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Is there specificity in a defensive mutualism against soil versus lab nematodes, *Dictyostelium discoideum* farmers and their bacteria?

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ABSTRACT

Background: The social amoeba *Dictyostelium discoideum* is a soil dwelling microbe, which lives most of its lifecycle in the vegetative stage as a predator of bacteria and as prey for nematodes. When bacteria are sparse, amoebae aggregate into a multicellular fruiting body. Some clones of *D. discoideum* have agriculture (Brock et al, 2011). They carry bacteria through the social stage, eat them prudently, and use some bacteria as defense against non-farming *D. discoideum* competitors. *Caenorhabditis elegans* preys on *D. discoideum* in the laboratory but does not encounter it in nature because *C. elegans* lives on rotten fruit. The nematode *Oscheius tipulae* is abundant in the soil.

Questions: Do the defensive bacteria farmers carry also protect farmers from nematodes? Is this protection specific to nematodes that reside with *D. discoideum*?

Hypotheses: Many organisms evolve defensive mutualisms against predators. *D. discoideum*'s natural habitat is populated with nematodes. Therefore, we hypothesize that farming *D. discoideum* clones use non-food bacteria for protection from nematodes. We predicted higher fitness of farmers than non-farmers in the presence of nematodes. We also predicted to see this change of fitness only in the presence of the soil nematode, *O. tipulae*.

Organisms: Amoeba, *D. discoideum*, nematode *Caenorhabditis elegans* and *Oscheius tipulae*, bacteria *Klebsiella pneumoniae* and *Burkholderia xenovorans*

Methods: We compared spore production of *D. discoideum* farmers and non-farmers with and without nematodes. We also looked at nematode proliferation in the presence of farmers, non-farmers, *K. pneumoniae* and *B. xenovorans*.

Results: Overall, farmer *D. discoideum* produced fewer spores when compared to non-farmers. There was a decrease in the spore counts in the presence of nematodes for both farmers and non-farmers. There was a significant decrease in the percentage change in spore production for the farmers in the presence of soil nematodes but not lab nematodes. Nematode proliferation with the lab nematode and soil nematode did not vary in the presence of farmers, non-farmers, *K. pneumoniae* or *B. xenovorans*.

Conclusion: The non-food bacteria farmers carry do not provide defense against nematodes. In fact, it was a disadvantage for farmers to carry bacteria since the soil nematode decreased spore production for farmers when compared to non-farmers. However, the results between the lab nematode and the soil nematode are different enough to conclude that different species of nematodes respond differently to *D. discoideum* as a food source.

Keywords: defensive mutualisms, *Dictyostelium discoideum*, *Caenorhabditis elegans*, *Oscheius tipulae* bacteria

Introduction

In symbiotic mutualisms, both partners of different species benefit from evolved interactions (West and Herre 1994; Sakai 2002; Way 1963; Hooper and Gordon 2001; Bronstein 1994; White and Torres, 2009). For example, pea aphids (*Acyrthosiphon pisum*) provide shelter and protection to bacteria *Buchnera aphidicola*. The bacteria, in exchange, produce nutrients the aphids lack in their diet (Moran *et al.*, 2005). There are some cases, classified as protection mutualisms or defensive mutualisms, in which the host in the interaction is protected from predators, pathogens, or biotic and abiotic stressors by the symbiont (White and Torres, 2009).

A classic example of a defensive mutualism is *Pseudomyrmex* ants which ward off predators of *Acacia* trees, receiving in return food and shelter (Janzen, 1966). Another classic defensive mutualism example is the relationship between bacteria and fungus-growing ants (Currie *et al.*, 1999). Sometimes the ants' fungus gardens are threatened by a parasitic fungus, *Escovopsis*. Bacteria, of the genus *Pseudonocardia,* are found on the cuticle of these ants and have been shown to suppress the growth of *Escovopsis* (Currie and Stuart, 2001; Currie *et al.*, 1999; Currie *et al.*, 1999; Poulsen *et al.*, 2009), but see (Kost *et al.*, 2007; Mueller, 2012).

Another example of a bacteria-eukaryotic protective mutualism is between bacteria and beetles. The bacterium, *Pseudomonas* sp., has been shown to be associated with the pederin producing beetle, *Paederus sabaeus* (Kellner, 2002; Oliver and Moran, 2009; Kellner, 2001; Piel, 2002). Piel (Piel 2002) discovered that the genes that are used to produce pederin are actually located on a cluster found in the genome of a *Pseudomonas* that is only found in pederin producing beetles. Pederin is a toxin that can create painful lesions on human skin (Oliver and Moran, 2009; Gelmetti and

Grimalt, 1993). Not only can pederin harm humans, but it harms beetle's predator wolf spiders (Kellner and Dettner, 1996; Oliver and Moran, 2009).

In this study, we tested for the presence of a defensive mutualism between bacteria and the social amoeba *Dictyostelium discoideum* that can deter predators. *D. discoideum* is a soil dwelling microbe which lives most of its life as single-celled amoebae that eat bacteria (Raper, 1937; Kessin, 2001). When bacteria are sparse, the social stage begins and thousands of amoebae aggregate to form a slug. The slug develops into a fruiting body in which 20% of the cells die to form a sterile stalk to hold aloft the other cells as fertile spores in a fruiting body (Kessin, 2001; Strassmann and Queller, 2010). A recent paper (Brock *et al.*, 2011) revealed that amoebae from many clones of *D. discoideum* not only prey upon bacteria, but also farm them in a kind of primitive agriculture. The bacteria are not completely consumed by the amoebae; instead, some are kept alive through the social stage, transported to new locations with *D. discoideum* spores, and seeded out to start new prey populations (Brock *et al.*, 2011). The farming clones of *D. discoideum* carry several different food bacteria, including the one we provide as food in the lab, *Klebsiella pneumoniae*. They also carry some bacteria that are not good food, but instead produce effective weapons against non-farmer clones. (Brock *et al.* 2013; Stallforth *et al*. 2013). *D. discoideum* strains that are capable of farming bacteria thrive in environments where preferred bacteria are scarce, but these strains do less well in lab conditions when bacteria are plentiful (Brock *et al.* 2011).

Thus, some of the bacteria farmed by *D. discoideum* harm competing non-farmer clones and keep them from eating both the protecting bacteria as well as the host's edible farmed bacteria. But it is uncertain whether this is the evolved function of the symbiosis. In particular, it is not obvious how the bacteria might evolve to harm non-farming clones of *D. discoideum* without also harming their host of the same species. One possibility is that the protecting

bacteria originally performed a different function. For example, they may have initially provided protection against predators.

We hypothesize that one of these non-food bacteria *Burkholderia xenovorans*, provides protection against predators. A likely predator is a nematode, since they have been demonstrated to eat *D. discoideum*(Kessin *et al.,* 1996). Nematodes are multicellular, eukaryote soil-dwelling predators of *D. discoideum* amoebae and bacteria. We tested two nematode species, the model organism *Caenorhabditis elegans* and a wild-collected nematode, *Oscheius tipulae* found in soil samples also containing *D. discoideum*. *C. elegans* has a sequenced genome, a three day life cycle in the laboratory, and reproduction by self-fertilizing hermaphrodites. It occurs in soil and on rotting fruit (Caswell-Chen *et al.,* 2005; Félix and Braendle, 2010). It has been shown to eat *D. discoideum* (Kessin *et al.,* 1996). *O. tipulae* is the most common soil nematode (Félix, 2006) and may therefore be a natural predator of *D. discoideum*.

We were interested in *B. xenovorans* because *Burkholderia* species have been shown to be toxic to nematodes (O'Quinn *et al.* 2001). Additionally, *B. xenovorans* does not serve as food. It is found in several different *D. discoideum* farmer clones and it harms non-farmer clones (Brock *et al.* 2013). We tested the bacteria protection against nematodes hypothesis that *D. discoideum* clones have an evolved mutualism with *B. xenovorans* at least partly to provide defense against predation by nematodes. We predicted that *B. xenovorans* carrying clones, farmers, would have higher fitness in the presence of nematodes than those *D. discoideum* clones that do not carry the bacterium, non-farmers. We measured fitness as spore counts in the presence and absence of nematodes. Additionally we measured nematode proliferation on farmers and non-farmers. We predicted that proliferation will decrease in the presence of *D. discoideum* farmer clones. To test our hypothesis that bacteria directly harms nematodes, we measured nematode proliferation in

the presence of the non-food *B. xenovorans* and the lab food *K. pneumoniae*. We predicted that nematode proliferation will decrease in the presence of *B. xenovorans*.

MATERIALS AND METHODS

Study animals

We used *C. elegans* N2 hermaphrodites donated from the Nonet lab at Washington University in St. Louis. We reared N2 strains on Nutrient Growth Media (NGM) (2.5g bacto peptone, 3g NaCl, 1ml of 5mg/ml cholesterol, 25ml 1M KPO_4:pH6.0, 1ml 1M $CaCl_2$, 1ml 1M $MgSO_4$ in 1 liter of ddH_2O) on 60mm diameter plates seeded with *K. pneumoniae*. We extracted *O. tipulae* isolates from plating diluted soil onto Hay agar plates (15g of cut grass per 1.5L, 1.5 g KH2PO4, 0.62 g Na2HPO4, and 15g agar per liter of hay infusion) with charcoal and *K. pneumoniae*. We reared the *O. tipulae* from soil isolates on 60 mm diameter water agar plates (1% agar in water + 5µg/ml cholesterol) (Barriere, 2006) seeded with *K. pneumoniae*. We sequenced the internal transcribed spacer region of the ribosomal DNA of *O. tipulae* using the methods described in Félix *et al.* (2001) paper to identity our wild-collected nematodes. We chose L4 nematode larvae from plates with different developmental stages for both species in order to start with un-fertilized young adults.

We used all of the strains of *D. discoideum* clones previously used in the Brock *et al.* (2011) paper. In our hands, only 7 of the sub-cloned strains were farmers. We determined this by testing all clones for ability to carry bacteria through the social stage as spores. We placed nine individual fruiting bodies on a single SM/5 nutrient agar plate (2g peptone, 0.2 g yeast extract, 2g glucose, 1.9g KH_2PO_4, 1.3g K_2HPO_4, 0.2g $MgSO_4$anhydrous, 17g agar per liter of ddH_2O) at 9 different spots and waited about four days to reveal amoebae proliferation, something that

could only occur if the spores carried bacteria with them. We called clones farmers if over 70% of the individual sori showed bacteria growth. We called clones non-farmers if fewer than 15% showed bacteria growth. There was only one intermediate value. In fact, of the 72 different spot tests of 9 fruiting bodies each, only 7 different instances showed nonconforming spots, and four had only a single nonconforming spot, two had 2 of 9 nonconforming and one had three of nine nonconforming, indicating the robustness of the test. We grew *D. discoideum* clones on SM/5 nutrient agar and used *K. pneumoniae* as their food source (100mm diameter plates). We harvested spores after a week and used them for the experiment.

Experimental Design

We had six experimental conditions (Figure 1). We plated nematodes with the food bacterium *K. pneumoniae* (Figure 1 Condition 1), or with the putative defensive bacterium *B. xenovorans* (Figure 1 Condition 2). Our bacteria directly harms nematodes hypothesis predicts that *B. xenovorans* will have a negative direct effect on nematodes. Therefore, condition one should yield a greater number of nematodes when compared to condition two.

Next, we plated farmer clones carrying both non-food and their food bacterium *K. pneumoniae* without (Figure 1 Condition 3) and with nematodes (Figure 1 Condition 4). We similarly plated non-farmer clones and their food bacterium *K. pneumoniae* without (Figure 1 Condition 5) and with nematodes (Figure 1 Condition 6). Our bacteria protection against nematodes hypothesis assumes that nematodes are predators of *D. discoideum*; therefore, conditions 3 and 4 allows us to determine the effect of nematodes on farmers, while, conditions 5 and 6 allows us to determine the effect of nematodes on non-farmers. According to our bacteria protection against nematodes hypothesis, the effect of nematodes on farmers should be more

negative than the effect on non-farmers, which should be protected by their bacteria. Additionally, we compared nematode proliferation between conditions 4 and 6. According to the bacteria protection against nematodes hypothesis, we should have a higher number of nematodes in condition 6 than in condition 4 since the nematodes should be harmed and unable to reproduce when in contact with farmers and their bacteria.

We used seven farmer clones and 17 non-farmer clones. We plated 10 L4 hermaphrodite nematodes per 23.75 cm^2, $1x10^4$ *D. discoideum* spores per 23.75cm^2 and 50µl of *K. pneumoniae* (OD_{600} = 1.5) or *B. xenovorans* (OD_{600} = 1.5) depending on the treatment or control. For example, for the first treatment we plated 10 L4 hermaphrodites per 23.75 cm^2 and 50µl of *B. xenovorans* (OD_{600} = 1.5). SM/5 nutrient agar plates were used for the *C. elegans* experiment. Hay agar plates were used for the *O. tipulae* experiment. We could not use the same type of agar plate for both species of nematodes because *O. tipulae* did not grow well on SM/5 plates presumably because of the overwhelming amount of nutrients compared to wild conditions. Therefore, we used the more nutrient poor hay agar plates, which more closely mimic the nutrients found in the soil.

We collected data on nematode number and *D. discoideum* spore production after five days to restrict our results to the first generation of nematodes. For each *D. discoideum* clone and nematode combination, all six treatments were performed twice. These were done in blocks of eight *D. discoideum* clones at a time. After chilling the plates on ice for about 10 minutes to stop nematode movement and ensure accurate counting, we counted nematodes using a Nikon SMZ 1500 dissecting scope. We placed a transparent grid (2.54cm x 2.54cm) with 10 random marked squares (0.506cm x 0.506cm) underneath the petri plate and counted nematodes only found on

the marked squares. We harvested *D. discoideum* spores from plates using 7ml of KK2 and a sterilized spatula and counted spores using a hemocytometer.

To visualize nematode feeding, we fed nematodes for about 4 hours on *K. pneumoniae* transformed to express green fluorescent protein (GFP) and *D. discoideum* spores and cells transformed to express red fluorescent protein (RFP). Afterwards, we paralyzed the nematodes with 10mM Na-azide and placed the nematodes on slides. We took pictures on a Nikon Eclipse E1000 with RFP and GFP filters.

Statistical Analyses

D. discoideum Spore Counts with and without Nematodes

For all statistical testing, we used the R project for statistical computing v2.14.1. To investigate the effect of nematodes on *D. discoideum*'s fitness, we used spore counts as our response variable. To measure the change of spore counts in the presence of nematodes we used percentage change in spore number ((presence-absence)/absence) x 100) We analyzed our data using the Mann-Whitney-Wilcoxon Test to see if there was a difference between the farmers and non-farmers.

Nematode Counts

To investigate the direct effect of *B. xenovorans* and *D. discoideum* on the fitness of nematodes, we looked for a significant difference between nematode counts in the presence of farmers and non-farmers, *B. xenovorans* and *K. pneumoniae*. The *C. elegans* and *O. tipulae* nematode counts were square root transformed to satisfy the assumptions for using a parametric

statistical test. We used an ANOVA to test for differences in nematode counts between nematodes fed on *B. xenovorans* vs *K. pneumoniae* and also farmers vs non-farmers.

Results

We observed active consumption of *K. pneumoniae* and *D. discoideum* cells by *C. elegans* (Fig. 2), which confirms our assumption for our bacteria protection against nematodes hypothesis. We observed the presence of labeled *K. pneumoniae* (Fig. 2A) and both spores and cells of *D. discoideum* (Fig. 2B, 2C) in the guts of *C. elegans*. However, we saw that *D. discoideum* spores stayed intact in the nematode gut, validating the result of Kessin *et al.* (1996). Therefore, nematodes are predators of *D. discoideum* cells, and potential dispersers of *D. discoideum* spores. We also observed the behavior of the nematodes. In the earlier stages of the nematodes' lifecycle, L1, L2, and L2 dauers can climb the stalks of the fruiting bodies and freely move around inside the sorus. This behavior was also witnessed and documented in Kessin *et al.* (2001).

D. discoideum Spore Counts with and without Nematodes

To directly test our bacteria protection against nematodes hypothesis, we compared farmers and non-farmers for the percentage change of spore counts in the presence of nematodes. Our hypothesis predicts that the change will be negative (nematodes harm spore production) but that it will be less negative for farmers because they are protected by their bacteria. The presence of either *C. elegans* or *O. tipulae* nematodes produced similar results. Counter to our prediction, farmers were actually more harmed by nematodes, significantly for one species and nearly significant and in the same direction for the other (*C. elegans*, $p=0.06$, Fig. 3A; *O. tipulae*, $p < 0.01$, Fig. 3 B).

Nematode Counts

We also wanted to test our bacteria directly harms nematodes hypothesis by quantifying whether there is a direct negative effect of non –food bacterium, *B. xenovorans* on *D. discoideum* predators, particularly nematodes. If *B. xenovorans* has a direct negative effect on nematodes, we expected the proliferation of nematodes to be lower when nematodes are fed strictly on *B. xenovorans* than on *K. pneumoniae*. For our bacteria protection against nematodes hypothesis to not be rejected, the proliferation of nematodes should be lower in the presence of farmers than non-farmers since farmers carry *B. xenovorans* that could potentially harm nematodes. *C. elegans* proliferation was consistent between the treatments (ANOVA: *K. pneumoniae* vs. *B. xenovorans* P=0.898, Farmers vs. Non-Farmers P=0.678, Fig. 5B). Similarly, *O. tipulae* proliferation was consistent between treatments (ANOVA: *K. pneumoniae* vs. *B. xenovorans* P=0.223, Farmers vs. Non-Farmers P = 0.09, Fig. 5B). Although the farmer versus non-farmer comparison approached significance, it was in the opposite direction to our prediction, with nematodes faring better on farmers.

Discussion

A previous study showed that farmer clones of *D. discoideum* are able to thrive in environments where non-farmers would do poorly, particularly when good food bacteria are scarce (Brock *et al.*, 2011). Therefore, there is a positive effect of carrying the bacteria through the social stage. We know that the bacteria are able to help in host conspecific competition and that they do so by producing compounds that harm competing non-farmer clones (Brock *et al.* 2013; Stallforth *et al.* 2013). Here we tested a related hypothesis. If *B. xenovorans* produces compounds that are harmful to non-farming *D. discoideum* clones, they might also serve as a weapon to fight

against predatory nematodes. If they protect against nematodes, then the protection against non-farmer clones might be a side-effect rather than an adaptation. However, the predator-protection hypothesis was not supported.

In order to measure *D. discoideum* fitness, we looked at percentage change of spore production as a measure of fecundity in the presence versus absence of predators. Both nematodes, *C. elegans* and *O. tipulae*, harmed fecundity for both farmers and non-farmers. Farmers, under the bacteria protection against nematodes hypothesis, should decrease less than non-farmers in the presence of nematodes. We did observe similar results when we used different species of nematodes. With *C. elegans*, we do not have a significant percentage change for farmers, which rejects our hypothesis. Furthermore, with *O. tipulae* we do have a significant percentage change of spore counts for farmers, however the decrease is much higher than non-farmers which also rejects our hypothesis. This allows us to conclude that the farmers' bacteria do not help protect the farmers against nematodes. On the contrary, the bacteria tend to increase the farmers' risk of predation. One hypothesis is that it could be due to the fact that the bacteria are harbored in the sorus. They may therefore attract the nematodes to this structure where they consume spores in addition to the bacteria. To further test our bacteria protection against nematodes hypothesis, we measured proliferation of nematodes on farmers and non-farmers. Neither *C. elegans* nor *O. tipulae* proliferation changed when reared on *D. discoideum* farmers or *D. discoideum* non-farmers. Therefore, the non-food bacteria do not protect *D. discoideum* farmers from predation.

As for our bacteria directly harms nematodes hypothesis, we found that for both species of nematodes, *C. elegans* and *O. tipulae*, proliferation did not change when reared on *K. pneumoniae* compared to in the presence of non-food bacteria, *B. xenovorans*. We can conclude

that the *B. xenovorans* do not harm the proliferation of *C. elegans* or *O. tipulae*, contrary to our prediction (Fig. 4). The non-food bacteria do not directly harm either nematode, *C. elegans* or *O. tipulae*, which is a contrast to a finding that *C. elegans* were killed by *Burkholderia psuedomallei* (O'Quinn *et al.*, 2001).

In conclusion, all predictions based on the hypothesis that carrying *B. xenovorans* protects farmers against nematode failed. Therefore, the bacteria carried by farmers are not used for protection against *C. elegans* and *O. tipulae*. This lack of an effect against predators makes it more likely that the true function of carrying *B. xenovarans* is the previously demonstrated effect in aiding the farmer's competition between conspecifics (Brock *et al.* 2013; Stallforth *et al*. 2013).

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Conditions	*K. pneumoniae*	*B. xenovorans*	Farmers	Non-Farmers	Nematodes
 1	✓				✓
 2		✓			✓
 3	✓		✓		
 4	✓		✓		✓
 5	✓			✓	
 6	✓			✓	✓

Legend	
K. pneumoniae	
B. xenovorans	
Farmers	
Non-Farmers	
Nematodes	

Figure 1. Design of the experiment with complete list of controls and treatments. We had two plates for each control and treatment which were done simultaneously for a total of 40 plates per block. We performed each block twice.

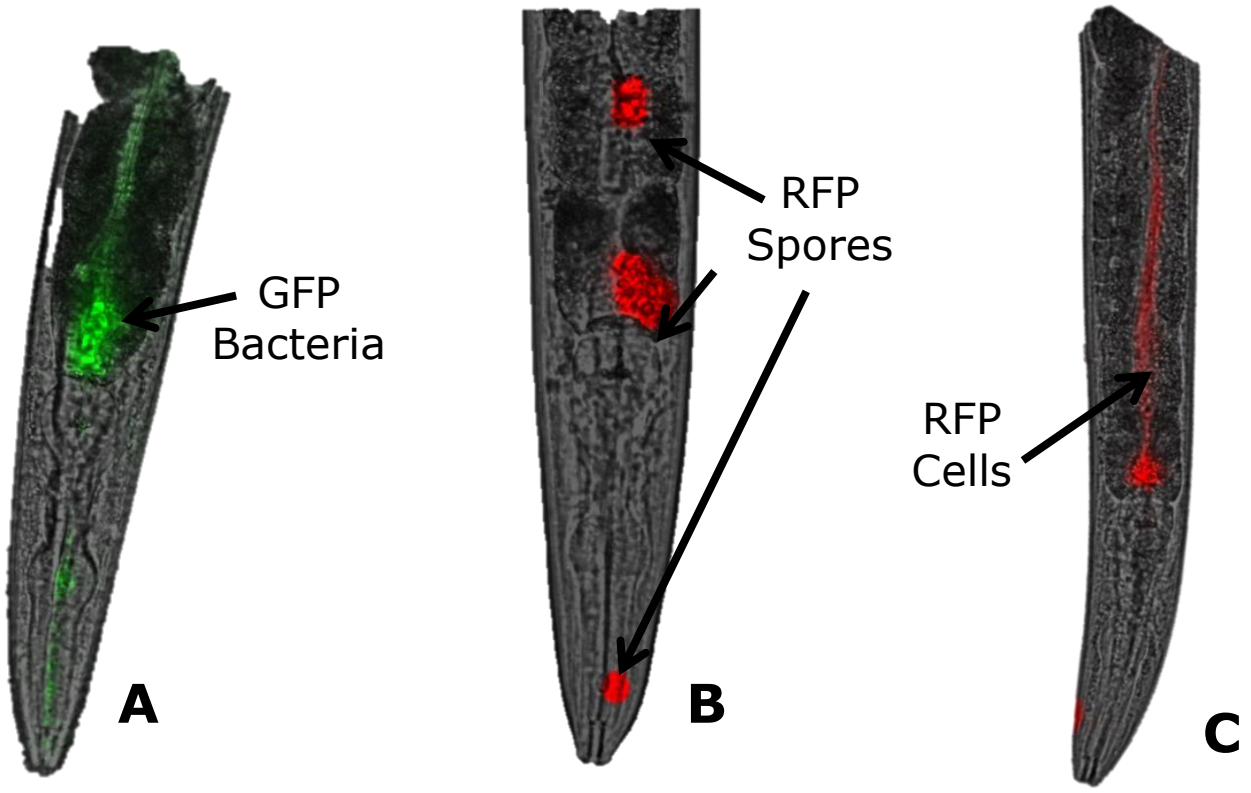
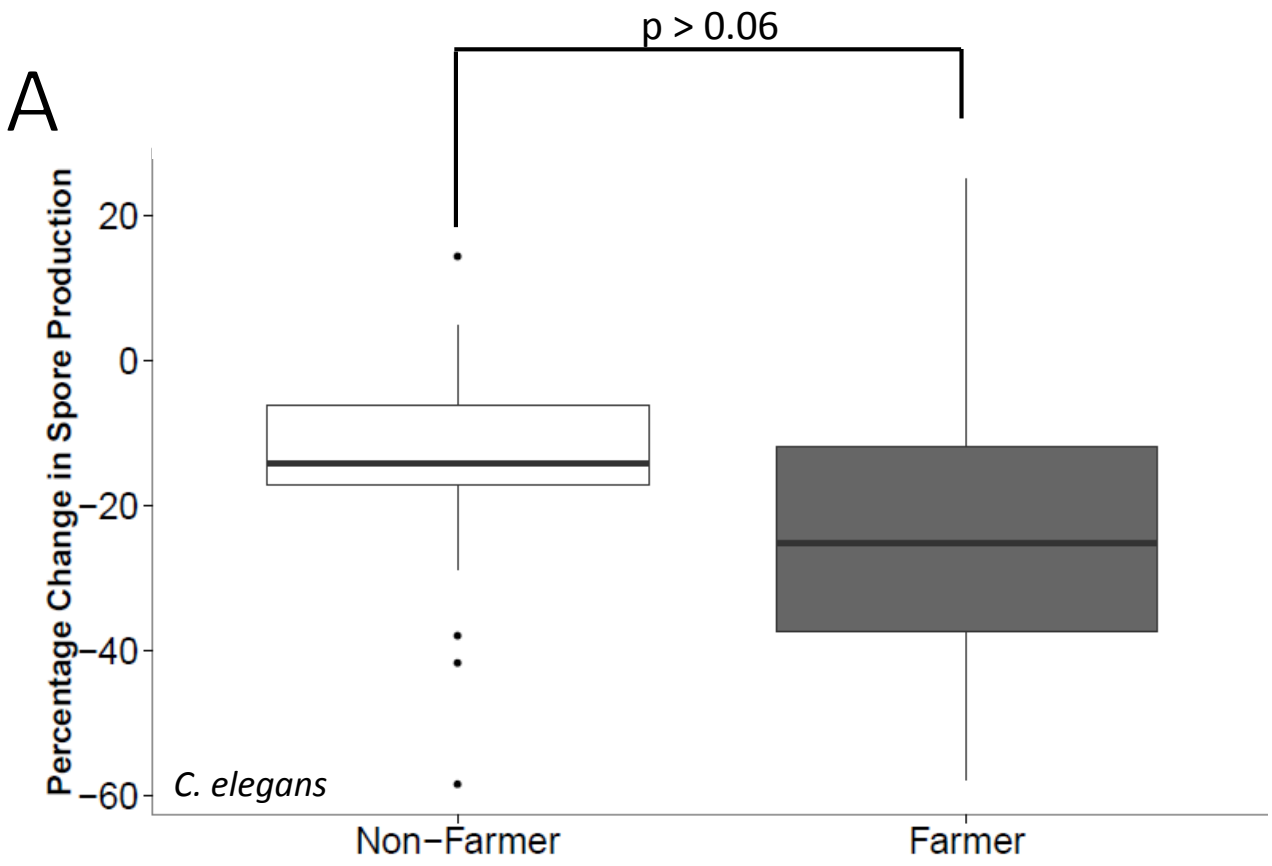


Figure 2. *C. elegans* Digestion after 3 hours. (A) *C. elegans* fed on GFP labeled *K.pneumoniae*. (B) *C. elegans* fed on RFP labeled *D. discoideum* spores. (C) *C. elegans* fed on RFP labeled *D. discoideum* cells.


A
p > 0.06
Percentage Change in Spore Production
20
0
−20
−40
−60
C. elegans
Non−Farmer
Farmer

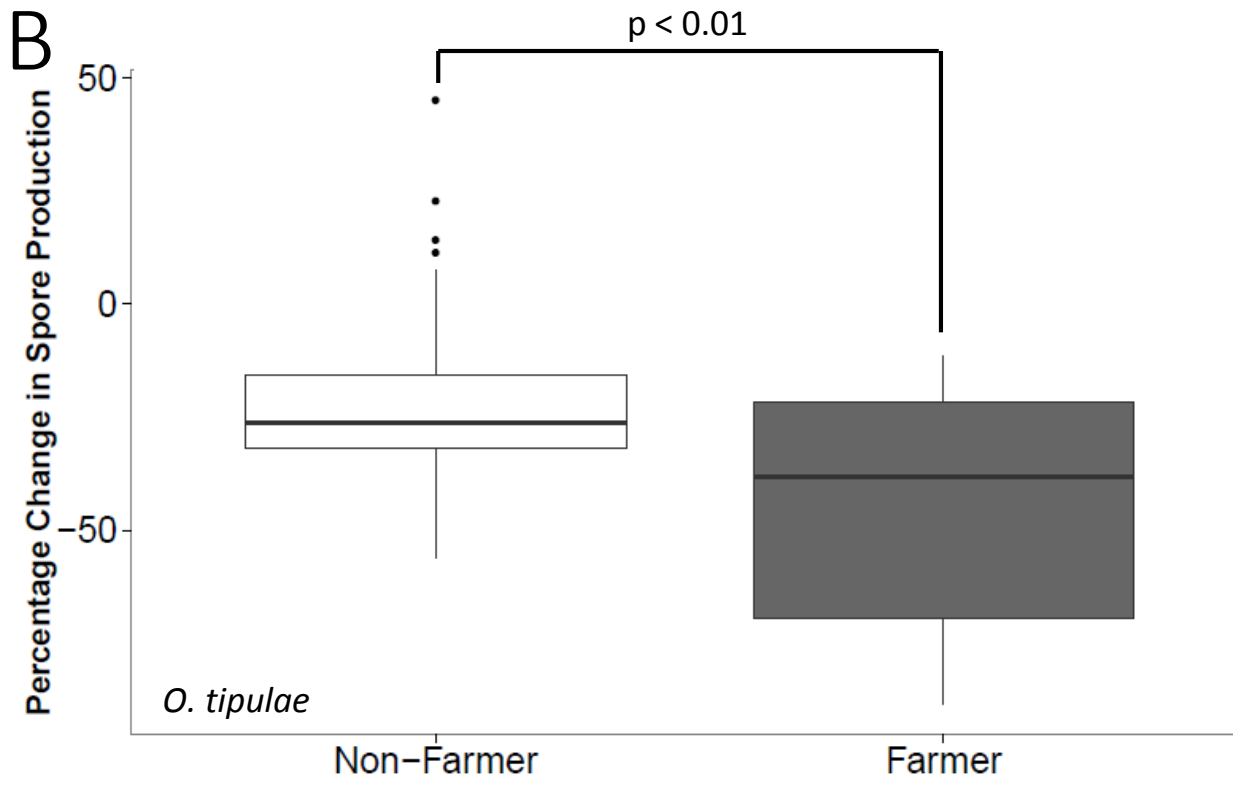
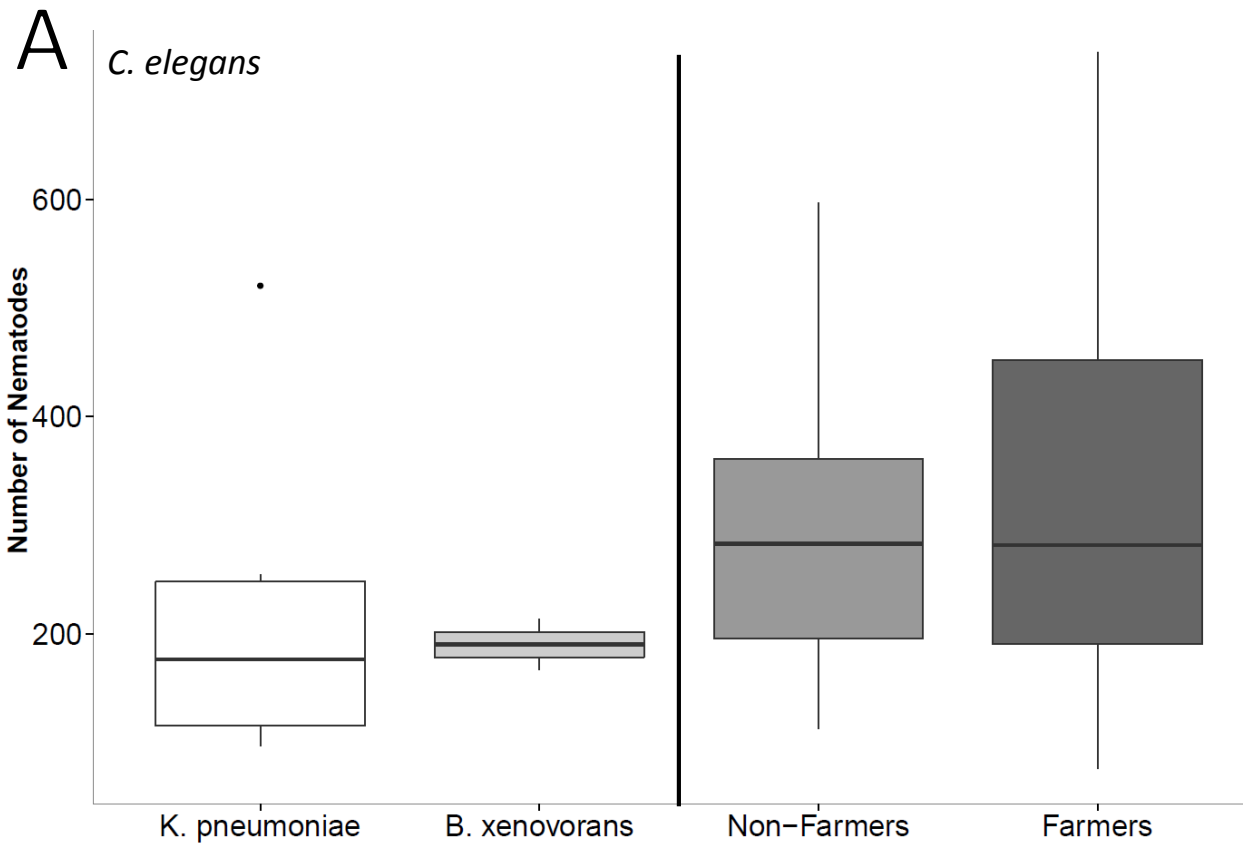

B
p < 0.01
Percentage Change in Spore Production
50
0
−50
O. tipulae
Non−Farmer
Farmer

Figure 3

Average percent change in spore production (±SD) of spores of non-farmer (N = 17) and farmer (N=7) *D. discoideum* clones in the presence of nematode predators *C. elegans* (A) Mann-Whitney-Wilcoxon Test, Non-Farmer vs. Farmer W=184, p>.06. *O. tipulae* (B) Mann-Whitney-Wilcoxon Test, Non-Farmer vs. Farmer W=134, p<.01.


A
C. elegans
Number of Nematodes
600
400
200
K. pneumoniae
B. xenovorans
Non−Farmers
Farmers

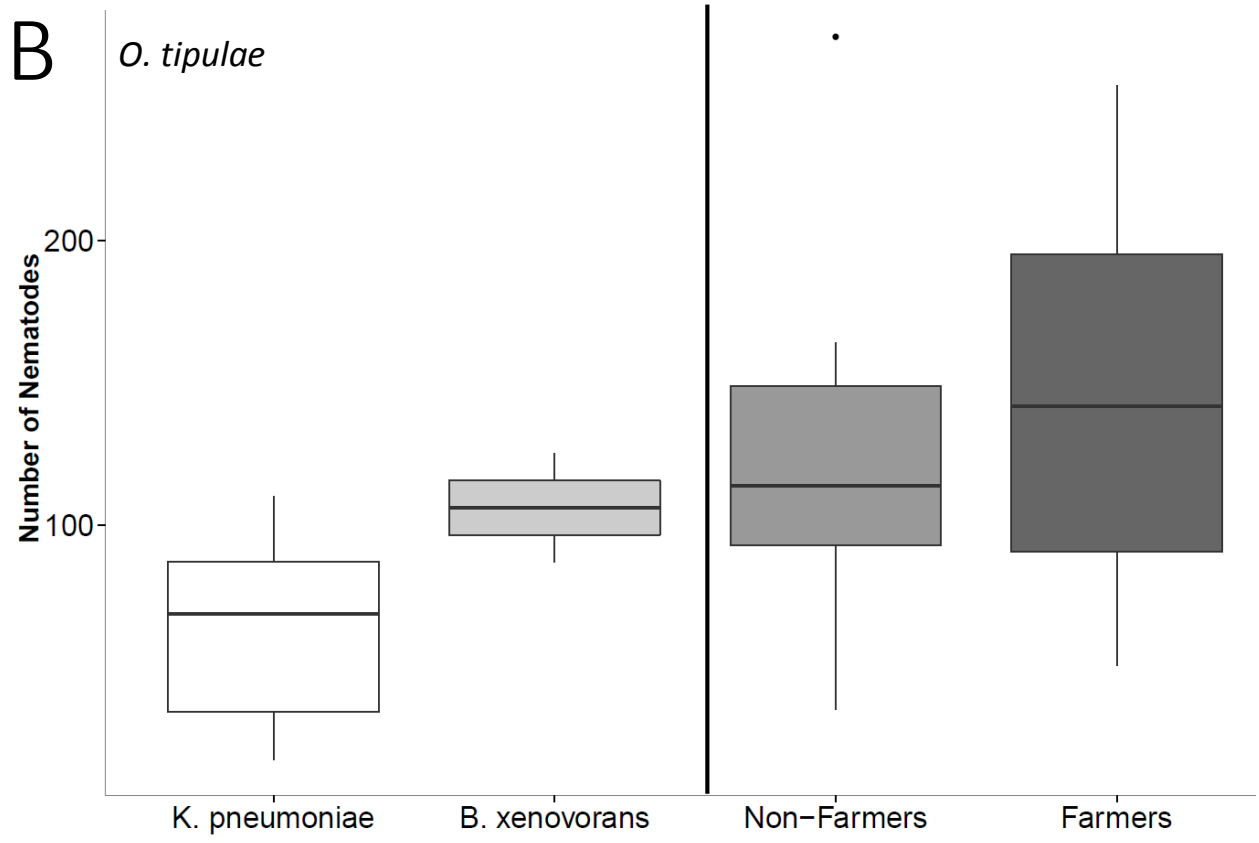

B
O. tipulae
Number of Nematodes
200
100
K. pneumoniae
B. xenovorans
Non−Farmers
Farmers

Figure 4.

Nematode counts (±SD) of *C. elegans* (A) and *O. tipulae* (B). Farmers n=7, Non-Farmers n=17, Bx n=2, *K. pneumoniae* n=6. We replicated and duplicated each treatment and control. We performed an ANOVA. For *C. elegans*, *K. pneumoniae* vs. *B. xenovorans*, P=0.898, Non-Farmers vs. Farmers P=0.678. For *O. tipulae*, K. *pneumoniae* vs. *B. xenovorans* P=0.223, Non-Farmers vs. Farmers P=0.09.