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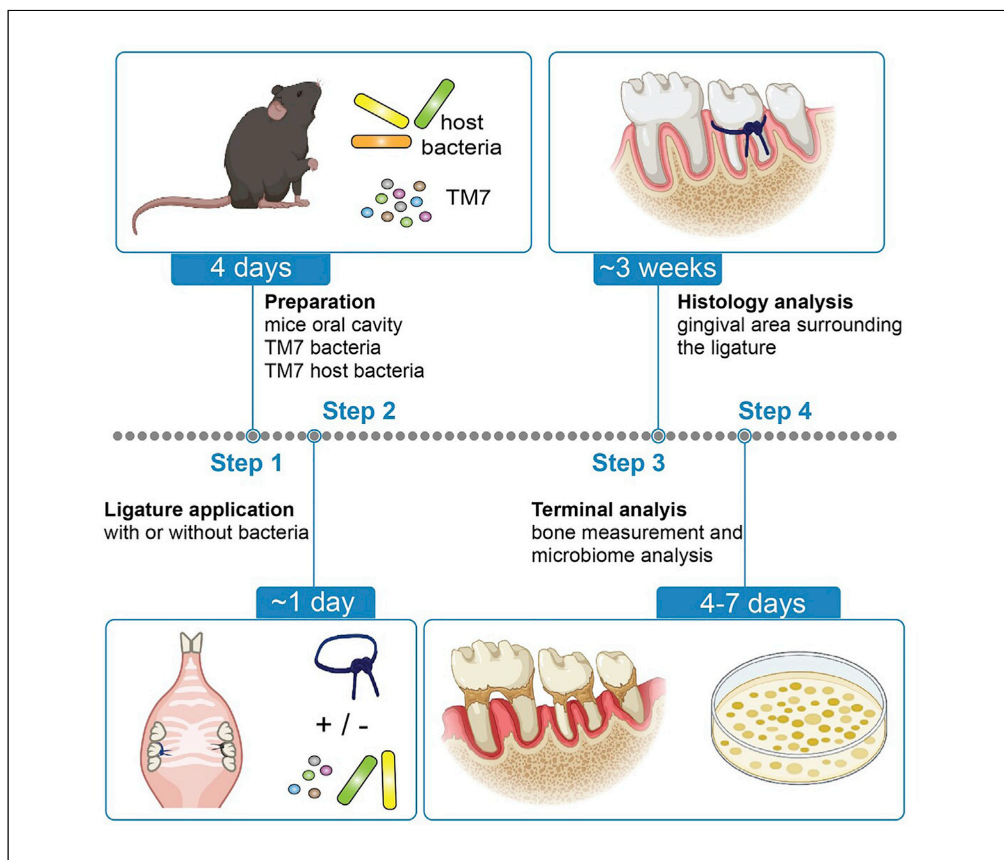
Protocol

Ligature-induced periodontitis mouse model protocol for studying Saccharibacteria



This protocol outlines the process of preparing Saccharibacteria (TM7) and applying ligature with and without TM7 onto a mouse molar, and measuring the subsequent bone resorption and inflammation. This ligature model is particularly useful in studying the pathogenicity of specific bacteria that do not typically colonize the mouse oral cavity. This is especially true in the case of TM7 bacteria that prefer to grow on the surface of other bacteria.

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Highlights

Ligature-induced periodontitis mouse model protocol for studying Saccharibacteria (TM7)

Beetle defleshing approach to allow for bone analysis

Detailed microbial, bone, and immunohistochemistry analysis techniques

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Ligature-induced periodontitis mouse model protocol for studying Saccharibacteria

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SUMMARY

This protocol outlines the process of preparing Saccharibacteria (TM7) and applying ligature with and without TM7 onto a mouse molar, and measuring the subsequent bone resorption and inflammation. This ligature model is particularly useful in studying the pathogenicity of specific bacteria that do not typically colonize the mouse oral cavity. This is especially true in the case of TM7 bacteria that prefer to grow on the surface of other bacteria.

For complete details on the use and execution of this protocol, please refer to Chipashvili et al. (2021).

BEFORE YOU BEGIN

This protocol describes the usage of Saccharibacteria strain TM7x and *Actinomyces odontolyticus* strain XH001 to conduct a ligature-induced periodontitis model in mice (Abe and Hajishengallis, 2013; Chipashvili et al., 2021). A similar protocol also has been utilized for other human and mouse oral bacteria as well (Jiao et al., 2013; Lin et al., 2014; Marchesan et al., 2018). The ligature method is used to mimic the occurrence of inflammatory periodontitis in a mouse model. The addition of bacteria allows for the differentiation of pathogenic versus commensal bacteria using inflammation and bone loss as a readout (Lin et al., 2014). This protocol will cover the bacterial preparation, ligature tying, as well as the termination and downstream sample processing/analysis focusing on the microbial community, bone resorption, and histology.

Over the years, the ligature-induced periodontitis method has mainly been established to study periodontal disease initiation and progression in the eukaryotic host, especially studying the immune response (Abe and Hajishengallis, 2013). However, there are few instances where it was used to study bacterial pathogenicity (Hoare et al., 2021; Li and Amar, 2007). The addition of bacteria to the ligature is based on the fact that ligature alone induces inflammation and bone loss only to a certain extent and saturates after 8–9 days (Marchesan et al., 2018). This disease initiation process can be further exacerbated by the application of pathogenic bacteria. As with any other rodent model, the ligature model does have its drawbacks, and we detailed them later in our methods. This study is unique in that we successfully incorporated ultra-small TM7 bacteria such as the TM7x strain, and clearly illustrated that ligature induced periodontitis model can aid in determining the pathogenic nature of these unique bacteria.

Growing bacteria and preparing ligatures

Timing: 3 days

Caution: Use standard bacterial sterilization techniques to clean any areas where bacterial work will be done (Siddiquee, 2017). Make sure to use a Bunsen burner or biosafety cabinet when working with bacteria.

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1. XH001 monoculture and TM7x/XH001 coculture in our protocol is grown in Brain Heart Infusion (BHI, BD Biosciences) (Bor et al., 2018), so make sure to prepare the appropriate amount of BHI (or bacteria-specific medium) needed for each experiment.
2. Prepare blood plates by using BHI with 5% v/v defibrinated sheep blood (Northeast Laboratory Services) and 1.5% agar (Fisher Scientific). Add blood after cooling the autoclaved medium.

3. XH001 and TM7x/XH001 culture preparations
   a. In a 15 mL conical tube (Fisher), inoculate 20–30 μL Actinomyces odontolyticus subsp. actinosynbacter strain XH001 monoculture (McLean et al., 2016) and TM7x/XH001 coculture into 1.0 mL BHI from frozen stock. Stocks were previously made by having an optical density measured at wavelength 600 nm (OD600) equal to 15. An OD600 of 15 is equivalent to approximately 10^9 CFU per milliliter for XH001 cells. We used the Genesys 30 spectrophotometer (Thermo Scientific) and disposable cuvettes (Fisherbrand) for our OD600 measurements. Cells were previously frozen in BHI with 18% glycerol (Sigma) at −80°C.
   b. Let the cultures grow at 37°C in a microaerophilic chamber (Don Whitley Scientific, 2% Oxygen, 5% Carbon Dioxide, Nitrogen balance) for 24 h.
   c. Expand the culture by passaging the 1.0 mL cultures into 9 mL of BHI. Incubate 24 h in the microaerophilic chamber.
      i Using a phase-contrast microscope (Nikon), observe the cultures every passage to confirm that there is no contamination. TM7x grows on a rod-shaped XH001, and typically contaminations will appear as cocci in our case (Figure 1F).
   d. Take 2 mL from the expanded cultures and passage into 18 mL of BHI. Incubate in the chamber for 24 h. Keep the remaining 8 mL as a backup inoculum in case there is contamination.

Note: We passage a minimum of two times to acquire homogeneous culture.

   e. Measure the OD600 for the cultures. XH001 and TM7x/XH001 typically grow to OD600 of 0.8–1.0.
   f. Centrifuge 20 mL at 17,000 *g* for 20 min using a benchtop centrifuge (Eppendorf), remove the supernatant, and resuspend the pellet in ~0.5 mL of Phosphate-Buffered Saline (PBS) (Corning) to have an equivalent OD600 of 15. The bacteria are typically 30-fold concentrated.

4. Isolation of TM7x bacteria on their own
   a. TM7x/XH001 coculture is inoculated and expanded to 400 mL BHI, similar to step 1.
   b. On the day of TM7x isolation, the larger coculture is aliquoted to 40 mL in conical tubes and centrifuged at 5,600 *g* for 10 min. This step allows most of the larger XH001 cells (>1 μm) to be pelleted so that free-floating smaller TM7x cells (200–300 nm) can be filtered easily.
   c. Supernatant containing free-floating TM7x is collected and filtered through 0.45 μm Stericup filtration system (Millipore).
   d. TM7x bacteria that pass through the membrane are centrifuged at 80,000 *g* for 1 h to pellet the small bacteria.
   e. Resuspend pelleted TM7x in 0.5 mL of PBS, which contains about $10^{9\text{-}10}$ bacteria.

5. Prepare and aliquot Carboxymethylcellulose (CMC) (Sigma) solution.
   a. Place a beaker with 100 mL of PBS on a magnetic stirrer (Thermo Scientific). Add a large stir bar and begin stirring and lightly heating the solution (~40°C). Gradually add 3 g of CMC powder.

⚠ CRITICAL: This process may take several hours, and the solution will be extremely viscous.

   b. Once the CMC is dissolved, aliquot ~0.5 mL of the mixture into microcentrifuge tubes (Denville).

Note: To facilitate aliquoting the viscous CMC solution, widen the tips of P1000 pipette tips (Thomas) by cutting the ends.

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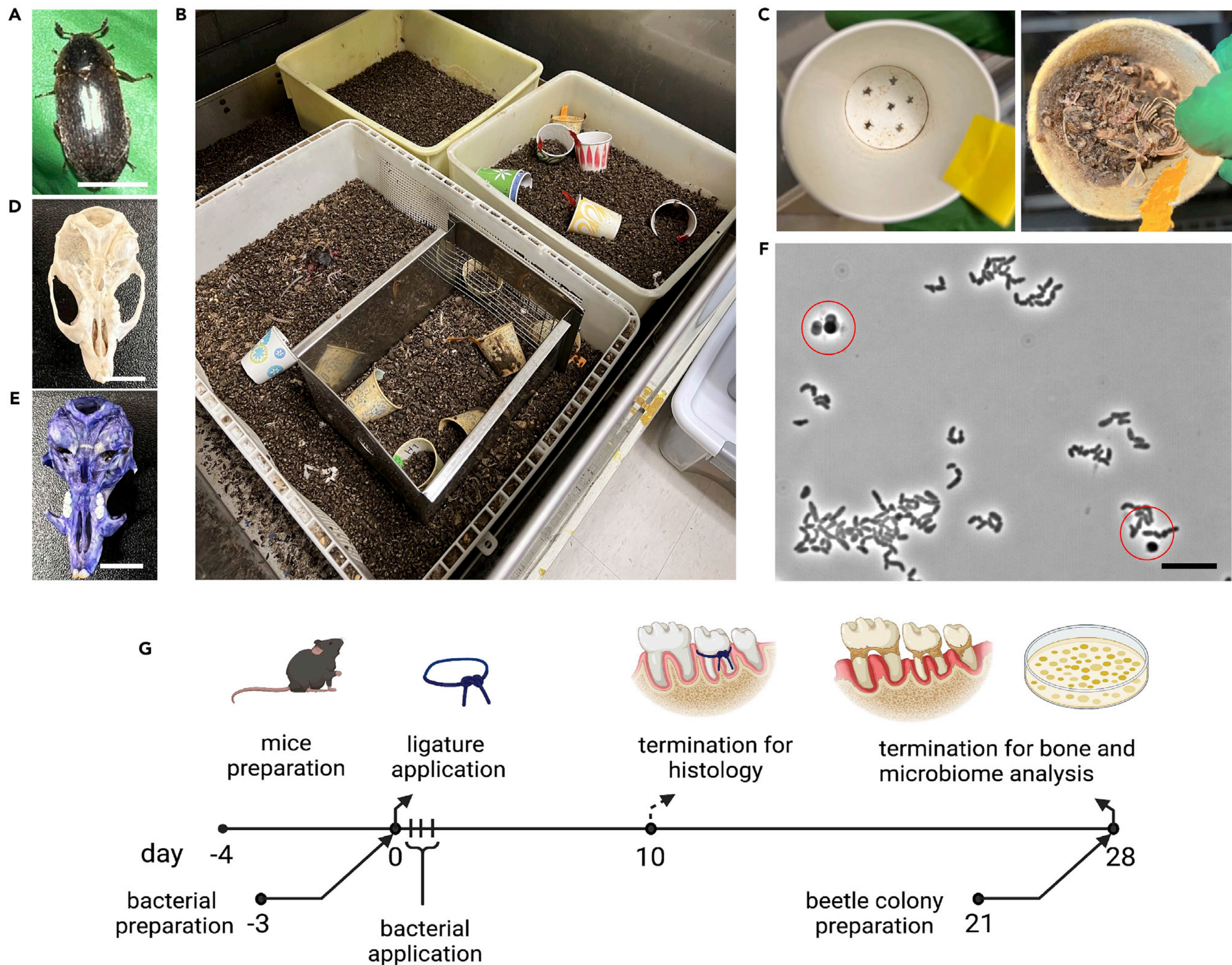

Figure 1. General preparation

(A) Dermestid beetle. Scale bar is 3 mm.

(B) Defleshing beetle setup containers.

(C) The mini-cup ensures that the skulls are not lost in the container. It has holes to allow free movement of the beetles.

(D) De-fleshed and bleached skull. Scale bar is 5 mm.

(E) Skull stained with toluidine blue. Scale bar is 5 mm.

(F) Phase-contrast image of possible contamination of XH001 culture. Taken at 100× magnification. Scale bar 10 μm.

(G) Timeline schematic of the ligature experiment. Solid lines represent the timeline of experiments that can be conducted on the same mice, while broken lines represent experiments that need to be set up separately. The ligatures were applied on day 0.

6. Applying desired bacteria on the ligature (Figure 1G)
   a. It is recommended to set up multiple groups for each set of experiments to include necessary controls. 0.5 mL of resuspended XH001, TM7x/XH001, TM7x alone, or PBS (control) are mixed with 650 μL of CMC solution by gently pipetting up and down. PBS control serves as a baseline to determine if the addition of bacteria on the ligature induces more bone loss.
   b. Cut a 6-0 size silk ligature (Teleflex 6-0) into 10–15 cm pieces. Each piece is enough for two ligatures during the tying process. Since mouse molars are very small, approximately 1 mm mesiodistally, ligatures larger than 6-0 size can cause unnecessary additional damage.
   c. Carefully insert the silk string into the bacteria/CMC mix. Leave a small segment out of the mixture to allow for easy extraction (Figure 2A). In one tube, 1–5 ligatures can be placed without tangling. Prepare one tube per experimental group with five ligatures each.

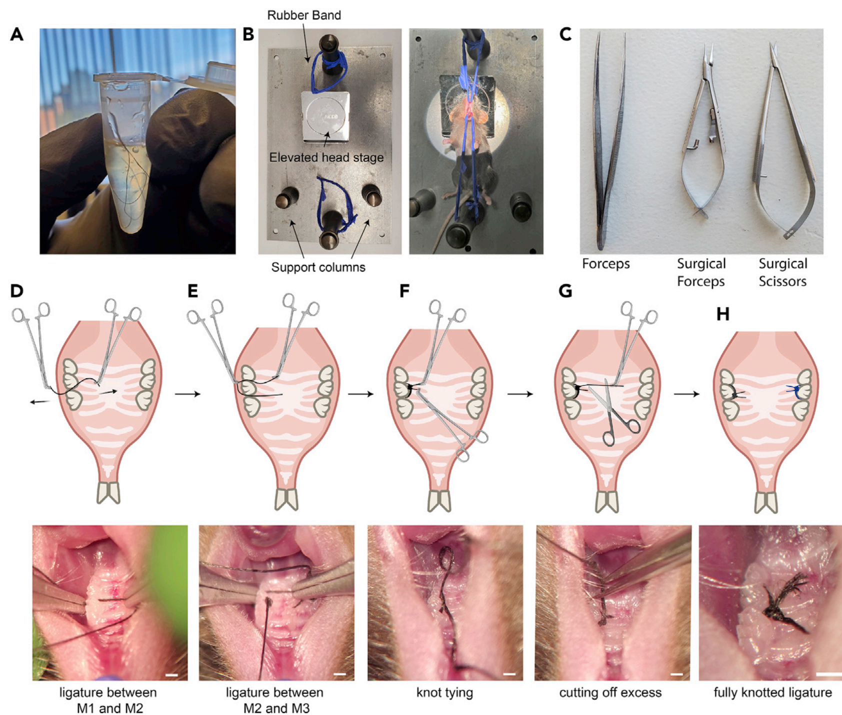

Figure 2. Ligature application on the mouse maxillary second molar

(A) CMC solution mixed with bacteria in a microcentrifuge tube. One silk ligature string is placed inside.

(B) Mouse stabilizing jaw retraction board without a mouse (left). Rubber bands are attached to support columns. Jaw retraction board with a mouse in position (right). Jaws are opened by rubber bands attached to upper and lower incisors.

(C) Main tools used for ligature tying procedure (D–H) Ligature tying procedure. Scale bar 1 mm.

(D) Placing ligature between M1 and M2.

(E) Placing ligature between M2 and M3.

(F) Tying the surgeon's knot.

(G) Cutting off excess ligature string using surgical scissors.

(H) Finished product of ligature tying procedure.

d. Let the ligatures soak in the mixture for at least 30 min before moving onto the ligature-tying procedure (see step-by-step method details section part 1).

⚠ CRITICAL: The ligature tying process takes approximately 5–10 min per ligature, so plan accordingly.

Preparation of animals and procedure area

Timing: 4 days

7. Order 8–9 weeks old female or male C57BL/6J mice from The Jackson Laboratory.
8. Let mice acclimate to the animal facility for 3–4 days before starting the ligature experiment. This will also homogenize and stabilize the mouse oral microbiome.

⚠ CRITICAL: The mice need to be housed in specific locations depending on the bacterial biosafety levels. For example, if the bacterial pathogen is biosafety level 2 and above, the mice need to be kept in a facility that has biosafety level 2 or higher standards.

9. Obtain post-operative care materials per your institute's ethics protocol.

10. Sterilize all surgical materials and the procedure room in the animal facility on the day of the experiment.
11. Prepare anesthetic by mixing ketamine (100 mg/mL) (COVETRUS) and xylazine (20 mg/mL) (COVETRUS) with PBS to get the final concentration.

⚠ CRITICAL: Acquire approval from your institution's ethical committee for ligature tying and anesthesia application procedures prior to starting this protocol.

12. Prepare 14% Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) (Sigma) solution in distilled water (ddH$_2$O).
13. Prepare a beetle colony for defleshing (Figures 1A–1C) (Sommer and Anderson, 1974)
    a. Beetles (Kodiak Bones & Bugs) (Figure 1A) are housed in a secondary container (Figure 1B)
    b. The colony is regularly fed with fresh meat to have sufficient numbers for the actual experiment.
    c. Small paper cups (Fisher) with holes will be used to incubate the different groups of skulls (Figure 1C).
    d. Defleshed skulls will have no tissue covering the bone (Figures 1D and 1E).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Purified anti-mouse Ly6G antibody (1:500)	BioLegend	Cat#127601; PRID: AB_1089179
AP goat anti-rat IgG (Mouse Absorbed) polymer kit, Alkaline Phosphatase	Vector	Cat# MP-5444
Biological samples		
Murine oral ligature	from this study	N/A
Murine gingival tissue	from this study	N/A
Murine maxilla with associated molar teeth	from this study	N/A
Critical commercial assays		
MasterPure Gram Positive DNA purification kit	Epicentre	Cat# MGP04100
Blue Substrate Kit, Alkaline Phosphatase	Vector	Ref# SK-5300
ImmPRESS- AP Goat Anti-Rat IgG (Mouse Adsorbed) Polymer Kit, AP	Vector	Ref# MP-5444
Experimental models: Organisms/strains		
Mouse: C57BL/6J (males or females 8–9 weeks)	The Jackson Laboratory	Stock #000664
TM7x/XH001 bacteria	From this study	He et al. (2015)
Dermestid Beetles	Kodiak Bones & Bugs	N/A
Chemicals, peptides, and recombinant proteins		
Toluidine Blue O	Sigma-Aldrich	198161-5G
Carboxymethylcellulose sodium salt	Sigma-Aldrich	Cat# C4888
Ketamine	COVETRUS	Cat# 056344
Xylazine	COVETRUS	Cat# 200-529
Lysozyme	Sigma-Aldrich	Cat# L6876
Ethylenediaminetetraacetic acid disodium salt dihydrate	Sigma-Aldrich	E5134
Phosphate-buffered Saline	Corning	Cat# 21-040-CV
Glycerol	Sigma-Aldrich	Cat# 56-81-5
Isoflurane	Patterson Veterinary	Cat# 07-893-1389
Citrate buffer	Fisher Scientific	Cat# 50-525-47
Xylene	StatLab	Cat# 8400-1
10% buffered formalin	Fisher Scientific	SF100-4
Hydrogen Peroxide	Fisher Scientific	H325-100
Eosin	Epredia	Cat# 71204
Hematoxylin	Polysciences, Inc.	Cat# 24245
Agar	Fisher Bioreagents	BP1423-500

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Phase-contrast microscope	Nikon	E400
15 mL conical tube	Fisher Scientific	Cat# 50-145-8829
Benchtop Centrifuge	Eppendorf	Cat# 5430 R
Magnetic Stirrer	Thermo Scientific	Cat# SP18425Q
Microcentrifuge tubes	Denville	Cat# C2170
Paper Cups	Fisher Scientific	Cat# S05166
P1000 Pipette tips	Thomas Scientific	Cat# 1145N16
Glass beads for plating	Fisher Scientific	Cat# 71-013-3
Adhesive Microscope Slides	Thermo Scientific	Cat# 3039
Microaerophilic chamber	Don Whitley Scientific	A35
28G Syringe	BD Biosciences	Ref# 329461
Oral infection blunt end needle	PetSurgical	AFN2425C
Surgical microscope	Seiler	Revelation
Surgical scissors	Fine Science Tools	Item No. 15000-00
Surgical Forceps	Fine Science Tools	Item No. 12060-01
Dissecting microscope	Vision Engineering	Lynx EVO
Bead Beater	MP	FastPrep -24
Stock tube	Fisher brand	Cat# 02-682-558
Glass beads for bead beating	Sigma-Aldrich	Cat# G8772
Platform Shaker	New Brunswick Scientific	Innova 2000
Ruler	Fisher Scientific	Cat# 12-000-152
Red modeling compound	Hasbro	F1981
Tissue Cassette	Fisher Scientific	Cat# B8551739BE
Paraffin	Fisher Scientific	Cat# 22-900-700
Microtome Blades	C.L. Sturkey	D554D50
Microtome	Leica Microsystems	RM2245
Coverslip	Fisher Scientific	Cat# 12542A
Mounting media	Vector	H-5000
Kimwipe	Fisher Scientific	Cat# 06-666
Hydrophobic barrier pen	Vector	H-4000
Humidified slide chamber	Fisher Scientific	Cat# 50-112-3683
Defibrinated sheep blood	Northeast Laboratory Services	SB100
Absorbent points	Kerr	13966
Stericup filtration system	Millipore	Cat# S2HVU01RE
Genesys 30 Spectrophotometer	Thermo Scientific	Cat# 840277000
Cuvette	Fisher brand	Cat# 14-955-127
Software and algorithms		
FIJI	NIH Image	https://imagej.net/Fiji

STEP-BY-STEP METHOD DETAILS

Part 1: Ligature application on the mouse maxillary second molar

Timing: 2–4 h

Tying the ligature is the most critical step of the entire experiment. It must be done efficiently, without causing harm to the mouse. This step will take some practice to master. If possible, use a pre-euthanized mouse to practice on before moving on to one that is alive. The greatest danger in this step is accidentally puncturing the buccal mucosa with the forceps during the ligature tying process. Depending on the severity of the puncture, this can cause bleeding. Figure 1G

⚠ CRITICAL: If the ligatures are tied wrong or loosely, the majority of them will fall off during the incubation time as the mice chew on them. It is not ideal to keep checking the ligature

once applied since that will stress the mice. Therefore, robust ligature application and secure tying are critical.

1. Administration of Ketamine and Xylazine anesthesia
   a. Weigh the mouse by putting a container on a scale and zero it. Put the mice in the container to determine their weight.
   b. Move the mice to the procedure area where the microscope (Seiler) is set up.
   c. Aspirate anesthesia mixture in the syringe (BD Biosciences) to prepare before handling mice
   d. Scruff the mouse in one hand to immobilize. Intraperitoneally (i.p.) inject the mixture of Ketamine (87.5 mg/kg) and Xylazine (12.5 mg/kg). This means a 20-g mouse will receive 0.1 mL of the mixture.
   e. Place the mouse in a cage and wait 4 min for the mouse to enter a complete anesthetic state. You may check this by tail or foot pinch movement.
2. Once the mouse is in a complete anesthetic state, open the jaw and position it in the stabilizing jaw retraction plate (Figure 2B).
   a. Position microscope so that the second maxillary molar is visible
3. Use surgical forceps (Fine Science Tools) to pull out a ligature string from the microcentrifuge tube with CMC and bacteria mix.
4. Grasp the ligature with the tips of the forceps and move into the oral cavity of the mouse.

⚠ CRITICAL: Using the wrong forceps will cause the ligature to slip during the tying process (Figure 2C).

5. Proceed with the ligature tying procedure (Figures 2D–2H, Methods video S1).
   a. Use the second pair forceps to grab the ligature approximately 1.0 cm away and position the two forceps on the gingival and palatal sides of the mouth
   b. Position the ligature between the first and second molars, and with a rocking motion, gently press down on the ligature so it gets lodged between the teeth (Figure 2D).

⚠ CRITICAL: If too aggressive during this step, the forceps will lodge into the side gingiva or buccal mucosa and cause bleeding. If this happens, use a sterile gauze pad to soak up the blood until it stops. If this mouse is going to be used for observing the inflammatory response in the gingival area, it should be excluded from the experiment since this could interfere with the results. If these types of adverse events happen often, the operator should do more practice before handling experimental mice.

   c. Release the forceps, and grab the ligature again, this time on the buccal side (Figure 2E).
   d. Repeat step "b" for the second and third molars. The loose ends of the ligature should be facing the palatal side (Figure 2E).
   e. Tie a surgeon's knot on the palatal side of the tooth. Make sure it is tight by pulling on either side (Figure 2F). Use scissors (Fine Science Tools) to cut off the excess silk ligature, approximately 3 mm away from the knot (Figures 2G and 2H).
   f. Using a blunt-ended needle (PetSurgical), apply 20–50 μL of additional bacteria to the ligature from the bacteria/CMC mix.

6. Remove the mouse from the stabilizer plate, and place it face down under a controlled heat source, and closely monitor until it awakes and returns to normal behavior.

⚠ CRITICAL: check mice to make sure they are moving around the cages and resuming normal behavior before putting them back in the cages.

Alternatives: Many of our basic laboratory reagents, supplies and equipment can be replaced by other equivalent laboratory supplies. There are a few items that we strongly recommend

using the indicated supplies from the key resources table such as the 6-0 ligature and surgery grade forceps.

Part 2: Additional bacterial inoculation on the ligature (follows part 1)

Timing: 3 days

For 3 days after the initial ligature tying, bacteria will be applied on and near the ligature to ensure colonization (Figure 1G).

7. Mix bacteria with OD600 of 15 in PBS with 3% carboxymethylcellulose (CMC) solution 1:1 in order to make a solution (~10^9 CFU/mL) that can be easily applied to the mouse oral cavity. All bacteria, including free-floating TM7x are prepared fresh (see preparation of animals and procedure area section).
8. Place the mice in an isoflurane induction chamber adhering to the IACUC safety guidelines. Use a flow rate of 0.5–1.0 L per minute at a 5% isoflurane balanced with oxygen.

> CRITICAL: mice will wake up quickly once removed from the chamber, so be swift and quick when applying bacteria in the following step. If necessary, a nose cone can also be used to allow for this procedure to be done while the mouse is continually under anesthesia.

9. Perform oral infection by using a blunt-ended needle (PetSurgical).
   a. Open the mouth using forceps.
   b. Apply 20–50 μL of the solution to the side of the oral cavity where the ligature is located, making sure to cover the gingival areas around the molars.
10. Remove the mouse from anesthesia and monitor recovery. Decontaminate the induction chamber by spraying it with 70% ethanol.
11. Repeat steps 7–10 on the next two consecutive days.

Part 3: Experimental endpoint—Microbiome analysis (follows part 2)

Timing: 4 days

For trial termination, there are several possible follow-up procedures based on the desired readout. This procedure describes the microbiome analysis that can be performed on the ligature.

12. Keep mice housed for 28 days after ligature application (see parts 1 and 2).
13. Euthanize the mouse by using a CO_2 chamber, following procedures approved by your institution.
14. Sterilize surgical scissors and forceps to avoid any contamination.
15. Remove the mandible of the mouse by cutting it out to expose the maxilla.

16. Remove the ligatures by using the scissors to make one cut through the ligature and then use forceps to pull it out in one piece. If there are any leftover strings, be sure to collect them as well. It is critical that all of the ligature is collected.
    a. Place extracted ligature in 150 μL PBS in microcentrifuge tubes, place on ice before moving to the next step.

> CRITICAL: Since this step may involve the collection of many ligatures, using ice will ensure that there is no significant bacterial growth during incubation.

b. Vortex the microcentrifuge tube for 5 min and use 10 µL of the mixture to make serial dilutions of 10^{1-5}.
c. Plate onto BHI blood agar plates by bead (Fisher) spreading method. These plates allow for the growth of the intended *Actinomyces* species.
d. Incubate in a microaerophilic chamber for three days before counting small and large colonies (Figure 3A). The small colonies will represent the *Actinomyces* bacteria that were added to the ligature. The other large colonies are members of the rest of the commensal mouse oral microbiota.

⚠ CRITICAL: counting colonies too early will make it difficult to differentiate the added *Actinomyces* from the commensal microbiota.

17. Use the remaining 140 µL for DNA extraction
    a. Mix 140 µL ligature resuspension with 150 µL of Tris-EDTA (TE) buffer from the MasterPure DNA isolation kit (Epicentre) and transfer to a stock tube (Fisherbrand) containing glass beads (Sigma).
    b. Add lysozyme (Sigma) to the sample, reaching a final concentration of 2 mg/mL. Incubate for 1 h at 37°C.
    c. Disrupt the cells by bead beater (MP) for 3 × 30 s each at 6 m/s speed with 1 min short pauses at 4°C.
    d. Isolate gDNA using MasterPure DNA isolation kit. Follow manufacturer's protocol.
    e. Isolated gDNA can then be used for 16S rRNA sequencing. We used the Zymo sequencing core facility, which has a turnaround time of 3 weeks (not included in overall procedure time).

For this experiment, each ligature can be used to generate independent 16S rRNA relative abundance data. If needed, two or more ligatures can be combined in one genomic DNA isolation method. The isolated genomic DNA can be submitted to a standard 16S rRNA sequencing facility such as the Zymo sequencing core. These core facilities generate relative abundance OTU tables, which are easy to interpret and can be used with standard graphing software such as Excel or Prism to display the outcome. From our experience, TM7x alone group does not form colonies since TM7x cannot grow without their host bacteria. Therefore, there is no need to plate ligature microbiome for this group unless the researcher is interested in learning how the total bacterial load on the ligature changes.

Part 4: Experimental endpoint—Bone analysis (follows part 2)

⏲ Timing: 2–3 weeks

This procedure follows part 2 of the step-by-step method details section. The same mice can be used for both part 3 and 4 of this method. Ligature can be removed and used in part 3 while remaining tissue and maxilla can be used to quantify bone resorption (Figure 1G).

18. Keep mice housed for 28 days after ligature application (see part 1 and 2).
19. Euthanize the mouse by using a CO_2 chamber.
20. Using scissors, cut off the head of the animal from the body, and remove skin/fur from the skull. Any tooth with a missing ligature should be noted and excluded from bone analysis.

Note: At this point, the ligatures can be removed for microbiome analysis (see part 3).

21. Add the separated skulls in small paper cups with holes and place them in a beetle colony (Figures 1A–1C).
22. Remove tissue by using a beetle colony (*Dermestes vulpinus*) for 2 weeks.

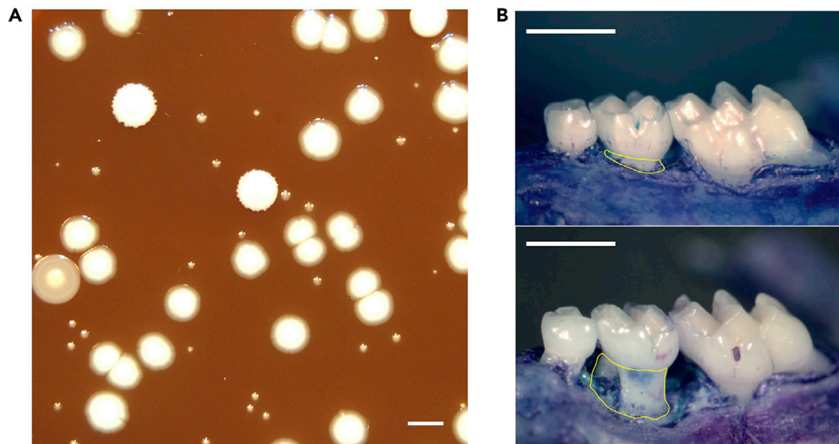

Figure 3. Quantification methods

(A) Colonies grown on a blood agar plate. ''small'' colonies are from the *Actinomyces* used in the experiment. ''Large'' colonies come from the murine commensal oral microbiota. Scale bar is 2 mm.

(B) Images of defleshed and stained molars. The region of interest is shown in yellow. Scale bar is 1 mm in length. Top image shows an example of low bone loss. Bottom image shows an example of high bone loss.

Note: Defleshing of the bone structures by beetles is an efficient method compared to chemical or mechanical means (Hall and Russell, 1933).

⚠ CRITICAL: Using the beetles for less than 2 weeks will risk incomplete flesh removal. Also, using the beetles for too long will risk excessive bone degradation from digestive acids.

23. Remove the maxilla from the beetle colony, and quickly clean with double distilled water (ddH_2O).
24. Bleach the maxillae by washing in 5% hydrogen peroxide (Fisher) for 8 h. Place on a platform shaker (New Brunswick Scientific) to make sure they are mixed well (Figure 1D).
25. Stain with 1% Toluidine Blue O (Sigma) in water for 5–10 s (Figure 1E).

⚠ CRITICAL: Staining for more than 10 seconds will cause over-staining and will make downstream image analysis difficult.

26. Photograph specimens by using a dissecting microscope (Vision engineering). Refer to Figure 3B to determine optimal magnification. While taking the image, it is recommended to have a ruler (Fisher) in the frame and on the same plane as the teeth. This will enable an accurate scale determination.

Note: we use red modeling compound (Hasbro) to hold the maxilla sideways and the ruler on the bottom (Figure 3B).

27. After establishing the scale of the image, measure the area from the cementoenamel junction to the alveolar bone crest of the second molar using FIJI software by drawing an area of interest (Figure 3B).
28. The area is quantified in mm^2. A larger area signifies a greater amount of bone loss.

For this procedure, the number of ligatures per group is mainly determined by the bone loss area measurement. We recommend determining the sample size by conducting a pilot experiment followed by a power analysis of the bone area that is lost. In our case, 8–10 ligatures/bone area measurement was enough to distinguish a significant bone loss difference between XH001 and TM7x/XH001 groups.

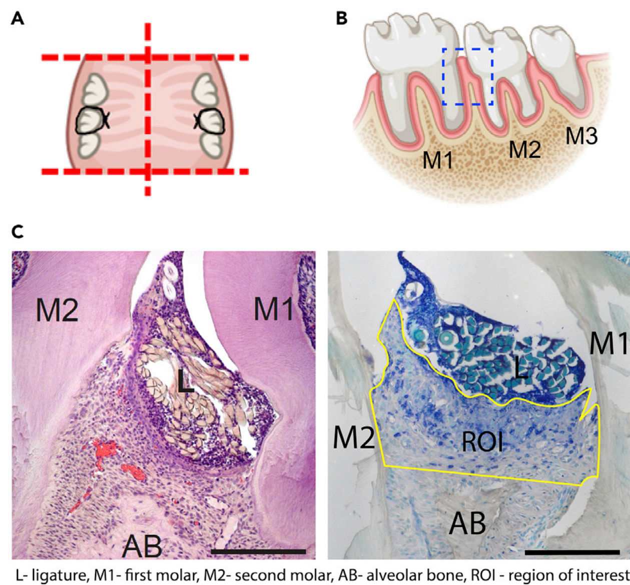

Figure 4. Histology and immunohistochemistry

(A) Diagram for removal of mouse palette for FFPE preparation.

(B) Cross-section view of the intended area of interest for histology sectioning (blue box).

(C) Immunohistochemistry for H&E (left) and Ly6G (right) stains. Scale bars represent 100 mm.

Micro-CT 2D scanning followed by 3D reconstruction can be an additional way to determine bone resorption in ligature experiments. This analysis can be costly, however, it can help decrease the number of animals used in two ways: (1) bone analysis can be more efficient so it requires fewer maxillae to see differences, and (2) histology can be combined with bone analysis since micro-CT scan does not damage fixed gingival tissues. In addition, micro-CT scans can produce more detailed output compared to 2D image analysis, such as 3D bone analysis and bone mineralization. We did not include micro-CT analysis in our protocol since it is technically challenging and has been covered elsewhere (Park et al., 2007).

Part 5: Experimental endpoint-histology analysis (follows part 2)

Timing: ~11 days

In this procedure, ligature and the tissues are kept intact so we set up an independent mouse experiment from parts 3 and 4, and we sacrifice the mice on day 10 (Figure 1G).

29. Keep mice housed for 10 days after ligature application (see parts 1 and 2).
30. Euthanize the mouse by using a CO_2 chamber.
31. Remove the entire oral palette by using a standard razor blade to cut around the molars (Figure 4A) and place in a cassette (Fisher) into 10% buffered formalin (Fisher) for 16 h in a volume at least twenty times that of the tissue sample.

CRITICAL: This step can be difficult to achieve without destroying the maxillary structure. Refer to Mizraji et al. (2013) for a detailed protocol of this process.

32. Transfer the cassettes to a container with 14% EDTA for decalcification for 10 days in a volume at least twenty times that of the tissue sample. This is done at 20°C–24°C. Use a plate rocker or platform shaker to gently mix the samples and replace the solution every 2–3 days.

33. Run the samples in a standard histology tissue processor using xylene (StatLab) as a clearing agent, and embed them into paraffin (Fisher) blocks with the buccal side of the maxilla facing the bottom of the mold. This ensures that the sections will be cut on the sagittal plane.

⚠ CRITICAL: Make sure that the tissue is embedded as level as possible. Avoid any movement during the paraffin solidification process, as any rotation of the maxilla will make it difficult to cut a section in the region of interest (see below).

34. Trim blocks until the tissue is visible, and place the block in an icy water bath for 30 min.
35. The aim is to reach an area in between the molars, with as much of the tooth root showing as possible. See Figure 4B for a diagram of the recommended cross-section area. See the troubleshooting section for possible issues during this step.
36. Using a microtome (Leica), cut sections at 5-micron thickness onto positively charged microscope slides (Thermo Scientific). These slides can now be used for downstream histology and immunohistochemistry analysis.

⚠ CRITICAL: Make sure the microtome blade (C.L. Sturkey) is sharp and that the sections are coming out smoothly. Make sure there are no wrinkles in the tissue when mounting them on the slides.

Hematoxylin and Eosin staining (follows after sectioning)

37. Bake slides for 1 hour at 60°C (Figure 4C).
38. Deparaffinize and rehydrate sections: 3 × 5 min Xylene, 2 × 5 min 100% ethanol, 1 × 3 min 95% ethanol, 1 × 3 min 70% ethanol, 1 × 5 min ddH_2O.
39. Hematoxylin (Polysciences, Inc.) stain for 1 min 15 s, and rinse in ddH_2O 3 times.
40. Place in bluing reagent (Epredia) for 1 min, and rinse in ddH_2O 3 times.
41. Eosin (Epredia) staining and dehydration: 1 × 30 s Eosin, 3 × 2 min 95% ethanol, 3 × 2 min 100% ethanol, 3 × 5 min Xylene.
42. Coverslip (Fisher) slides using Xylene-based mounting medium (Vector).

Neutrophil staining (follows after sectioning)

43. Bake slides for 1 hour at 60°C (Figure 4C).
44. Deparaffinize and rehydrate sections: 3 × 5 min Xylene, 2 × 5 min 100% ethanol, 1 × 3 min 95% ethanol, 1 × 3 min 70% ethanol, 1 × 5 min ddH_2O.
45. Perform antigen retrieval by placing slides in pH 6 citrate buffer (Fisher) at 98°C for 20 min. This can be done in an oven or a double boiler.
46. Take slide container with citrate out of boiler/oven and place it into a container with cool running tap water and let slides cool down for 20 min.
47. After slides are cooled down, rinse in distilled water for 5 min.
48. Take slides out of the water and dry area around the tissue using a kimwipe (Fisher).
49. Use a hydrophobic barrier pen (Vector) to draw a small circle around the tissue on each slide.
50. Block endogenous peroxidases by adding 3% hydrogen peroxide (Fisher) onto the sections for 10 min.
51. Wash sections in PBS two times.
52. Incubate in normal goat serum for 20 min.
53. Remove serum from slides and add on the primary antibody (Ly6G antibody, BioLegend) diluted 1:500. You should only need about 150 μL per slide. Leave primary antibody on for 1 h at 20°C–24°C. Keep slides in a covered humidified chamber (Fisher).

Note: It is recommended to use a negative control in which no primary antibody is added.

54. Wash slides in PBS two times
55. Apply secondary antibody and subsequently the blue substrate (Vector) following the manufacturer's protocol.
56. Dehydrate through graded alcohols and clear with xylene: 3 × 2 min 100% ethanol, 3 × 5 min Xylene.
57. Coverslip with xylene-based mounting medium.

Ly6G positive cells should be quantified at 200× magnification, with the region of interest being the gingival connective tissue between the root surface of the first and second molars (Figure 4C), from the epithelial border down to the alveolar bone crest (Xiao et al., 2017). The ligature should be excluded from quantitative analysis. Again, the number of regions of interest quantified per group is determined by a pilot study followed by power analysis. In our case, a sample size of 6–8 per group was sufficient to determine the difference between XH001 and TM7x/XH001 groups.

EXPECTED OUTCOMES

The three main outcomes of this protocol are a detailed look at the microbial community composition, innate immune responses in the tissues surrounding the ligature, and alveolar bone resorption.

Analyzing the microbial community of the ligature by 16S rRNA sequencing will show the relative abundances of bacteria that are occupying the ligature at the end of the experiment. However, this does not show the sheer number of bacteria at the endpoint of the experiment. For this, we can analyze the absolute reads from the sequencing data, or ligature bacteria can be plated on an agar plate to quantify colony forming units for each type of bacteria. To do this, optimal media and culture conditions of the bacteria must be known. Many native oral bacteria will not grow on these plates, so the colony-forming units that are determined in this assay are an underestimate of the total bacteria present on the ligature.

Histology and immunohistochemistry serve as a visual aid in measuring the level of inflammation, tissue degradation, and immune response that each experimental group is generating. The histology should show polynucleated immune cells such as neutrophils. There will also be a degradation of the epithelial tissue bordering the ligature.

Bone resorption analysis by two-dimensional analysis allows for determining a differential effect of each experimental group. The bone loss is occurring as a result of the massive amount of local inflammation around the site of the ligature. Bone loss will be shown by an increase in the distance and area between the cementoenamel junction to the alveolar bone crest of the second molar (Chipashvili et al., 2021).

LIMITATIONS

Our protocol describes the application of bacteria on a ligature in a murine model of periodontitis in order to deduce the pathogenicity of the bacteria in the context of human periodontal disease. However, there are several limitations of this model which do not allow for the study of human periodontitis in a robust manner. One such limitation is that human oral microbes may not always be able to colonize inside a murine ligature model. Even if they do colonize and are able to persist throughout the duration of the experiment, their interaction with the endogenous murine oral microbiome will be unpredictable. Also, since the immune system of a mouse is different from that of a human, the immunological response could vary drastically. However, using a humanized mouse model may show a response more representative of human periodontitis. Another important limitation is that our model is not designed to show disease progression. In a real-life context, human periodontitis develops gradually over months and years. The immune response of blunt oral trauma does not represent progressive degradation. Even without adding any bacteria, the introduction of a ligature into the oral cavity induces a significant level of inflammation and bone loss. This response is

considerably large and introduces noise into the readout of the experiment. This noise can make it difficult to discern the effects of any addition of bacteria.

Observing the immune response in the mouse tissue can be difficult in this model. Due to the limited tissue, it is difficult to do protein analysis and more suitable to monitor mRNA expression by RT-qPCR. It may be necessary to pool together the gingival tissue of several mice to get any significant results for protein analysis. It is for this reason that histology and immunohistochemistry are recommended. After tissue fixation, the bone of the maxilla and teeth need to be decalcified to make it possible to section. Using a formic acid based decalcifier may be more beneficial to keeping a higher tissue immunoreactivity; however, we found that it was destroying the connective tissue of the gingiva, resulting in poor tissue sections. EDTA takes a longer time, but it preserves the tissue better.

This protocol is focused on studying the subgingival microbiome of the mice, but we found that sampling the mouse subgingival microbiome was very difficult. We attempted using absorbent points (Kerr), but we found that the resulting microbial load is very low, and it is almost impossible not to have contamination from the saliva.

TROUBLESHOOTING

Problem 1

Difficulty retaining grip during ligature tying procedure (step 5).

Potential solution

Keeping a paper towel nearby during the procedure can be very helpful. If the ligature starts to slip out of the grip of the forceps, it may be because the forceps are too wet. Use a paper towel to dry the jaws of the forceps. It is also important to use the correct forceps, as using non-surgical forceps will cause additional problems.

Problem 2

Ligature is often loose and falls out during the experiment (step 5).

Potential solution

Making sure the ligature is tight during the tying procedure is a critical element of the protocol. Avoid any extra slack on the knot during the surgeon's knot step. Make sure the first knot is completely tight before applying the second knot. Also, it may help to turn the ligature and wedge the knot in between the second and third molars of the animal. This must be done consistently throughout every animal in the experiment.

Problem 3

Difficulty orienting tissue and finding the area of interest during tissue sectioning (steps 33–36).

Potential solution

Success in sectioning relies heavily on the accuracy of embedding. It is imperative that the tissue is embedded at an angle that allows for an even cutting of the teeth. During the sectioning process, it is recommended to periodically take one section on a slide and add one drop of hematoxylin to it and check it under the microscope. Doing this will show whether the tissue is being cut on the correct plane. Most modern microtomes allow for small adjustments to be made to correct the sectioning plane. This is done by changing the angle of the block holder.

Problem 4

The tissue is breaking apart during sectioning (step 36).

Potential solution

This can be caused by insufficient decalcification or a -warm formalin-fixed paraffin-embedded (FFPE) block. During the decalcification process, it is possible to check if the sample is sufficiently decalcified. This is achieved with an ammonium oxalate test. Take 5 mL of used decalcifying fluid (at least 1 day old), and neutralize it by adding drops of ammonium hydroxide., When the solution reaches a pH above 7, add 5 mL of saturated aqueous ammonium oxalate. After 30 min, if there is no precipitate, this means that the last change of EDTA solution was free of calcium ions (ref). If a precipitate is seen, more decalcification is necessary. It is not possible to fix the level of decalcification after the tissue has been processed and embedded. The experiment must be redone with a slightly longer decalcification period. A warm FFPE block can cause the section to be brittle and break apart during the cutting process. To fix this, place the FFPE block into ice slush at least once every 5 min while cutting. It is also possible to use refrigerating block holders as well as refrigerating aerosols for this issue. These methods will keep the block cold during cutting.

Problem 5

Skulls are not fully defleshed after beetle colony treatment (step 22).

Potential solution

When preparing the skull, make sure that all the skin and fur are completely removed before placing it in the beetle colony. When placing the sample into the beetle colony, it is recommended to manually add a few beetles to the container in order to initiate the process. Check on the skull samples every 3–4 days to monitor the defleshing.

Problem 6

Determining appropriate throughput and experimental duration (see "before you begin").

Potential solution

It is important that a reasonable throughput and experimental time are determined before beginning this procedure. Because different bacterial species – and even strains – will have a varying level of pathogenicity, it is recommended to conduct a pilot study to monitor the bone degradation rate. If the bone is degraded too much during the experiment, the molar will completely detach from the maxilla, making it impossible to take measurements. It is also important to take into consideration the number of ligatures being tied for a single experiment. Depending on the intended output, the termination and sample collection process may take up to twenty minutes per mouse. Sample collection can be sped up by having multiple people working in tandem.

Problem 7

Low yield of genomic DNA from ligatures (step 17).

Potential solution

This can be solved by combining two or more ligatures from the same group and extracting gDNA from this pooled sample. It is also possible to change the gDNA isolation method or to do sequencing at a deeper level.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Batbileg Bor (bbor@forsyth.org).

Materials availability

Please see a detailed list of biological reagents that were generated from this study in our main manuscript (Chipashvili et al., 2021).

Data and code availability

Please see detailed code and 16S rRNA analysis in our main manuscript (Chipashvili et al., 2021).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101167.

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AUTHOR CONTRIBUTIONS

Conceptualization, B.B. and O.C.; methodology, B.B. and O.C.; investigation, B.B. and O.C.; writing – original draft, O.C.; writing – review & editing, B.B. and O.C.; funding acquisition, B.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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