Microbiome Experiment Aug 2022

Probiotic list

Probiotic #1 Proteobacteria *Pseudomonas* RBS5.4 at 6 x 10^7 cells/ml stock

Probiotic #2 Proteobacteria *Stenotrophomonas* THA2.2 at 6 x 10^7 cells/ml stock

Probiotic #3 Proteobacteria *Pseudomonas* RBS5.11 at 6 x 10^7 cells/ml stock

Probiotic #4 Cocktail, 6 x 10^7 cells/ml each probiotic

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | RSB5.4 (P1) | THA2.2 (P2) | RSB5.11 (P3) | Cocktail (P4) | Controls |
| 1 week harvest | n = 12 | n = 12 | n = 12 | n = 12 | n = 12 |
| 3 week harvest | n = 6 | n = 6 | n = 6 | n = 6 | n = 6 |

90 *Xenopus laevis*

Called in Geneious: ProbioticXenoAug2022. Did on home computer

ASV18 matches RSB5.4 (P1)

ASV9 matches THA2.2 (P2)

ASV10 matches RSB5.11 (P3)

**Questions:** Does probiotic exposure impact the immune system and resident microbiome, and is this consistent across probiotic strains? Do probiotics persist after exposure ceases?

TIMELINE:

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Sunday | Monday | Tuesday | Wed | Thur | Friday | Saturday |
|  | **Aug 15th**  Day 0  Swab frogs  Start treatment | 16th | **Aug 17th**  Day 2, Treated with probiotic or sterilized RO water (controls) | 18th | **Aug 19th**  Day 4,  Treated with probiotic or water. 8 hrs later transferred to new non-treated water | 20th |
| **Aug 21st**  Day 6,  Treated with probiotic or sterilized RO water | Aug 22nd | **Aug 23rd**  Day 8, Swab all + tissue harvest (n = 12 per trmt). | 24th  Put RNAlater tubes in freezer | 25th | 26th | 27th |
|  | **Aug 29th** | 30th  Day 15, swab frogs |  |  |  |  |
| Sept 4th | Sept 5th  LABOR DAY | **Sept 6th**  Day 22,  Remaining animals swab + tissue harvest ( n = 6 per trmt) | **Sept 7th**  Put RNAlater tubes in freezer |  |  |  |

* Start experiment Aug 15th
* Keep bacteria in fridge unless in use
* Rinse frogs with sterile RO water, swab (20 strokes on entire ventral side only) all frogs at start of experiment prior to treatment (Day 0).
  + Label tanks (P1F1 for probiotic1-frog1, P2F1 for probiotic2-frog1, P3F1, P4F1, CF1)
  + Put labels on tubes (P1F1D0, P1F2D0, etc)
* Expose frogs every other day to treatment (Days 0, 2, 4, 6) 1 ml of probiotic into each individual tank (treatment) or sterilized RO water (controls) added to terrarium. At Day 6, all frogs treated for 8 hours in same water since start of experiment. Then after 8 hours of final bath on Day 6, water change. All water dumped and new water added to same aquarium.
* Aug 22nd (Day 8) Swab all (n = 90), euthanasia and tissue harvest (n = 12 x 5 trmts = 60 animals)
  + 5 tubes per frog and one skin in 24-well plates (P1F1W1 swab, P1F1W1 sk later)
    - 1 swab (Carly) – swab
    - 1 large skin in RNAlater (Amy) – sk later
    - 1 skin in protease inhibitor (Barney) – sk prot
    - 1 skin in Trizol (Leon) – sk tri
    - 1 skin in 24-well plates (Leon, histology)
  + PLACE RNAlater tubes in fridge for 24 hours and then into -20 or -80.
  + Place all other boxes into freezer with appropriate labels.
* Aug 29th (Week 2) Swab remaining frogs
  + Put label on tubes (P1F1W2, P1F2W2, etc as above),
* Sept 6th (Week 3) swabs followed by euthanasia and tissue harvest
  + 5 tubes per frog and one skin in 24-well plates
    - 1 swab (Carly) – swab
    - 1 large skin in RNAlater (Amy) – sk later
    - 1 skin in protease inhibitor (Barney) – sk prot
    - 1 skin in Trizol (Leon) – sk tri
    - 1 skin in 24-well plates (Leon, histology)
  + PLACE RNAlater tubes in fridge for 24 hours and then into -20 or -80.
  + Place all other boxes into freezer with appropriate labels.

**APPENDIX**

Growing bacteria methods

Bacteria out of cryopreservation. Into monthly stock. 5 ml from monthly stock into 150 ml and grown at 17 °C for 68 h. The amount needed for inoculation was then washed twice by centrifugation (4500 rpm, 10 min) to remove detrimental metabolites and re-suspended in sterile RO water. Kept in fridge until used each day.

From Muletz et al. 2012 – soil inoculated with 1.5 ml of the J. lividum suspension (2.9 x 107 J. lividum CFUs/dry g of soil).

From Becker et al. 2021 - 100 mL of a solution containing the genetically modified Diaphorobacter 63F:vio was administered every other day for 10 days using a dose of 375,000 cells/mL for the first application, 750,000 cells/mL for the second, and 1.5 million cells/mL for the third, fourth, and fifth applications. The gradual increase of dose was conducted to reduce the likelihood of an immune shock reaction to the probiotics [32].

200 ml in tanks. **Need tank dimensions.** 6 x 10^7 cells/ml stock will be added. Exposure in 200 mL is 300,000 cells/ml when 1 ml is added.

Carly to do: grow up bacteria, calculate cell counts per ml with hemocytometer, label each probiotic according to number as above

Carly brings: probiotics + sterile RO water 150 mls each (need about 75 ml if treated 4 times), RNAase away, tube labels, swabs, sterile RO water for rinsing, rinse bottles

Rationale for bacterial choices

RSB5.4 – yes use! *Pseudomonas* – used in Gabe’s paper, 99% inhibition, inhibits most Bd strains. Matches ASV 37. Found on four sal species 2018 + Xenopus + Ambystoma. Harry & Keshab also looking at this one (antifungal against Culvularia lunata also, no chitase activity). Hub bacteria in Randall’s paper in cinereus, bislineata, monticola. Transferred to X. laevis

THA2.2 – *Stenotrophomonas* – 100% inhibition, Harry & Keshab found chitinase activity. Hub bacteria in Randall’s paper in cinereus and viridescens, matches ASV15. Found on four sal species 2018 + Xenopus. Transferred to X. laevis

RSB5.11 – Pseudomonas – 100% inhibition, Harry says prefer RSB5.11 strain because it produces 3 different lipopeptides, i.e. pseudodesmin (showed the best anti-fungal activity from all our tested lipopeptides) and the new di-lipopepetide and tolaasin (a very

large lipopeptide). So possibly 3 compounds that can mediate some activity (or work

synergistically ?).

Brian - Pseduomonas can be pathogenic (that may stimulate an undesired immune response), and did not stick on our last probiotic experiment on golden frogs.