Higher order interactions in bacterial communities

**Background**

Some *Acetobacter* and *Lactobacillus* pairs exhibit mutualistic growth; when both are cocultured, they grow more than the pairwise sum of their monocultures. This is because *Lactobacillus* produces lactic acid, which *Acetobacter* (likely) uses as a carbon source, promoting growth. *Acetobacter* stimulates *Lactobacillus* growth by means that are currently being elucidated; previous research suggested *Acetobacter* produced amino acids that *Lactobacillus* used, but current (Robert’s) research indicates that the acetate produced by *Acetobacter* acts as a growth signal for *Lactobacillus*.

Our *Lactobacillus brevis* (*Lb*) strain genomes have been sequenced and shown to be unusually short, suggesting that their metabolic pathways are supported by environmental nutrients and small molecules. These nutrients could be obtained by coopting into other mutualistic relationships; essentially, *Lb* potentially cheats the system by taking nutrients from the network without providing any.

**Goal**

My goal is to:

1. Find higher order interactions between an *Acetobacter*, a *Lactobacillus* (likely *L. plantarum*), and *Lb*
2. Once a higher order interaction is found, determine the mechanism with which *Lb* interacts with the system

**Experiments for Goal 1**

Higher Order Assay Protocol

Plate each *Acetobacter* strains with *Lp* and *Lb* in a 96 well plate in chemically defined media (CDM). Controls could be: running without *Lp*, running without *Lb*, running without both. *Lp* and *Lb* strains can be varied to search for strongest interactions, but the previously isolated starvations strains will likely be used.

Question: does *Lb* interact with any *Acetobacter*/*Lp* pairs non-additively? That is, does *Lb* participate in a higher order interaction that it would not otherwise without both *Acetobacter* and *Lp* present?

Anticipated results:

* Carrying capacity is the same as the sum of *Lb* and *Acetobacter* + *Lp* carrying capacities: no higher order interactions occur, or growth of *Lb* counteracts deficits in *Acetobacter* + *Lp* growth (plating will determine this)
* Carrying capacity is less than the sum: higher order interaction possible; *Lb* is likely “cheating” the *Acetobacter*/*Lp* interaction and taking nutrients without providing any. *Lb* consumes the acetate growth signal (or amino acids), leaving less for *Lp*, which in turn produces less lactic acid, reducing the effect on *Acetobacter* and etc.
* Carrying capacity is more than the sum: higher order interaction possible; *Lb* somehow contributes to the *Acetobacter*/*Lp* interaction, or benefits significantly more from *Acetobacter* benefits so that its growth overcomes deficits in *Acetobacter* + *Lp* growth (plating will determine this) – this out competition of *Lb* [seems most likely](https://www.nature.com/articles/s41396-021-00986-y)

Day 1:

* For each of the 7 *Acetobacter* species:
  + Make MYPL agar plates and streak species from glycerol stocks
* For each of the 2 *Lactobacillus* species:
  + Make MRS agar plates and streak species from glycerol stocks

Day 3:

* For each of the 7 *Acetobacter* species:
  + From agar, culture species in liquid MYPL media (5 mL)
* For each of the 2 *Lactobacillus* species:
  + From agar, culture species in liquid MRS media (5 mL)
* Make CDM; at least 15 mL required per plate

Day 4:

* Transfer 1 mL from liquid culture into 4 mL 50% CDM

Day 5:

* Transfer 3 mL from 50% CDM culture into 12 mL 100% CDM

Day 6:

* From liquid culture, transfer 1 mL each to 2 microcentrifuge tubes and centrifuge at 2000 RCF for approx. 30 sec
* Remove supernatant and dilute the remaining bacteria in PBS to 0.1 OD600; at least 300 µL for *Acetobacter*, 2 mL for Lactobacillus are required
* In a 96 well helper plate:
  + One column (8 wells) should contain 300 mL of each *Acetobacter* species in one well; bottom well is blank for control
  + One column containing 300 mL *Lp* in each well
  + One column containing 300 mL *Lb* in each well
* In a 96 well experimental plate, use a multichannel pipette to add to each column:
  + Add 140 µL CDM to each well
  + Add 20 µL *Lp* to each well except last row
  + Add 20 µL *Lb* to each well except last row
  + Add 20 µL *Acetobacter* to each well except last row
* Apply EasyBreathe to top of plate; poke holes in each well for oxygen diffusion
* Measure initial OD600 in each well
* Place the experimental plate in a gravity convection oven at 30°C

Day 8:

* Measure final OD600 in each well
* Analyze to determine any higher order interactions
* Swab one well in each row and culture on 3 plates (1 MYPL, 2 MRS)
* Treat the MRS plates with antibiotics: one with carbenicillin (200 µg/mL), one with kanamycin (200 µg/mL)

Day 10:

* Use colony counter to determine fractions of final growths

Migration Experiment

Use Josh’s migration/invasion experiments to determine to what degree *Lb* competes with the *Acetobacter*/*Lp* dynamic. Kan/Carb can be used to distinguish *Lp*/*Lb* respectively.

**Experiments for Goal 2**

Nutrient Assay Protocol

Plate *Lb* against various CDMs. Best *Lb* strain should have been determined from Goal 1. CDMs can just be a concentration gradient of carbon or nitrogen; alternatively, we can mine the *Lb* genome to determine the nutrients *Lb* is lacking in its metabolic pathway and make CDM focusing on that nutrient. Controls: no bacteria, no *Acetobacter*

Question: which nutrients is *Lb* dependent on the *Acetobacter*/*Lp* interaction for?

Anticipated results:

* *Lb* grows normally across gradient: *Lb* is not dependent on the nutrient
* *Lb* grows proportionately with nutrient concentration: *Lb* is likely dependent on nutrient; hopefully, it does not grow without the nutrient
* *Lb* grows inversely proportional with nutrient concentration (grows more with less nutrient): nutrient somehow inhibits growth (unlikely)

Day 1:

* Make MRS agar plates and streak *Lb* from glycerol stocks

Day 3:

* From agar, culture *Lb* in liquid MYPL media (5 mL)
* Make 2 sets of CDM with maximum nutrient concentration; at least 3 mL are required for each
* Make blank CDM; at least 15 mL are required

Day 4:

* From liquid culture, add 1 mL to 4 mL 50% CDM

Day 5:

* From CDM culture, add 3 mL to 12 mL 100% CDM

Day 6:

* From *Lb* liquid culture, transfer to 1 mL each to 2 microcentrifuge tubes and centrifuge at 2000 RCF for approx. 30 sec
* Remove supernatant and dilute the remaining bacteria in PBS to 0.10 OD600; at least 2 mL are required
* In a 96 well helper plate, add 300 µL to 7 of the 8 wells in a column; add 300 µL ‘blank’ (no nutrient) CDM to the bottom well for control
* For each half of a 96 well experimental plate use a multichannel pipette to:
  + Add 180 µL blank CDM to all columns except for the first
  + Add 180 µL maximum nutrient CDM to the first row
  + Use another 180 µL maximum nutrient CDM for column dilution; mix the maximum with the blank in the second row, and then use 180 µL of now half nutrient CDM to dilute the third row, etc. Leave the last row blank
  + Add 20 µL *Lb* from the helper plate to each column
* Measure initial OD600 in each well
* Place the experimental plate in a gravity convection oven at 30°C

Day 8:

* Measure final OD600 in each well
* Analyze to determine if *Lb* is nutrient deficient

Gene Knockouts

Use gene knockouts to determine the specific metabolic pathway in *Acetobacter*/*Lp* that *Lb* targets. Given data from the nutrient assay, we can confirm that *Lb* gets the nutrient from the mutualistic relationship between *Acetobacter* and *Lp*.