Protocol for Preparing Lactobacilli MRS Agar Plates

1. Mix 49g of Lactobacilli MRS Agar with 700mL of deionized water. Swirl 2000mL flask vigorously to mix the powder into the water as homogeneously as possible.
2. Bring solution to boil in order to dissolve agar.
3. Cover container with aluminum foil and autoclave immediately. Autoclave at 121˚C for at least 20 minutes on liquid cycle. Be sure to add water to the autoclave basin before starting the cycle.
4. Remove container from autoclave. Swirl solution to see if any particles rise from bottom. Swirl until have a homogenous solution.
5. Allow glass container to cool in a 50˚C water bath until solution reaches 50˚C.
6. Turn on bunsen burner. Put plates in stacks of 4-5 to make a total of 28 plates. Pour 25mL media into each plate (until bottom of plate is full). Make sure to pour media near flame in order to limit contamination. If air bubbles seem to appear in plates, flame bubbles. Move quickly, will congeal in <5 minutes.
    a. stacking plates helps to limit condensation.
    b. Can pour in laminar flow hood without burner
7. With lid on plate, allow agar to harden overnight. Once agar has set, tape around edges of dish. Label dish with date and write "Lactobacilli." Store plates upside down at 4˚C.

Protocol for Preparing Lactobacilli Broth Media

1. Weigh out 5.5g of Lactobacilli powder. Mix with 100mL of deionized water.
2. Dispense 10 ml into (x) tubes. Cap with aeration caps. Save (x) ml in small reagent bottle with loose cap.
3. Cover container with aluminum foil and autoclave immediately. Autoclave tubes and bottle at 121˚C for no more than 20 minutes on liquid cycle. Be sure to add water to the autoclave basin before starting the cycle.

Day 1: Protocol for Preparation of *Lactobacillus coryniformis subsp. torquens*

1. Open vial according to enclosed instructions.
2. Withdraw approx. 0.5-1.0mL of the prepared Lactobacilli Broth and use to rehydrate the entire pellet.
3. Aseptically transfer the rehydrated pellet back into the broth tube. Mix well.
4. Pipette 100µg (0.1mL) of suspension into 5 tubes with 10mL of broth. Apply aeration caps.
5. Incubate tubes at 37°C in an atmosphere of 5% CO2 for 24 to 48 hours.

Day 2: Continuation of *Lactobacillus coryniformis subsp. torquens*

1. Use a spectrophotometer to ensure that the OD600 is ~1. Repeat for each tube.
    a. Pipette 2.5mL of solution out of two tubes. Then pipette 2.5mL of glycerol into the respective tubes to make a 25% glycerol solution. Harvest 1mL samples from the tubes and freeze immediately at -37˚C.

b. Use the cells from one tube to inoculate 10 plates. 3 plates with 50µL, 75µL, and 100µL respectively. One plate with no cells. Incubate inverted plates at 37°C in an atmosphere of 5% CO2 for 24 to 48 hours. Loosen screw caps of all test tubes during the incubation period. This test will be used to determine the optimal lawn size.
c. Pipette 100µL from one tube into two new tubes. Streak 2 plates with cells [will be used later for PCR]. Incubate tubes and inverted plates at 37°C in an atmosphere of 5% CO2 for 24 to 48 hours.

Day 3: Continuation of *Lactobacillus coryniformis subsp. torquens*

1. Follow the procedure from above to determine if the OD600 for tubes from 1c from Day 2 are at ~1.
2. Analyze the plates with varying amounts of cells and determine the volume of cells for optimal lawn growth.
3. Use hockey stick spreader to spread liquid cultures of *Lactobacillus coryniformis subsp. torquens* on agar plates using the optimal volume cultures at OD~1. Make 19 plates.
4. Perform serial dilutions using the following volumes.

Dilution #	Concentration (ug / mL)	Volume of previous dilution (uL)	Volume of Buffer Added (uL)	Total Volume (uL)	Volume of this dilution applied to disc / plate (uL)	Final Mass on Disk / Plate (ug)
Stock	100	N/A	N/A	500	5	0.5
1	10	200	1800	2000	5	0.05
2	1	200	1800	2000	5	0.005
3	0.2	200	800	1000	5	0.001
4	0.16	200	50	250	5	0.0008
5	0.1	200	120	320	5	0.0005
6	0.08	200	50	250	5	0.0004
7	0.05	200	120	320	5	0.00025
8	0.01	200	800	1000	5	0.00005

5. Apply 5 µL of bacteriocin solution to a sterile filter paper disk. Allow to dry in a sterile petri dish with cover corresponding to dilution (will use a total of 9 petri dishes). Control disks are prepared with the same buffer used to make bacteriocin dilutions

6. For the bacteriocin plates (in triplicate), put one disk in each quadrant. Make 3 plates with lawns and NO bacteriocin, and 3 plates with bacteriocin and no lawn (neg control), and three untreated plates. Each of the plates with the dilution series will have a control disk.
7. Store the remaining volumes of each dilution at -20. Be sure that the buffer is sterile.

8. Grow plates at 37 °C. Record images of plates every 12 hours.

9. Measure zones of clearing over time as well as presence of aberrant colonies.

10. Use colony PCR to confirm that aberrant colonies are *Lactobacillus coryniformis subsp. torquens* and not contamination.

Protocol for colony PCR