-knead the M:D solution and pooled feces mixture for 3-5 minutes or until completely homogenized.

- 3.9 Aliquot 45 mLs of the fecal mixture into sterile 50 mL conical tubes until all of the mixture has been dispensed. 

- 3.10 Within the conical tubes, centrifuge the fecal M:D mixture at 400g (or RCF) for 30 mins at 4°C (Centrifuge 5810 R, 15 amp version, Eppendorf, Enfield CT, USA). Balance the centrifuge by loading the centrifuge rotor symmetrically and ensuring opposite tubes are the same weight. Store filled conical tubes awaiting centrifugation at 4°C.  

- 3.11 After centrifugation, using a transfer or serological pipette, remove supernatant to a 1:0.75 ratio of fecal sediment to supernatant. Avoid disturbing the sediment layer while decanting. 

Example for 45 mL fecal saline mixture with 10 mL of sediment:
Remove 17.5 mL of supernatant to leave 17.5 mL of solution within the sediment tube.

- 3.12 Vortex the remaining supernatant with the fecal sediment until the mixture is completely homogenized. Pour the resulting fecal solution into the integrated strainer element of a sterile Stomacher™ Circulator Strainer bag (Seward™ Strainer bag for Stomacher™ 400, Fisher Scientific, Catalog # 14-285-22).

- 3.13 Using a fresh clean glove, gently detach the integrated strainer element from the plastic component of the Stomacher™ bag. Clip or hold the end of the strainer element to ensure no spillage of fecal material. Gently squeeze the strainer element until liquid is no longer produced and the retained material feels chalky and dry.

- 3.14 Aliquot 45 mL of the filtered fecal suspension into sterile 50 mL conical tubes and centrifuge the fecal material at 400g for 15 mins at 4°C.   

- 3.15 After centrifugation, using a transfer or serological pipette, remove supernatant to a 1:0.75 ratio of sediment to supernatant. Avoid disturbing the sediment layer while decanting. 

- 3.16 Vortex the fecal sediment with the remaining supernatant until homogenized, then immediately freeze at -80°C.   

Note: FMT slurry must be frozen at -80°C for at least 48 hours prior to lyophilization.



Protocol 4: Fecal Lyophilization and Encapsulation of Lyophilized Fecal Slurry for Oral Capsular FMT

4 Fecal Lyophilization and Encapsulation of Lyophilized Fecal Slurry for Oral Capsular FMT

- 4.1 Remove frozen fecal slurry from -80°C freezer, remove lids from conical tubes.
- 4.2 Wrap open end of conical with KimWipe, secure with rubber band, and place in lyophilizer (such as FreeZone Benchtop Freeze Dryer, Labconco, Kansas City, MO, USA). 
- 4.3 Lyophilize at -87.5°C and 0.005 mBar for 48 hours. 
- 4.4 After 48 hours, remove conical tubes from lyophilizer.
- 4.5 Homogenize and grind the dried FMT material using a clean mortar and pestle at room temperature.
- 4.6 Encapsulate homogenized FMT by filling capsules individually. For bulk lyophilized FMT capsule production, use capsule filling device (Profiller 3600 Capsule Filling System, Torpac Inc., Fairfield, NJ, USA) with the desired capsule type and size.

For dogs: Size 0 gelatin or delayed-release capsules are recommended.
For cats: Size 4 gelatin or delayed-release capsules are recommended.
- 4.7 Store lyophilized FMT capsules at 4°C (or room temperature) until ready for use. 

Protocol 5: Standardized FMT Preparation for Fecal Slurry without Centrifugation

5 Standardized FMT Preparation for Fecal Slurry without Centrifugation

- 5.1 Personal protective equipment (PPE) must be worn at all times while handling feces. Minimum PPE includes a lab coat, goggles, and gloves. A face mask is also recommended to protect the nose and mouth from any possible splash of biohazardous materials while processing.

5.2 For dogs, the owner collects feces immediately following defecation using a clean plastic bag. Feline owners are instructed to collect naturally voided feces from the litter box within 12 hours of defecation using a clean plastic bag.



5.3 The owner labels the plastic bag and stores feces in a standard refrigerator (4°C) within 15 minutes of defecation (for dogs) or immediately upon collection (for cats). Owners store feces at 4°C for no more than 24 hours prior to delivery to a BSL-2 laboratory.



5.4 Owners should deliver refrigerated feces on ice to a BSL-2 laboratory for processing. Feces should be weighed (grams) and processing should be started immediately upon arrival to laboratory (within 24 hours of original defecation).



Note: If processing feline feces, use a metal spatula to remove litter material prior to weighing feces.

5.5 Combine all feces from a single donor into a sterile round bottom Stomacher™ 400 Classic Standard bag™ (Seward™, round bottom Stomacher™ bag for Model 400, Fisher Scientific, catalog # 14-285-284). Seal the bag using a standard Stomacher™ bag clip and place the sealed bag on ice. Gently hand-knead the bag until feces is homogenized.

Note: If additional testing (i.e. pathogen testing, 16S rRNA amplicon sequencing, Dysbiosis Index) of raw feces is desired, use a sterile spatula to transfer 1-gram aliquots of homogenized pooled feces into sterile cryovials. Store filled cryovials at -80°C until future use.

5.6 Add sterile 0.9% saline to the Stomacher™ bag with homogenized feces at a ratio of 1:4 feces to sterile saline (4x weight of feces in grams = mL of saline to add to feces)

Example: If processing 100 g feces
 $100 \times 4 = 400$ mL saline added to feces

5.7 Pour the resulting fecal solution into the integrated strainer element of a sterile Stomacher™ Circulator Strainer bag (Seward™ Strainer bag for Stomacher™ 400, Fisher Scientific, Catalog # 14-285-22).

5.8 Using a fresh clean glove, gently detach the integrated strainer element from the plastic component of the Stomacher™ bag. Clip or hold the end of the strainer element to ensure no spillage of fecal material. Gently squeeze the strainer element until liquid is no longer produced and the retained material feels chalky and dry.

5.9 If planning to freeze fecal slurry, add sterile glycerol (Millipore Sigma, Sigma-Aldrich, Catalog # G9012) to the fecal slurry, to a final concentration of 10% glycerol.





Example: Fecal slurry volume = 40 mL

$40\text{mL} \times 0.11 = 4.4 \text{ mL glycerol}$

Add 4.4 mL glycerol to 40 mL fecal slurry to reach a final concentration of 10% glycerol

Note: This final fecal slurry can be: (1) administered immediately via enema, endoscopy, or nasogastric tube; (2) encapsulated and frozen at -80°C for later use as frozen FMT capsules (see section 2.1.1); or (3) stored at -80°C for later use as a fecal slurry.

- 5.10 If stored at -80°C , rapidly thaw filtered fecal suspension in a 37°C water bath for 30-45 minutes and keep warm until administration. Once thawed, FMT product should be utilized within 12 hours.

