**Experiment:**

Purpose: Test and identify the volatile profile emitted from microbes that affect the foraging behavior of SWD flies.

Hypothesis: The susceptibility of fruit (blueberries) to spotted winged drosophila is influenced by the microbiome of the fruit.

Test: record and compare the volatile profile of attractive and repellent effects of fruit associated bacteria.

* Attractive: Bacillus subtilis, Pantoe aggiomerans
* Repellent: Frigoribacterium faeni, Enterobacteriaceae sp.
* Fly associated bacteria: Bacillus amloliquefacians
* Control: Blueberry juice

For the preliminary study we will capture the profiles of 3 bacteria

* Pantoe aggiomerans (fruit associated attractive)
* Enterobateriaaceae sp. (fruit associated repellent)
* Blueberry control (reaction blanks for each bacteria)

**Two-choice trap assay** *(Wong et al.)*

Two-choice trap assays were set up as shown in Figure S1D. Flies were food deprived for 3h before being transferred to the test cages (350 3 260 3 150 mm). Each cage contained two traps (transparent cups, 34mm diameter) of 1ml MRS medium diluted in water, one seeded with the bacterium (AP or LP) and the other unseeded. Experiments were conducted at the same time of the day (between noon – 2pm) at room temperature, 12h:12h light-dark cycle. The number of flies in the test traps and outside the traps (no choice) was scored after 24 and 48h. Pilot tests were performed at different food deprivation length (3h, 6h and 18h) and we observed no difference in the proportion of flies entering traps 24h or 48h after (data not shown). Thus, the shortest length (3h) was chosen for the experiment. Experiments were conducted in biological replicates of arenas with multiple flies (mean number of flies per arena = 19.4; median = 17; Figure 3B), where n refers to the number of arenas (see STAR Methods).

Bacteria growth and inoculation protocol (*Wong et al.*):

* Preparing glycerol stocks
  1. Obtain cultured bacteria
  2. Prepare a sterile 50% glycerol solution
  3. Aliquot 500uL of glycerol solution into glycerol tubes
  4. Aliquot 500uL of bacteria culture into tube prepared with glycerol
  5. Store at -80C

**Bacteria culture Day**

* Setup traveling tray with:
  + 6-loops, 6-culture tubes, 2-pipets, 1-pipet bulb, marker, tube rack, media broth
  + gloves, bleach, and EtOH
  + N2 into freezer box into styrofoam box then pull microbe
* Clean biohood with bleach then EtOH then clean everything with EtOH before placing it into the hood
* Culture bacteria from glycerol stock:
  + Obtain desired bacteria for testing from glycerol.
    - Remove glycerol stock from -80C freezer and transport to biohood frozen (dry ice or liquid N2)
  + Inoculate selected bacteria into 6mL of liquid medium (LB/MRS/TSB, dependent upon which one it was isolated on originally).
    - Prepare culture tube with 6mL of broth media
    - With a loop, scrape the glycerol stock tube and place loop into culture tube (repeat as needed).
    - Return stock tube to freezing conditions **QUICKLY**
  + Incubate the sample in the shaking incubator for a 24-hour period at 32℃.
  + Prepare inoculation and sampling schedule

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| **CULTURE Start: 12/17/2018 0900** | | | |
| **TIME** | **12/18/2018** | **12/19/2018** | **12/20/2018** |
| **0800** | SPME BLANKS |  |  |
| **0845** | RESUSPEND EMPTY JAR |  |  |
| **0900** | INOCULATE EMPTY JAR | VENT EMPTY JAR | VENT EMPTY JAR |
| **0945** | RESUSPEND CONTROL |  |  |
| **1000** | INOCULATE CONTROL | VENT CONTROL | VENT CONTROL |
| **1045** | RESUSPEND REP1 |  |  |
| **1100** | INOCULATE REP1 | VENT REP1 | VENT REP1 |
| **1101** | VENT EMPTY JAR |  |  |
| **1145** | RESUSPEND REP2 |  |  |
| **1200** | INOCULATE REP2 | VENT REP2 | VENT REP2 |
| **1201** | VENT CONTROL |  |  |
| **1245** | RESUSPEND REP3 |  |  |
| **1300** | INOCULATE REP3 | VENT REP3 | VENT REP3 |
| **1301** | VENT REP1 |  |  |
| **1345** | RESUSPEND REP4 |  |  |
| **1400** | INOCULATE REP4 | VENT REP4 | VENT REP4 |
| **1401** | VENT REP2 |  |  |
| **1445** | RESUSPEND REP5 |  |  |
| **1500** | INOCULATE REP5 | VENT REP5 | VENT REP5 |
| **1501** | VENT REP3 |  |  |
| **1601** | VENT REP4 |  |  |
| **1701** | VENT REP5 |  |  |

**Inoculation Day**

* After 24-hours, aliquot 200uL of replicate into 5 wells of a 96well plate to obtain an absorbance reading.
* Prepare container intended for bacterial inoculation (sampling container)
* Pellet bacteria and resuspend in saline solution
  + Centrifuge culture tubes at 8,000 rpm for 10 minutes.
  + Remove supernatant
  + Add 1.5 ml of 1% saline to the culture tube
  + Use pipet and mix bacteria pellet of each sample.
* Add the total volume of each resuspended culture tube to the sample container
* Add 6 ml of blueberry juice into the sample container
* Incubate sample container tore in a 32C incubator, and sample at 2 hours, 24 hours, and 48 hours after initial inoculation.

**Volatile collection**: we will focus on SPME to conduct the preliminary investigation. After the results are provided to the granting agency we will compare SuperQ and SPME.

* When cleaning reusable items DO NOT USE ACETONE
* SPME rotated through samples daily
* SPME fibers not to exceed 50 runs before replacement.

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| --- | --- | --- | --- |
| Bacteria *n* | Day 0 | Day 1 | Day 2 |
| Rep 1 | SPME-1 | SPME-2 | SPME-3 |
| Rep 2 | SPME-2 | SPME-3 | SPME-1 |
| Rep 3 | SPME-3 | SPME-1 | SPME-2 |
| Rep 4 | SPME-1 | SPME-2 | SPME-3 |
| Rep 5 | SPME-2 | SPME-3 | SPME-1 |
| Control | SPME-3 | SPME-1 | SPME-2 |
| Empty Jar | SPME-1 | SPME-2 | SPME-3 |

* Sample size n=5, Sample times: 2-hours, 24-hours, 48-hours
* Clean 4-oz ball jars vials after use
* Ball jars and lids: 100 Celsius

Control preparation and the determining permeation time, exposure time, and SPME fiber

* Test control sample of 1% saline solution in blueberry juice on each polymer type (mono-, di-, and tri-)
  + Incubator of room 258, 30C, vent time = 10s
  + Permeation and exposure trials:
    - Permeation = 5 mins, exposure = 5 mins
    - Permeation = 5 mins, exposure = 3 mins
    - Permeation = 5 mins, exposure = 2 mins
    - Permeation = 10 mins, exposure = 5 mins
    - Permeation = 15 mins, exposure = 5 mins
* **Tripolymer at 10P:5E** JTB-E02-(181-184) for details

GC-MSD Methods

* SPME: no solvent delay needed and faster setup
  + Condition new SPME for 30m on Gladerial
    1. Method: SPME.CONDITION.M
  + Run 2 min blanks on SPME’s daily
    1. Datafile: JTB-E02-pg-line; lab book: project name-SPME name
    2. For the first SPME: Load method🡪insert SPME🡪start run🡪start a 5 minute timer🡪pull SPME and replace
    3. Remaining SPME’s: insert SPME🡪 set 2MINUTE timer🡪pull SPME and replace
       - SPME instrument method:
         * Lego: Legolas\_40\_4.M
         * Gladerial: Gladerial\_DB1\_40\_4.M
* SuperQ: solvent delay and flow can capture heavier volatiles
  + How to clean a SuperQ (setup in Shawn's)
    1. 600ul milli-q water
    2. 600ul MeOH
    3. 600ul DCM
  + Collect and run initial DCM rinse daily before use
  + Method:
    1. Leggo: Leggolas\_40\_4\_#
    2. Gladerial: Gladerial\_DB1\_40\_4\_# (this method will need to be created with the added solvent delay)

Data Mining: After polymer selection has been made and runs have been completed on both the DB-Wax and DB-1 columns complete the following steps

1. Opening data
   1. Load data file🡪Integrate (use default set integration parameters)🡪select Percent Report🡪select Spectrum then “Library Search Report” 🡪set library to “NIST” 🡪select ok, then select “Library Search Report” 🡪check screen report only🡪select correct integration parameter (the default one: ) and select “Apex” 🡪”ok”
   2. File🡪export to .csv file🡪”current file with current data” 🡪generate library search results🡪”ok” and take note of “appended” message
2. Building database
   1. Open file explorer and open the “Results” .csv file you just made
      1. Compare the retention times between the library search results and the integration results
      2. Copy and paste “RT”, “Area PCT”, and “Library” columns to the end of the integration results section and remove the remaining columns in lower results
      3. Same main database as a separate file, in .xlxs extension, and in a different location. As more data is collected it will be added to this main database.
3. Walking through peaks:
   1. Complete these steps on the DB-WAX and the DB-1
   2. Zoom into a region containing peaks
      1. Make a small “left click” box inside the peak to select the peak🡪make a small “left click” box at the baseline then navigate to the SUBTRACT option🡪double “right click” in the spectra area to obtain the identification result of the peak
      2. If the selected peak has a higher match rate than the match in the database file then replace the chemical name in the database with the newly found chemical name. Also add peak information for all peaks that were manually integrated and identified
4. Calculating RI’s: DO NOT SAVE OVER MACROS! EVER!
   1. Complete these steps on the DB-WAX and the DB-1
   2. Open “Huge list of VOCs” file🡪navigate to ”WAX” sheet🡪open main database🡪file🡪open🡪browse🡪navigate to DB-WAX macro, highlight it, and select open🡪”enable” it once the file opens as needed then minimize it
   3. Navigate to database🡪make an empty column next to chemical name column🡪select all the RT in question🡪press ctrl + g to calculate the RI values