BIOAG PROJECT PROGRESS OR FINAL REPORT TEMPLATE

TITLE: Biodiversity and the Natural Suppression of Human Pathogens

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Cooperator(s) & Role(s) in project: We have established a geographically-dispersed network of cooperating mixed-vegetable growers located across WA, OR, and CA (Table 1, Fig. 1). These growers are cooperating with the on-farm components of the proposed research.

KEY WORDS: BIODIVERSITY, FOOD SAFETY, HUMAN PATHOGEN, GAP REGULATIONS, MIXED-VEGETABLE FARMING, INTEGRATED LIVESTOCK

ABSTRACT Restoring livestock to mixed-vegetable farms allows on-farm fertilizer production and the sale of high-value meat products. Likewise, diversifying farms with native plants provides habitat for pest-killing birds and predatory insects. Unfortunately, both practices carry the risk of unintentional contamination of produce by human pathogens (e.g., *E. coli* O157:H7), transmitted through livestock feces or those of vertebrate wildlife drawn to native plantings. Currently, the only way to manage this risk is to remove all natural vegetation from the farm, which disrupts natural pest control, or to install deer-fencing around the entire farm perimeter, which is expensive and cannot exclude all vertebrate carriers of human pathogens. Arthropods and microbes that eat feces likely reduce this risk, but little is known about coprophage biodiversity, impacts, or conservation. Working on farms varying in their levels of livestock integration, we proposed to: (1) Quantify biodiversity of feces-feeding arthropods (e.g., dung beetles, flies) through intensive field sampling; (2) Assess functional-genetic diversity of soil microbes using next-generation sequencing approaches, focusing on genes likely to be active in feces digestion; and (3) Relate biodiversity among coprophagous arthropods and microbes to rates of feces removal and *E. coli* suppression. Our ultimate goal is to provide vegetable growers with practical ways to reduce the risk of harboring human pathogens on their farms, by conserving and augmenting beneficial coprophagous insects and microbes. We addressed BIOAg goals by developing "*biologically-intensive approaches to sustainable management of soil quality, whole farm food systems, livestock and animal health, and organic wastes.*"

PROJECT DESCRIPTION

Restoring livestock to mixed-vegetable farms can greatly enhance sustainability by providing on-farm fertilizer production and high-value meat products. Likewise, diversifying farms with native plants provides habitat for pest-killing birds and predatory insects (Gurr et al. 2012). Unfortunately, both practices carry the risk of unintentional contamination of produce by human pathogens, transmitted through livestock feces or those of vertebrate wildlife drawn to native plantings. Indeed, raw vegetables have been responsible for many foodborne outbreaks in recent years - including events in Washington (Ackers et al. 1998, Jay et al. 2007, Wendel et al. 2009, Laidler et al. 2013). Particularly damaging is contamination with pathogenic *E. coli* strains (e.g., *E. coli* O157:H7; Ackers et al. 1998, Atwill 2008, Wendel et al. 2009), which causes symptoms including bloody diarrhea, vomiting, and abdominal cramps; death usually results from renal failure and loss of red blood cells (Montenegro et al. 1990, Rabatsky-ehr et al. 2002, Newell et al. 2010). A vast range of animals are known to shed enterohemorrhagic *E. coli* in their feces, including species both domesticated (e.g., cattle, sheep, goats,

pigs, cats, and dogs) and wild (e.g., deer, rats, rabbits, wild pigs, and starlings) (Hancock et al. 1998, Rabatsky-ehr et al. 2002, Nielsen et al. 2004). These animals can act as vectors of pathogenic *E. coli* directly, by fecal deposition onto crop plants, or indirectly, through contamination of surface waterways or soil (Jay et al. 2007). Once an *E. coli* outbreak is traced to a particular farm (e.g., Jaquith Strawberry Farm, Washington County, Oregon, in 2011), resulting lawsuits can lead to that grower's bankruptcy.

To manage the risk of human-pathogen contamination, growers are increasingly being pressured, by processors and government regulators, to (1) remove all natural vegetation from the farm, which likely decimates natural pest control, and/or (2) to install deer-fencing around the entire farm perimeter, which is expensive, harmful to wildlife conservation, and cannot exclude all vertebrate carriers of human pathogens (Beretti & Stuart, 2008). It is troubling that neither of these practices has been shown to actually reduce the risk of pathogen contamination (Stuart 2006). Arthropods and microbes that eat animal feces likely reduce the risk of *E. coli* contamination, but little is known about coprophage biodiversity, impacts, or conservation. A diverse community of flies and beetles feed on animal dung, and are known to be critical for removing feces from the environment and fostering the return of nutrients to the soil (Kim 1993). There is some evidence that these insects also suppress *E. coli*. (Jones 2013). Likely, fecal degradation by insects is complemented by the action of soil-microbial communities. Farm soils, in particular those managed to encourage greater soil biodiversity through the incorporation of animal manures and avoidance of broad-acting soil fumigants (e.g., De Fede et al. 2001, Mäder et al. 2002, Reganold et al. 2010), house a diverse community of bacterial competitors and antagonists. These include bacteriophages specific to bacteria expressing the O157 antigen of pathogenic *E. coli* O157 (Kudva et al. 1999). Identifying specifically if and how natural pathogen suppression operates, and identifying key players, would form an obvious first step towards managing farms to enhance pathogen suppressiveness.

We requested funds to build a functioning interdisciplinary team, and to generate critical preliminary data, in support of a USDA-NIFA grant proposal. We felt that the broadly interdisciplinary nature of our project would be attractive to major federal funding agencies, and indeed that proved to be the case: our USDA-ORG grant was funded last year for $498,235 over 3 years.

We proposed three tightly inter-related, integrated research objectives:

(1) Measure biodiversity of coprophagous insects and their rate of feces consumption.

(2) Measure soil quality, microbial functional diversity and feces degradation.

(3) Relate biodiversity of coprophagous insects and microbes to rates of feces removal and degree of *E. coli* suppression.

OUTPUTS

Work Completed:

<u>Objective 1. Measure biodiversity of coprophagous insects and their rate of feces consumption.</u>

In the 2014 field season, PhD student Matt Jones was able to sample dung beetles, flies and isopods on over 30 farms from central California to northern Washington (Table 1) using feces-baited pitfall traps. Dung beetles (Coleoptera: Scarabaeidae) (>800 collected in total) were captured on the vast majority of farms across the entire study range. Interestingly, we have found that the invasive dung beetle species *Onthophagus nuchicornis* dominates beetle communities from Oregon north, while a more-diverse community of native coprophagous beetles is found in California. Feces-feeding flies (>43,000 collected in total) were captured on every farm in high densities, with dominant taxa including house flies (Diptera: Muscidae) and blow flies (Diptera: Calliphoridae). Isopods (>700 collected in total) were captured inconsistently on a smaller number of farms. We have sorted and cleaned pitfall-trap catches, and we now are working to identify these species to the lowest-possible taxonomic level (our fly identifications are being led by co-PD Headrick). Once identifications are complete, we will be able to calculate coprophage richness and evenness for each of our cooperating farms.

Region	System Type	Farm Name
Washington	Organic	Terry's Berries LLC
Washington	Organic	Highland Farm West
Washington	Organic	Boistfort Valley Farm
Washington	Organic	Punkin Center Farm
Washington	Organic	Wild Hare Organic
Washington	Organic	Mercer 2 Organic
Washington	Integrated	Little Eorthe Farm
Washington	Integrated	Heavenly Hill Harvest
Washington	Integrated	Willow Grove Gardens
Washington	Integrated	Cedarville Farm
Washington	Integrated	Helsing Junction Farm
Oregon	Organic	Gathering Together Farm
Oregon	Organic	Barking Moon Farm
Oregon	Organic	Laughing Worm Farm
Oregon	Organic	Circle H farm
Oregon	Organic	Mustard Seed Farm
Oregon	Integrated	OSU Organic Farm
Oregon	Integrated	Wintergreen Farm
Oregon	Integrated	Runnymede farm
Oregon	Integrated	The Little Homestead
Oregon	Integrated	Rose Hill Farms
Oregon	Integrated	Mulino Farm
California	Organic	CalPoly Org. Farm
California	Organic	Woodleaf Farm
California	Organic	Foster Ranches/Pinnacle
California	Organic	Springfed Organic Farm/nursery
California	Organic	Good Humus Produce
California	Integrated	Full Belly Farm
California	Integrated	Churn Creek Meadow Organic Farm
California	Integrated	Chico State Organic Vegetable Project
California	Integrated	Pozo Organic Farm
California	Integrated	Lemonade Springs

Table 1: Updated list of our cooperating growers. Farms are variously mixed-vegetable-only organic farms, or organic farms that integrate livestock and mixed-vegetable production.

In addition to trapping insects on each farm, we have been directly measuring rates of feces removal by the coprophage community. Briefly, we place 20g frozen pig feces “cubes” on the soil surface in the same location as the pitfall traps from the previous week. Arthropods are allowed to feed on presented feces for 7 days, after which feces is removed. We then calculate removal weights using dry mass. Feces removal rates differed dramatically among farms, ranging from 4%-97% removal over our 7 day period of feces exposure to the coprophages. We currently are in the process of searching for correlations between on-farm coprophage biodiversity measured during our insect sampling, and the rates of feces removal observed on each farm. Furthermore, we are working to create GIS maps of each farm, which will eventually allow us to search for landscape features associated with coprophage abundance and biodiversity, as well as rates of feces removal.

In the 2015 field season, we have somewhat increased our sampling network to include 32 farms. These include the farms sampled in 2014, along with 5 farms that are new to our network in 2015. It is early in our field season, with PhD student Jones expecting to spend several more months traveling to, and sampling from, the farms of our cooperating growers. Thus far, we have sampled each farm once with plans to visit each farm a second time during the fall Brassica planting season. Preliminary data from our 2015 trapping efforts indicate a catch volume similar to that seen in 2014 in terms of coprophage abundance and biodiversity. We again are measuring feces-removal rates on each farm, and will conduct GIS mapping of new collaborators' farms.

Objective 2: Measure soil quality, microbial functional diversity and feces degradation.

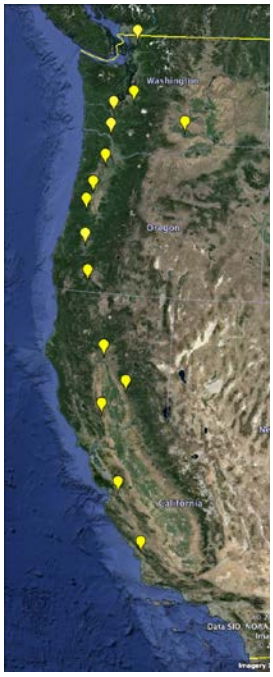

Fig. 1. Sites of our cooperating farms. Each yellow pin represents a pairing of one vegetable-only farm with one nearby integrated farm.

First in 2014, and now again in 2015, we are measuring a broad suite of biotic and abiotic soil characteristics. These include pH, % organic matter, particle size/soil texture, ammonium, and nitrate. We do this by taking 10-cm-deep soil cores at the exact site where pitfall trapping and above/belowground manure degradation experiments will occur. Soil cores (3 per farm) are homogenized/pooled in a sterile bucket and then mailed overnight (on ice packs) back to our laboratory in Pullman. Approximately 2 liters of soil are needed from each farm to complete all of the soil work. Once at the university, pooled soil samples are divided up and sent to the various collaborating labs responsible for different sub-components of our rigorous soil testing. For example, we have been measuring soil microbial activity using dehydrogenase testing, in collaboration with Lynne Carpenter-Boggs at WSU. Briefly, this biochemical assay measures a soil enzyme that reflects the potential net metabolism of the soil's microbial community. In essence, this is a broad measure of microbial life that spans the entire community. Initial results indicate dramatic differences between farms in soil microbial activity (0.56-7.32 ug TPF per g dry soil per h).

We have been collecting soil DNA soon after each soil sample arrives on campus. Microbial DNA is being extracted from a 0.25-gram aliquot of homogenized soil, processed following procedures of the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc.). DNA concentration is quantified using a Qubit Fluorometer (Life Technologies). DNA samples from the 2014 field season were processed and sent to BGI Americas, who generated DNA libraries targeting bacterial 16S ribosomal RNA v3/v6 hypervariable regions (Chakravorty et al. 2007, J Microbiol Methods) following high-throughput sequencing on the Illumia HiSeq 2000 Platform. We obtained raw data in the FASTQ format from BGI America in June 2015. We are currently processing these high-throughput sequencing data, and we plan to analyze bacterial diversity and taxa abundance. In turn, this will allow us to understand how microbial diversity (species richness and species evenness) correlates with the soil biological activity and manure degradation rates we are measuring in other components of our project (described above and next).

We are monitoring below-ground feces degradation by microbes with a methodology similar to that described above for our measurements of feces consumption by coprophagous insects. Briefly, we bury 20g cubes of frozen pig feces enclosed in mesh bags (fine mesh on bottom, course mesh on top) 10cm

below the soil surface. Mesh caging of this type prevents coprophagous insects from reaching the feces, while allowing contact with the soil and thus feeding by soil-dwelling microbiota. We allow soil organisms to feed on these feces for 2 weeks before recovering the mesh bags and remaining feces. Similar to above-ground feces removal calculations, we use dry mass removal to determine amounts being consumed by soil microorganisms.

Lastly, we are testing each soil sample for both generic *Escherichia coli* (meaning, typical *E. coli* strains that are not harmful to humans) and pathogenic (O157:H7) *E. coli*. For the first year of data, no pathogenic *E. coli* were found on any farm, and very low levels of generic *E. coli* were found across all of the farms in our sampling network. Initially, we thought we might be getting poor *E. coli* recovery due to sample degradation during handling; our initial sampling protocol involved overnight-shipping soils back to our lab on the WSU main campus, in well-ventilated containers and packed on ice packs, before testing for the presence of *E. coli*. To explore this possibility, during the 2015 field season we tried various adjustments to our protocol to focus on conserving any *E. coli* in the soil samples. For example, we explored adding media nutrients and antibiotics to subsets of our soil samples before shipment to campus. Through these efforts we found that our original technique provided the best survival of the *E. coli* in our farm soils, without plates becoming overgrown with fungi and undesirable bacteria. It appears, then, that our initial measures of low *E. coli* abundance were accurate, indicating that these bacteria simply do not often persist for long in the agricultural soils on our farms.

Ultimately, across all sub-components under Objective 2, we will have the data to search for correlations among soil health, biodiversity among soil-dwelling organisms, microbe-mediated feces degradation, and densities of *E. coli* bacteria.

Objective 3: Suppression of pathogenic *E. coli* O157:H7 by insects and microbes.

This Objective is designed to build upon the field work pursued under Objectives 1 and 2. Now that we have initial data from these aspects of the project as described above, fulfillment of the components of this Objective will form a key focus of the work we pursue with our newly-acquired USDA-ORG support.

Publications, Handouts, Other Text & Web Products: We have not yet secured sufficient data to pursue research-journal publications. Our successful USDA-ORG proposal is the main text product resulting from our BIO-Ag funding thus far.

Outreach & Education Activities: We plan to deliver a webinar on our project through eOrganic this coming fall, and are beginning work on eOrganic web content described in our USDA-ORG proposal. Project PhD student Matt Jones has given invited talks regarding this project at the following:

- The National Meeting of the Entomological Society of America in Portland, OR.
- The Center for Produce Safety annual research symposium in Los Angeles, CA.
- UC Berkeley.
- The Annual Meeting of the Lower Mainland Horticultural Improvement Association in Abbotsford, BC.

Furthermore, Matt is also scheduled to give invited seminars in the coming year including:

- The National Meeting of the Entomological Society of America in Minneapolis, MN (where he is also co-organizer of a symposium focused on detrital food web ecology).

- The Biology Department Seminar Series at California State University at Chico.
- The Hoes Down Harvest Festival at Full Belly Farm, Capay, CA.
- Chico State University's Sustainability Conference in Chico, CA.

IMPACTS

Short-Term: The central goal of our project is to provide organic mixed-vegetable farmers, including those that integrate livestock into their farming operations, with an ecological approach to reducing their food safety risks. Our project already is providing evidence that on-farm populations of coprophagous insects are contributing to rapid feces removal. Work in upcoming years of the project will focus on microbial degradation of feces, and the combined impacts of coprophagous arthropods and microbes on pathogenic *E. coli*.

Intermediate-Term: Organic growers are under increasing pressure to conform to regulations often presented under the heading of "Good Agricultural Practices" (GAP), which include practices designed to reduce food-safety risks. Harmful strains of *E. coli* and several other human pathogens are thought to be brought onto farms, at least in part, by livestock and vertebrate wildlife. To reduce these perceived risks, GAP regulations often mandate the complete removal of hedgerows and other non-crop vegetation from farms, with unknown harm to beneficial predatory insects, pollinators, and other species that rely on these habitats as refuges. Our project seeks to demonstrate that many of the more draconian GAP mandates are unnecessary, allowing growers to retain natural vegetation on their farms along with the many environmental benefits that these habitats provide.

Long-Term: The ultimate goal of our project is to provide new, transitioning organic growers with a roadmap for how to build naturally-pathogen-resistant farms. However, our project may also suggest to long-time organic vegetable growers that they could benefit from a second transition, moving towards livestock integration or the adoption of other practices found to build coprophage biodiversity.

ADDITIONAL FUNDING APPLIED FOR / SECURED

1. Bill Snyder is PD, with Tom Besser and John Reganold as Co-PDs: USDA-NIFA-ORG, "A natural approach to human-pathogen suppression: Can biodiversity fill the GAPs?" ($498,235 over 3 years).

2. Project PhD student Matt Jones has just been awarded a Fulbright Fellowship to expand this research into New Zealand.

GRADUATE STUDENTS FUNDED This project has entirely supported the PhD work of graduate student Matthew Jones, and partially supported the training of postdoctoral scholar Zhen "Daisy" Fu. In addition, undergraduate students Ashley Huhn (WSU), Andrea Watts (WSU), and Andrew MacDonald-Urango (CalPoly) have participated in project research.

RECOMMENDATIONS FOR FUTURE RESEARCH

Natural pest control is a cornerstone of organic agriculture. Wild insect-eating birds are important contributors, and many growers seek to enhance these benefits by maintaining hedgerows or other bird habitats. On the other hand, birds sometimes damage produce or act as key vectors of bacteria (e.g., E. coli, salmonella), viruses (e.g., West Nile) and parasites (e.g., fowl mites) harmful to humans or livestock.

Unfortunately, there have been surprisingly few holistic studies of wild birds' ecological roles, both good and bad, on North American organic farms. This leaves growers unable to predictably weigh the benefits and risks of encouraging or discouraging wild bird populations. Work is needed to: (1) Relate biodiversity of wild birds to farm-management practices, through intensive field sampling and GIS modeling; (2) Quantify the birds' impact on pest insects through non-invasive, molecular analysis of prey-DNA remains in bird feces; and (3) Assess the birds' risk of spreading pathogens and parasites that endanger food safety and human/livestock health.

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