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Cover: The cover illustration was drawn by Jonathan Frye, using homemade *Acer saccharinum* charcoal.

The quotation is taken from Wendell Berry's essay, *The Loss of the University*, (p. 82 in *Home Economics*, North Point Press, 1987) in which he references the book *Samuel Johnson*, by W. Jackson Bate (Harcourt Brace Jovanovich, 1977, p. 51) as follows: "Dr. Johnson told Mrs. Thrale that his cousin, Cornelius Ford, `advised him to study the Principles of everything, that a general Acquaintance with Life might be the Consequence of his Enquiries - Learn said he the leading Precognita of all things ... grasp the Trunk hard only, and you will shake all the Branches.'"

Cantaurus is an official publication of the Division of Science and Technology, McPherson College, McPherson, KS. The purpose of this journal is to publish the results of original research conducted by students majoring in the natural sciences at McPherson College. The research published herein represents partial fulfillment of the requirements for the B.S. degree at McPherson College.

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Prairie Restoration Effects on the Soil Properties of Retired Agriculture Fields in Central Kansas

Tanner Cardoza

ABSTRACT

Native prairies, characterized by their rich biodiversity and unique soil compositions, play a crucial role in carbon sequestration, water retention, and habitat provision for various species. This research project aims to investigate soil samples collected from retired agriculture fields in the process of being restored back to native prairie to assess the ecological health and viability of these ecosystems. Using a combination of field sampling and laboratory analysis, we aim to identify patterns in soil texture, soil organic matter, and microbial diversity among the different fields. Preliminary findings indicate significant variations in soil health metrics correlated with the state of restoration of the prairie fields. The soil texture was constant between the fields, however, the percentage of organic matter and microbial diversity varied at different depths. This research emphasizes the importance of soil health in the success of prairie restoration efforts and offers valuable insights for land management practices aimed at enhancing biodiversity and ecosystem resilience in restored habitats.

Keywords: *Biodiversity, soil texture, soil organic matter, microbial diversity, ecosystem resilience, prairie restoration.*

INTRODUCTION

Prairie restoration efforts are an essential step towards creating a sustainable and resilient ecosystem for future generations. Due to its crucial impact on soil quality, conserving biodiversity, and mitigating climate change, prairie restoration efforts have increased in popularity (Brye, 2008). Approximately 20% of Earth's grasslands have been converted to cultivated crops (Ramankutty, 2008), and the use of mechanized agriculture and intense tilling practices have highly depleted the carbon accumulation in the soil. The poor quality and altered food webs of these former agricultural soils acts as barriers to reestablishing diverse plant communities (McCullough, 2024).

Because prairie grasslands have been shown to hold great potential for sequestering soil carbon (Bai and Cotrufo, 2022), it is important that we maximize our efforts to restore abandon agriculture land back to native prairie. Higher soil moisture often favors greater plant and microbial diversity, while soil organic matter affects the water holding capacity of soil and nutrient retention. Because native grasses and plants have such deep root systems, they can help improve soil quality by preventing erosion, enhancing water infiltration, and promoting nutrient cycling. Increasing soil carbon can help reduce runoff and increase water-holding and cation exchange capacity, thereby increasing soil fertility (Weil and Magdoff, 2004).

Given that about 30% of the planet's land surface is covered in grasslands, the widespread cultivation has likely caused a massive carbon dioxide efflux from the soil to the atmosphere over the last two centuries, especially following mechanization (Lal, 2002). Since prairies absorb an immense amount of atmospheric carbon (Dempsey, 2022), they can help regulate climate change by removing large amounts of carbon dioxide emitted into the atmosphere by humans.

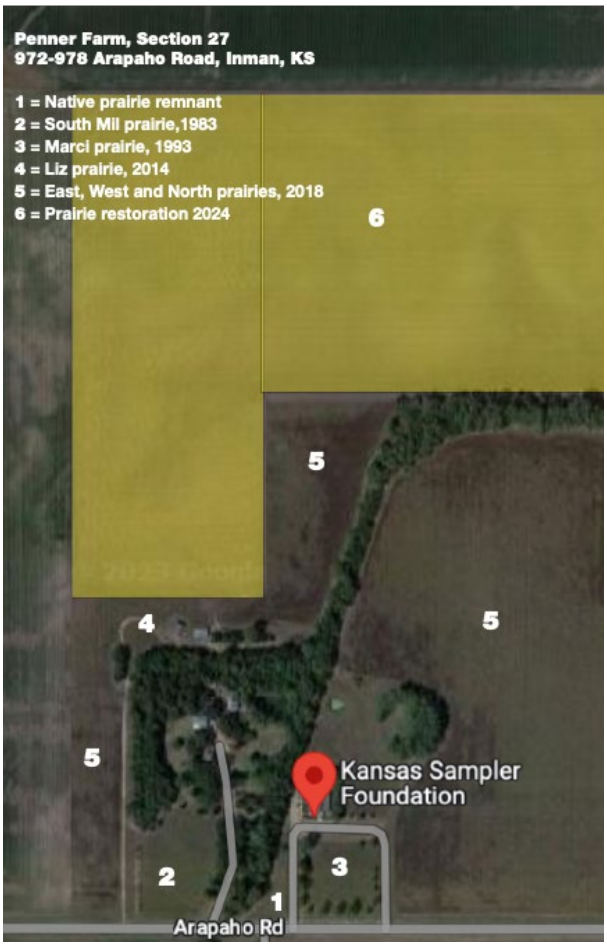
While these are just some of the major impacts of prairie restoration, they all have to do with the soil quality. Therefore, the objective of this study will be to investigate how soil quality varies with differences in the number of years since being converted from agricultural farmland to native prairie. I will be evaluating the soil organic matter percentage, soil texture, and microbial diversity of the soil in retired agriculture fields in Inman, Kansas at the Penner Family Farm on Section 27. This property contains 5 different fields that are currently in the restoration process in being converted back to native prairie from agriculture field.

MATERIALS AND METHODS

Soil Sample Collection

Soil samples were taken from the Penner Farm in Inman, Kansas. This site includes a chrono-sequence of retired agricultural fields in the restoration process of being converted back to native

prairie. A random point was selected in each field being tested. We then measured out one square meter and took samples from each corner. We used a sampling probe and a mallet or sledgehammer to drive the probe to deeper depths. Samples were collected down to 100cm and separated in Ziploc bags every 20 cm for each field.



Lab Methods

Before doing any tests with the samples, it was important that all moisture was removed from the samples. To do this, we separated the samples into 8-inch loaf pans then weighed them. They were then put in the oven at 104°C and reweighed every 24 hours until the weight was constant. Once dry, samples were then pulverized into a very fine sample.

To measure the soil organic matter (SOM) percentages of the soils in these fields, we put the samples through a loss on ignition method where they were oven dried and then heated to 400°C. They were then weighed again. The difference between the weights represented the total amount of organic matter in each sample.

Soil texture was also tested through a bouyoucos hydrometer analysis (Thien and Graveel, 2002). These results were then compared to those of the soil conservation service.

Microbial Biodiversity

To compare the microbial biodiversity of the samples from these sites, 1g subsamples were collected and mailed to CD Genomics for a metagenomic analysis. To get these samples, soil cores were split in half and soil was scraped from the middle of the core using sterile techniques to minimize the potential for contamination. In this analysis, DNA was extracted from the samples and then amplified and sequenced in ways to identify differences in microbial abundance and diversity among fields and depths.

RESULTS

Soil Textural Analysis

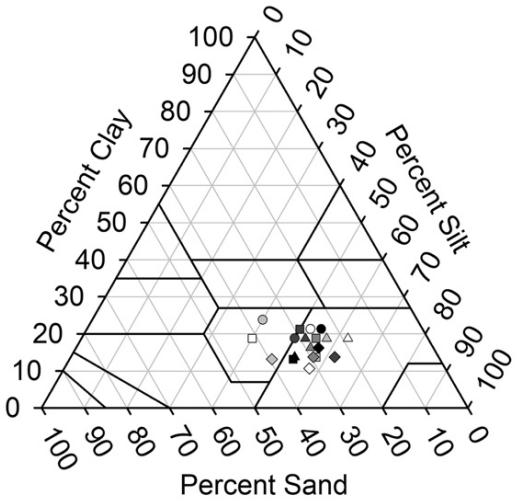


Figure 1. Soil texture triangle with results from bouyocous hydrometer analysis. Shapes indicate Field#, Shades of White to Black indicate depth. Triangle=Field#2, Circle=Field#3, Square=Field#5, Diamond=Field#6.

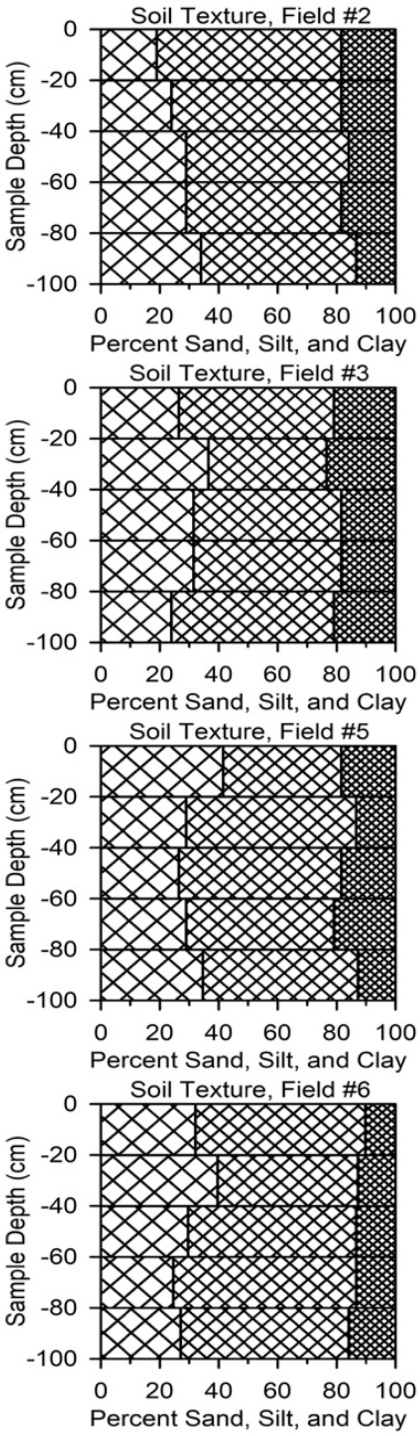


Figure 2. Soil texture results from bouyoucos hydrometer analysis.

Through the soil textural analysis, we learn that the soil samples were a silty loam.

Soil Organic Matter Analysis

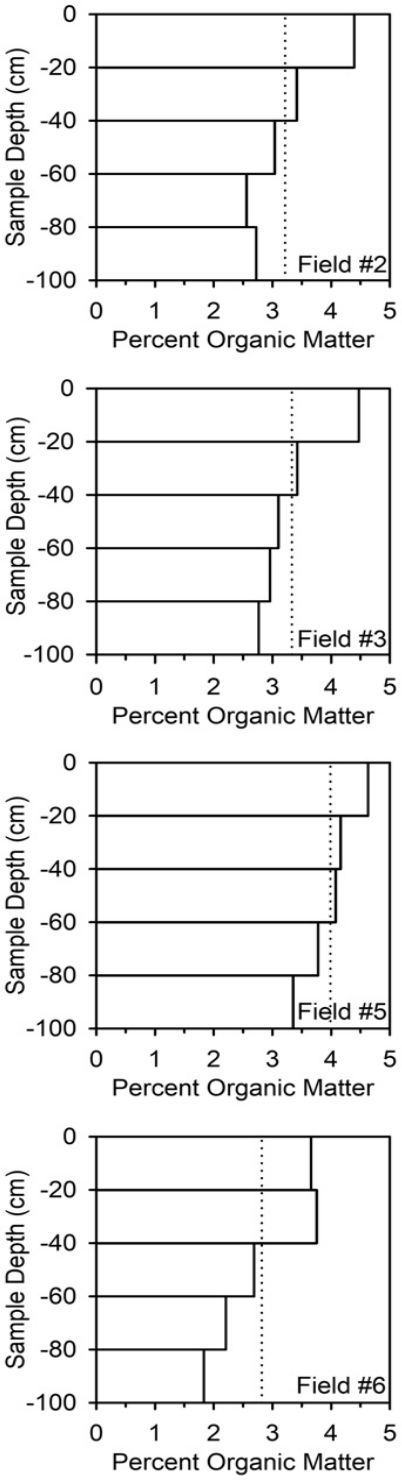


Figure 3. Percent SOM at different depths among fields.

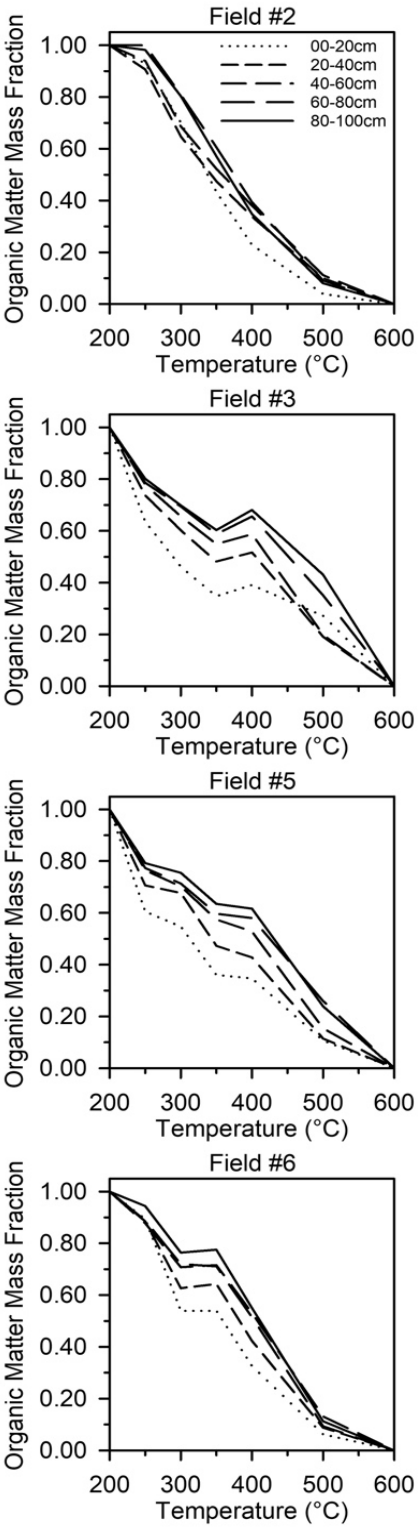


Figure 4. SOM fraction as temperature rises in loss on ignition procedure.

Through the soil organic matter analysis, we can see that there is typically more SOM closer to the surface of the fields. Also, as the temperature rose in the loss on ignition method, the SOM burned up quicker in the younger fields.

Metagenomic Analysis

Table 1. Statistics of alpha diversity. D=Deep S=Surface

Sample	Observed species	ace	Chao 1	Simpson	Shannon
PF2D	370	185.01	267.125	0.965965	6.432705
PF2S	252	149.6175	170.8333	0.951489	5.663282
PF3D	249	105.5072	101.0625	0.96336	6.099688
PF3S	300	100.7758	106	0.956316	5.874171
PF5D	128	34	30.14286	0.918068	4.028951
PF5S	346	129.367	138.7692	0.960399	6.132953
PF6D	292	113.8772	109.5263	0.959621	6.115831
PF6S	281	99.45875	99.71429	0.945549	5.530681

Four different metrics were calculated to assess the alpha diversity: Chao1 and Ace simply estimate the number of species in a community; Shannon and Simpson account for both richness and evenness of a community. The larger the Chao1, Ace and Shannon indices correspond to a smaller Simpson index value, indicating greater diversity of species.

The results of this analysis show that on average, there is a greater abundance of species in the older fields.

DISCUSSION

The results of the soil textural analysis were compared to the latest soil conservation service's report for the field and surprisingly varied, with the NRCS reporting the field to be more of a silty clay loam and my data showing more of a silty loam. Silty loam soil is ideal for prairie fields because it provides great drainage while retaining moisture and nutrients for the prairie plants to thrive(Bach, 2010).

Based on past research, I had expected for the fields that were further along in the restoration process to have significantly more SOM. While it was evident that the samples burned up quicker in the younger fields, it wasn't as clear which field necessarily had the greatest SOM content.

ACKNOWLEDGEMENTS

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The influence of predation risk on the behavior of the slime mold *Physarum polycephalum*

Summer Dorn

ABSTRACT

The acellular slime mold *Physarum polycephalum* is an emerging model organism in the study of the behavior of complex organisms, but the influence of predation risk on its foraging behavior is severely understudied in the literature. To help fill this gap, we conducted an investigation to determine whether *P. polycephalum* considers a perceived risk of predation when making foraging decisions. We allowed the temperate white springtail *Folsomia candida*, a known slime mold predator, to explore one half of an experimental enclosure for several hours before removing it from the environment and allowing *P. polycephalum* to explore the same enclosure. We also added two identical food spots to the enclosure at this time, one in the half explored by the springtail and one on the unexplored side. We recorded *P. polycephalum*'s chosen direction of exploration and found a statistically significant preference for the half of the enclosure which was not explored by *F. candida*.

Keywords: *Folsomia candida, foraging behavior, Physarum polycephalum, predation*

INTRODUCTION

One of the most difficult elements of biology to study in the laboratory is the behavior of complex organisms. In recent years, macroscopic slime molds have been proposed as a model system for the study of behavior (Latty & Beekman 2015), as it has become more evident that slime molds make decisions in fundamentally similar ways to more complex organisms.

The acellular slime mold *Physarum polycephalum* is an organism that demonstrates remarkable behavioral properties despite its apparent simplicity. For example, it can find the shortest path through mazes (Nakagaki et. al. 2000) and can select the optimal food source from among many options(Dussutour et al. 2010). It is also capable of habituation to stimuli (Boisseau et al. 2019) and retains that learning for a significant amount of time (Boussard et al. 2019). They are subject to speed-accuracy trade-offs (Latty & Beekman 2010c) and experience comparative valuation (Latty & Beekman 2010b), two irrational behaviors exhibited by more complex organisms, including humans (Tversky & Simonson, 1993), and it was the first non-neural organism demonstrated to solve the two-armed bandit puzzle (Reid et al. 2016). These features add to a growing body of evidence that decision-making shares a common mechanism across all eukaryotic organisms (Reid et al. 2014). If that is the case, and slime mold proves to be a useful model system, the study of behavior in controlled settings becomes much simpler.

All foraging decisions made by complex organisms are the product of a risk-reward assessment comparing the benefits (more or higher quality food) to the hazards (risk of predation, injury, conflict with conspecifics or with other organisms). The risk of predation is the single hazard which represents the greatest danger to the forager, and is the hazard most widely studied in the literature. In animals, risk assessment patterns across many different species are thoroughly understood, with quantitative models of behavior available (Brown & Kotler 2004). For example, the precise valuation the Eurasian blue tit *Parus caeruleus* gives to predation risk and other environmental factors when foraging has been determined mathematically (Todd & Cowie 1990). However, similar research in protists is nearly nonexistent.

Many species of mites, beetles, and nematodes are known to prey upon slime molds (Wheeler 1984), and it is known that *P. polycephalum* takes many other factors into account when deciding when and where to forage, including light exposure (Latty & Beekman 2010a), salt exposure (Smith-Ferguson et. al. 2020) and food quality (Latty & Beekman 2009). It is also capable of alerting conspecifics of potential dangers (Briard et al, 2020). This makes it very likely that *P. polycephalum* considers the risk of predation when determining its foraging behavior.

We conducted a behavioral study of *P. polycephalum* to determine whether it takes predation risk into account in its foraging behavior by placing it in a controlled environment, one part of which was previously explored by a predator species.

MATERIALS AND METHODS

Experimental procedures largely mirrored previous slime mold behavior experiments, examining the organism's choices between safe and risky food options in a controlled environment (e.g. Latty & Beekman 2010a).

Initial samples of *Physarum polycephalum* were ordered from Carolina Biological Supply. However, this colony was contaminated by an unknown fungus after the completion of the initial treatment, and a replacement colony was ordered to complete the remaining treatments. Because *P. polycephalum* was unavailable from Carolina at that time, the replacement colony was ordered from Liquid Fungi, a supplier of fungi and slime molds. Both colonies were cultured in closed plastic containers on a bed of paper towels with a supply of oat flakes as a food source. This bedding was replaced twice weekly, and the container was misted daily. The slime mold was kept at room temperature and in a low-light environment except when maintaining the enclosure or preparing samples for testing.

Many of the known predators of slime molds are difficult to obtain, exist in only some parts of the world, and/or are poorly understood. *Folsomia candida,* the temperate white springtail, was chosen as the best candidate for use in this study. It is an omnivorous species that will consume any biomass available to it, and which is used as a model organism in several fields. It is also globally distributed and easy to care for, and it is known to leave pheromone trails to communicate with conspecifics (Nilsson & Bengsston 2004).

Our springtail colony was ordered from FrogDaddy, a supplier of terrarium accessories (*F. candida* is used commercially in the construction of bioactive terrariums). A commercial supplier was chosen for being cheaper and more readily accessible than dedicated science suppliers of *F. candida*. The colony was kept in a sealed plastic container with coconut fiber substrate and food sources of uncooked rice and baker's yeast. Food sources were replaced weekly, and the enclosure was misted daily.

Behavioral studies of *P. polycephalum* typically use standard petri dishes as environments. 60 mm diameter petri dishes were used for our experiment. Experimental dishes used 2% plain agar gel as substrate. A sheet of plastic was cut into rectangular pieces of the appropriate size (60x10mm) and one was embedded in the agar gel to divide each encloser into two equal halves. The underside of each dish was marked with either a line or a small x to differentiate the two halves. Several individuals of *F. candida* were allowed to explore one half, while the barrier prevented them from accessing the other half.

Whether the springtails were allowed to explore the marked or unmarked half of the enclosure was alternated with each treatment: In the first, third, and fifth treatments, the marked side was explored. In the second and fourth treatments, the unmarked side was explored.

All organisms were moved into or out of the experimental dishes under a fume hood to minimize exposure to atmospheric contaminants.

Due to their small size, individuals of *F.candida* are extremely difficult to transfer into the enclosure directly without crushing them. They were instead moved by using forceps to collect a small piece of substrate containing several individuals, holding the piece over one half of the experimental dish, and shaking gently to dislodge several springtails. The dish was then closed and left overnight to give them time to thoroughly explore one half of the environment. The next morning, the springtails were removed from the dish by simply turning it over and gently tapping the underside several times.

After exploring *F. candida* were returned to their colony, the barriers were removed from the experimental dishes and two oak flake food spots were placed in each dish: one at the center of the half which was explored by *F. candida*, and one at the center of the unexplored half. Then, an oat flake colonized by one of our culture samples of *P. polycephalum* was removed with forceps and placed at the center of each experimental dish.

When a section is cut and removed from a sample of *P. polycephalum*, it becomes a fully functional individual of its own in minutes (Kobayashi et. al. 2006), so no further preparation was required. The samples were left to grow in a dark room for 24 hours before results were collected.

When *P. polycephalum* forages, it typically extends a single pseudopod in one direction at a time, called the 'exploring arm'. Which half of the enclosure each individual explored with its exploring arm was recorded to determine our results.

This exploration preference of each sample of *P. polycephalum* was recorded as either *springtail* if it favored the side explored by *F. candida*, *non-springtail* if it favored the side which had not been previously explored by *F. candida*, or *undecided* if the individual did not move from its original position. Very rarely, an individual of *P. polycephalum* would instead direct multiple pseudopods into both halves of the dish simultaneously. These individuals were also recorded under *undecided*.

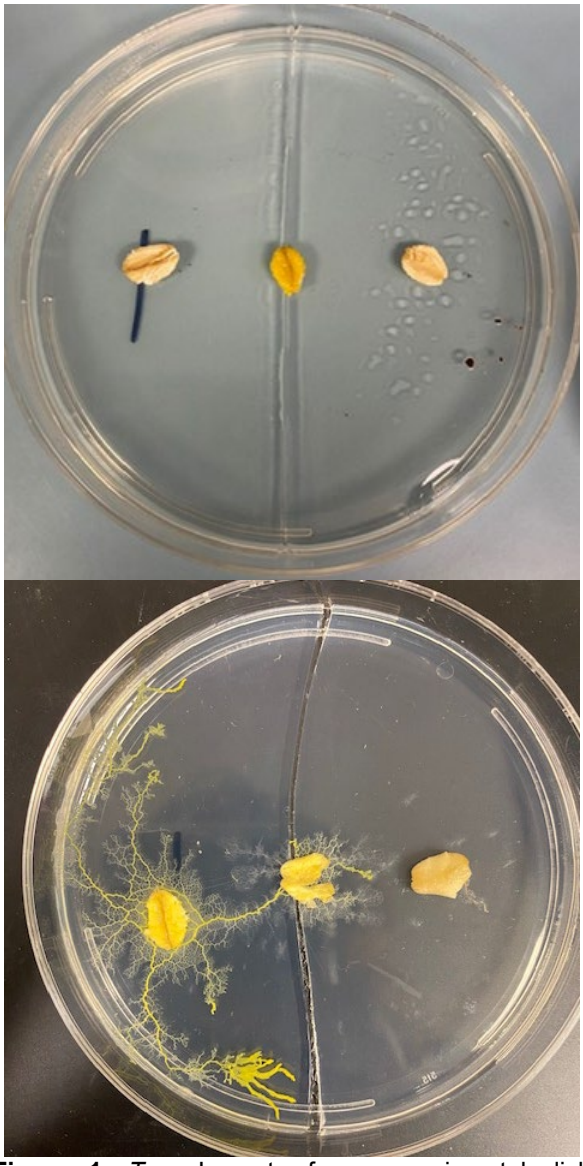

Figure 1. *Top*: Layout of an experimental dish immediately after a *P. polycephalum*-colonized oat flake was placed in the enclosure. *Bottom*: Another dish from the same treatment after 24 hours had elapsed. In this treatment, the unmarked (right) side was explored by *F. candida*

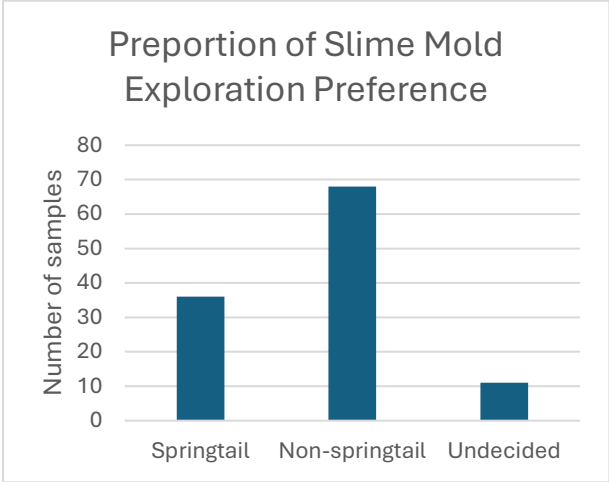

Figure 2. Bar graph representing our findings. From left to right, each bar represents the total number of *P. polycephalum* individuals which preferred the *F. candida*-explored half of their enclosure, which favored the unexplored half, or which did not favor either half.

RESULTS

Five treatments were conducted on different dates for a combined total of 115 samples. Of the samples, 36 favored the half of the enclosure explored by *F. candida*, 68 favored the side which was not explored by *F. candida*, and 11 were undecided. A graphical representation of our results can be found below.

We performed a series of χ^2 goodness of fit tests to determine the statistical significance of our results. We first combined the *springtail* and *non-springtail* results (all individuals which explored one half of the dish but not both) and compared them to the *undecided* results, finding a strongly significant preference for exploring one side or the other over either not exploring or exploring in multiple directions ($\chi^2 = 75.2$, df = 1, $P < 0.001$). We then compared the results for the *springtail* haft of the enclosure to those of the *non-springtail* half, excluding the *undecided* results, and found a significant preference for exploration of the *non-springtail* half of the experimental dish ($\chi^2 = 9.85$, df = 1, $P = 0.002$; Figure 1).

We also used χ^2 goodness of fit tests to look for variation in our results across the five days. (Table 1, below) We found significant variance in the likelihood of remaining *undecided* across treatments ($\chi^2 = 16.4$ df = 8, $P = 0.037$). However, we found no significant variation in preference for either the *springtail* or *non-springtail* halves of the dish across different days ($\chi^2 = 3.97$, df = 4, $P = 0.411$).

Table 1. The number of *P. polycephalum* individuals which preferred each exploration direction. Data is organized by the date each treatment was completed. Categories are *Springtail, Non-springtail*, and *Undecided*.

Date	*Spr.*	*Non.*	*Und.*	Total
10/17	1	9	5	15
12/16	11	20	3	34
12/18	14	18	3	35
12/30	6	12	0	18
01/03	4	9	0	13
Total	36	68	11	115

DISCUSSION

We found strong evidence that *P. polycephalum* is able to recognize when its environment has been explored by a predator and that it is more likely to avoid areas associated with the presence of a predator organism. This adds to the growing body of evidence that *P. polycephalum* is highly dynamic with its foraging behavior, bolstering its credibility as a model organism for complex decision making.

Several confounding variables may have influenced our results. Contamination of an experimental dish by airborne particles might influence *P. polycephalum*'s preferred exploration direction. This risk was minimized by only opening experimental dishes within a fume hood and performing all maintenance as quickly as possible. Also, the oat flakes used as food sources could not be made completely identical. Although care was taken to ensure each sample received two oat flakes of similar shape, size, and appearance, the slime mold may perceive some oat flakes as more desirable than others based on factors such as the precise nutritional value of each individual flake that cannot be readily controlled for.

The integrity of our experiment was reliant on the effectiveness of our constructed barriers at preventing *F. candida* from accessing the entire dish. In a total of four samples (three in the initial treatment and one in the second), we found that at least one springtail had slipped through a gap in the barrier in the course of its exploration. These samples were removed from the treatment and *P. polycephalum* was not permitted to explore them. The barriers used in these samples were then discarded and new ones were measured and constructed from the original plastic sheet to replace them in future treatments. No further 'escaped' springtails were observed after the second treatment, but it cannot be guaranteed that none of the springtail found their way into the other half of a dish but returned to the 'correct' side before next being observed.

The largest anomaly within our results, the significant variance in the number of *undecided* individuals across the five treatments, has several potential explanations. Firstly, our initial treatment, which had the highest number of *undecided* individuals, was also our only treatment to use our original colony of *P. polycephalum*. Colonies of *Physarum* species are known to have unique 'personalities,' with some colonies being more passive than others (Masui *et al* 2018). We may have exchanged a more naturally passive colony for a more active one in our later treatments. The relative passivity of different colonies is not known to alter the exploration preferences of the organism, only its willingness to explore in the first place, and therefore would not alter our other results. Additionally, the efficiency with which we were able to set up experimental dishes improved over time. In particular, the act of moving springtails into only one half of each dish proved initially challenging and took progressively less time per dish with each successive treatment. This reduced the amount of time each open dish was exposed to air and therefore the opportunity for atmospheric contamination that might alter *P. polycephalum*'s decision-making patterns.

Future research in this area should examine how the slime mold reacts to other predators besides *F. candida* and the relative weight of predation risk compared to other factors such as food quality or light exposure, with the ultimate aim of devising a more quantitative model of how *P. polycephalum* responds to predation risk.

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Effects of Pesticides on Honeybees and the Number of CCD Cases

Isaac L Hale

ABSTRACT

Honeybee colonies are in danger and are disappearing at an alarming rate. One major cause is the use of pesticides in fields and agricultural land. These chemicals which are often used to protect crops from pests can harm honeybees by affecting their ability to gather food, weakening their immune systems, and making it harder for their hives to survive. This research looks at how different types of pesticides, especially neonicotinoids (knee-o-nicko-ti-noids) and organophosphates, impact honeybee health. Environmental studies show that long-term exposure to pesticides can make bees less effective at collecting pollen, increases their chances of dying early, and weakens the entire hive as a whole. As a result, entire colonies can collapse and die. Understanding how pesticides contribute to this problem is important for protecting honeybees and keeping ecosystems healthy. This research highlights the need for better farming practices and pesticide regulations to help save honeybee populations.

Keywords: *Apis mellifera*, *CCD, Colony collapse disorder*

INTRODUCTION

Honeybees, also known as Apis mellifera*, play a major role in nature and agriculture by helping plants grow and thrive through the magical natural process of pollination. Without them and their source of pollination, many of the fruits, vegetables, and nuts we commonly eat and take for granted would no longer be available (Klein et al., 2007). One of the most important things honeybees do is collect pollen. The collected pollen is then used as food for the hive. Pollen is the key food source that gives the honeybees the proper nutrients that they need in order to maintain their health, reproduce, and keep the colony thriving with order (Di Pasquale et al., 2013). In recent years however, honeybee populations have been on the decline at an alarming rate. One of the main reasons for this is the increased exposure and introduction to different pesticides (Goulson et al., 2015).

Pesticides, especially neonicotinoids and organophosphates, are commonly used to protect crops from pests. On the flip side however, they can also cause major harm and endanger local honeybees. When bees come into contact with such pesticides while out collecting pollen or nectar, these chemicals can weaken their immune systems and impair their ability to navigate back to the hive. In some cases, the unwanted exposure can even lead to death (Tosi et al., 2018). Chronic exposure to pesticides has been linked to a deadly disorder called colony collapse disorder (CCD*). This disorder is a phenomenon in which an entire honeybee colony will disappear. In turn leaving behind their queen and any of the few immature bees that decide to stay behind with the queen/hive (VanEngelsdorp et al., 2009). This is a major concern because honeybees contribute to the pollination of over 75% of global food crops. This would mean that their decline could have some major shockwaves on local/nationwide food production, as well as serious complications in the environment's biodiversity (Potts et al., 2010).

Understanding how pesticides affect honeybee health is important for protecting these pollinators and the environment in which they inhabit and share with the rest of us. By studying how different pesticides impact honeybee foraging behavior, hive strength, and survival, researchers and environmentalist can work on solutions to reduce the harm to Apis mellifera* and keep their populations as stable/healthy as possible. This research will examine the connection between pesticide exposure and CCD* in order to better understand how to protect the world's honeybees.

MATERIALS AND METHODS

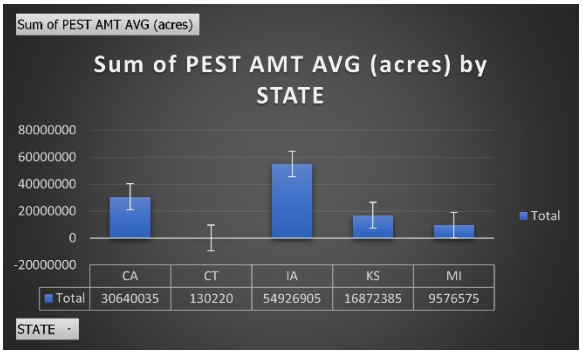
For this research, I used the USDA National Agricultural Statistics Service (NASS) Quick Stats website (https://quickstats.nass.usda.gov/) to gather information on honeybee colony losses and pesticide use across the five different states. This website provides real data on agriculture. Including data like the number of bee colonies that have collapsed, and the types of pesticides being used in different areas. To organize the data, I used Microsoft Excel to gather/sort the data and create tables that helped me inspect the results. On top of the USDA data, I also reviewed some scientific studies to better understand how pesticides/insecticides like neonicotinoids and organophosphates affect honeybee health (Goulson et al., 2015; Potts et al., 2010).

I searched the USDA Quick Stats database for information on honeybee colony losses and pesticide use from the past several years. I focused on three states with high agricultural activity since they tend to use more pesticides, and two states with lower

agricultural activity as a comparison variable. After collecting the data, I sorted it into categories based on the year, location, quarter of year, and pesticide application. I then used the USGS website for further pesticide data exploration and utilized Microsoft Excel to create visuals to see if there was a pattern between increased pesticide use and higher rates of colony collapse disorder (CCD). Once the data was organized, I compared my findings with scientific studies that explain how pesticides impact honeybees. Research shows that pesticides can weaken bees' immune systems and make it harder for them to navigate back to their hives. Or just like my research topic, can even lead to the collapse of honeybee colonies (Tosi et al., 2018; VanEngelsdorp et al., 2009). This research highlights why tracking pesticide use is important. As well as how better regulations could help protect our bees in the future.

RESULTS

The data that was collected from the contrasting states shows a correlation between the amount of pesticides used and the number of Colony Collapse Disorder (CCD) cases in honeybee hives. States with high pesticide usage tend to have higher CCD case numbers. For example, California reported 25,040 CCD cases, with 30,640,035 acres treated with pesticides. Likewise, Iowa experienced 13,570 CCD cases, with 54,926,905 acres of pesticide use.



As shown in Figure 1, pesticide application varies significantly by state, with Iowa and California reporting the highest amounts.

On the contrary, Connecticut showed only 870 CCD cases, with just 130,220 acres of pesticides applied. This suggests that areas with less pesticide usage may have much fewer CCD cases. Across all of the states, insecticides were one of the most commonly used types of pesticide. Which could contribute to the higher rates of CCD in those regions.
Overall, the findings suggest that the increased pesticide usage, like insecticides, is linked to higher CCD rates in honeybee populations across the different states.

DISCUSSION

The results of this study support the hypothesis that pesticide exposure plays a significant role in the occurrence of Colony Collapse Disorder. The states with higher pesticide usage like California and Iowa, have much higher CCD case numbers compared to states like Connecticut, which uses much less pesticides. This pattern aligns with previous research that suggests pesticides negatively affect and weaken honeybees' immune systems. As well as hindering their ability to find their way back to their home hive (Tosi et al., 2018).

One reason for this could be that insecticides in a way directly affect a honeybees' nervous systems. This in turn makes it far more difficult for them to find their way back to the hive. Over time, this can lead to the collapse of entire honeybee colonies. Even worse, the chronic exposure to pesticides can also make bees more susceptible to catching diseases. Which even further contributes to that of CCD for the hive (VanEngelsdorp et al., 2009).

This study shows the importance of reducing pesticide use and finding much safer alternatives in order to guard and save honeybee populations. If these trends continue, we might see even more vast declines in honeybee numbers. This could in turn have some major consequences for agriculture and the environment as a whole. More research and stricter pesticide regulations could help prevent any further harm to bee populations.

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Extracting Natural Dyes from The Mulberry Tree and Comparing the Dyes on Wool and Cotton Yarn

Karisa Hernandez

ABSTRACT

There has been an increase in the usage of natural dyes for textile products. Synthetic dyes have harmful chemicals in them that do produce very vibrant colors but are harmful to the environment and humans. Increasing the variety of natural dye products help makes the extraction and dying process of textile products greener. The purpose of the study was to extract dye colorants from different parts of mulberry trees and see the difference in how wool and cotton hold the color extracts for future use. The extracts were taken from 3 different parts of the mulberry tree: the leaves, bark, and roots. The extraction process also incorporated two different solvents (water and ethanol) to see if there was a difference in the color that was extracted. The leaves extract had darker extracts compared to the bark and roots. The use of both solvents did bring out different colors for each part of the tree. The usage of wool and cotton yarn was to compare how well protein and carbohydrates absorbed natural dyes since they are commonly used in clothing. The wool yarn looked to have taken in more of the colors from the dye extracts than the cotton yarn.

Keywords: *Natural Dyes, Mullberry Tree, Wool, Cotton, and Extractions*

INTRODUCTION

Dyes have been around for thousands of years and have recently been a huge topic in the science world. As many people know, long ago, these dyes came naturally mainly from plants, insects, and minerals. Natural dyes have been used to color clothing, food, different textile products and many other things. (Mansour, Yusuf 2018) Natural dyes have recently been studied more because they are more eco-friendly compared to synthetic dyes. Synthetic dyes are coloring agents that were chemically made, which produced a quicker way to dye products like textiles and to achieve the desired color wanted.

Recent research has been done that declares that the chemicals in synthetic dyes can be harmful to the environment and to people. Certain dye colors in foods have also been shown to have health risks and cause skin irritations to people. (Joshi, Kuriyal 2023)

There is a wide variety of different textile products and materials that are used in the process of using coloring agents. Some common types of materials that are dyed in assorted colors are wool and cotton yarn. Wool yarn is mainly made of protein, while cotton yarn is mainly made of cellulose. Wool is made from sheep and the fleece of their coat, while cotton yarn is made from the collection from cotton plants and all the impurities extracted from the cotton.

Mulberry trees come from the (*Morus)* genus. They are native to North America and Asia. (Britannica 2024) The mulberry trees do produce multiple color berries such as white, red, and black berries. They are a very popular food for silkworms. (Zhu L. 2014) The leaves also do provide good sources of protein, minerals, and fibers.

With the research of finding alternatives to natural dyes, there are so many articles about extracting pigments and dyes from plants and fruits. The goal of this research is to observe how the difference between wool and cotton yarn holds the pigments from different parts of the mulberry tree and to observe the colors that were extracted from each part. The mulberry berries have already been experimented with dyeing textile products. According to Padma (Vankar 2000), most of the time red colorants are found in the bark and roots of the mulberry tree. This red color comes from the pigment anthocyanin (Fig. 3) or known as anthraquinone dyes. In the mulberry leaves, the two major colorant materials found in the leaves are lutein (Fig. 1) and beta-carotene (Fig. 2).

Figure 1. Lutein structure

Figure 2. Beta-carotene structure

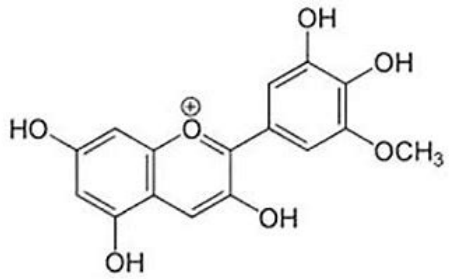

Figure 3. Anthocyanin structure

Dyeing textile products has been a method used for many years. Using plants and other options are more eco-friendly than using chemicals to dye. When dyeing fabrics and textile products, the rest of the plant goes to waste from not being used. Finding other color sources from things like roots or bark can make the whole plant more beneficial and useful. This leads to having a greener and safer process of dyeing materials and fabrics.

Mordants are substances that are incorporated in the process of dyeing fabrics and textiles because it allows the colors to get fixed on to the fabrics. Without mordants, there's a greater chance of the colorant fading and not fixing to the fabric. The mordants bond with the dyes which allows for better attachment of dye. The mordants used in the experiment were alum, ferrous sulfate, and tannic acid. They all help the dye attach to the fabric better, yet the different mordants do give off their own color and affect the results of the brightness and fastness of the dyes (Rohan 2024).

This study presents the exploration of extracting natural dyes from the leaves, bark, and roots of the Mulberry tree. The natural dyes will be tested on wool and cotton yarn material to see what colors come from the different parts and to test the color fastness of the natural dyes during the process.

MATERIALS AND METHODS

The leaves and bark from the mulberry tree were collected from a McPherson College professor's land located just outside of Mcpherson, Kansas. The roots were provided by another professor at McPherson College who had a newer growing mulberry tree which had easier access to the roots. A total of 61.41 g of leaves were picked. They were placed in a fridge once they were picked to keep them maintained. The bark and roots were then tested around the same time.

The mordants, which are substances that are used to set dyes onto the fabric to help the color stay in the fabric, and the equipment used during the experiment were available at McPherson College. The wool and cotton fabric that the dyes are tested on were purchased from amazon.

Extraction process. The leaves were washed to make sure that they were clean. They are dried and cut into small pieces and weighed for the total amount of leaves there are. The leaves were separated into 51.39 g and 10.02 g. The 51.39 g of leaves were placed into a 1000 ml hot water bath. It was boiled for an hour at 100°C and filtered out once cooled. The other 10.02 g was placed into 500 ml of ethanol and left to soak in the fridge. After an hour, the leave extract and ethanol were filtered into another clean beaker.

The bark from the mulberry tree has a similar extraction process like the leaf's extraction. 30 grams of bark were collected. The bark was cut into smaller pieces for the process. The bark is then placed into a Soxhlet apparatus to complete the extraction of the dye from the bark. 2 extractions were done, splitting the grams into two 15 g weights so that one extraction has water as the solvent and the other solvent would be ethanol. Each of the two liquids were collected into separate flasks. The extraction process was done by having the Soxhlet apparatus set up. The material was placed in the thimble and 1000ml of the solvents were placed into the round bottom flask at the bottom of the apparatus. The extractions were run for about 5-6 hours as referred to (Murani et. al. 2020) Soxhlet experiment.

The root of the mulberry tree was the last sample tested during the experiment. One of the science professors at McPherson College was able to provide a sample of roots from her mulberry tree that is still in a big pot. The roots extraction will be like the process of the bark extraction. The roots will be cut into smaller pieces. The powder is then placed and extracted using the Soxhlet extraction process. The roots weighed out to about 15 grams. They were also separated into 2 extractions to use ethanol and water as solvents. The process took about 5-6 hours in the extraction process. (Murani et. al. 2020)

Dyeing process. Once the extracts are collected, the dyes were used to dye pieces of fabric to observe what color comes out and how well the color can stay in the fabric. The extracts from the leaves, bark, and roots are all placed in beakers. Each mordant will have 2 containers, one that has wool and the other which contains cotton yarn. So, there will be 6 containers of material that consists of wool and alum, wool and tannic acid, wool and ferrous sulfate, cotton and alum, cotton and tannic acid, and cotton and ferrous sulfate. The mordants that are used and tested are tannic acid, ferrous sulfate, and aluminum sulfate. The amount of mordant used was 2.81 g of each mordant. 200 ml of water was mixed with the mordant. It was brought to a boil and dissolved in the water. The fabrics were soaked in heated tannic acid, ferrous sulfate, and alum for about 1 hour. Once the fabric had been soaked, it was rinsed in water about the same temperature as the fabric at the time. They were set to the side to air dry.

The process for dyeing each of the pieces of wool is the same for each extract. 5 ml of DI water was put

into a breaker as well as 50 ml of the dye extracts. About 5 grams of wool were weighed out and divided roughly into 6 groups. Each group of wool with the mordants was then split to have around .90 g of the material to get dyed in each of the extractions. The dyeing process for all the material is essentially done the same. The fabric is added to 55 ml of the extract mixture and heated for about an hour. After the fabrics are done dyeing, the pieces will then be washed and dried to determine whether the color was able to stay stuck to the fabric and what color was extracted from the parts of the tree.

RESULTS

The first extraction that was made was the leaves collected from the mulberry tree. Two different extractions were made to the leaves. One process was done with allowing the leaves to be boiled in D.I water, and the other process was done with the leaves soaking in ethanol in a cold temperature. The color that came from the water extraction was a dark brown/reddish color, while the ethanol extraction was a vibrant green.

The extraction of the bark was done a bit differently, where the Soxhlet extraction was used. The extraction was done twice. The bark was split in half by weight and one extraction was done with ethanol as the solvent and the other extraction was done with water as the solvents. The bark with ethanol extracted more of a cloudy orange color, while the bark and water extract were a clearer orange color.

The extraction of the roots was done the same exact way with both solvents being used. The roots and ethanol extract have more of a yellow tint to it almost like a pastel/light yellow, while the roots and water extract had more or an orange tint just a little lighter than the bark and water extract.

For the dyeing process, each extraction liquid was used on both pieces of wool and cotton. The wool and cotton material were dyed in different ways. One way was that they were both placed in only the dye extracts themselves. Another way was they were placed in 3 different mordant solutions (ferrous sulfate, alum sulfate, and tannic acid) and then placed in the dye extracts once they dried from the mordant solutions. The wool materials in each of the extracts observed looked a little brighter (Fig. 4), while I think the cotton material collected more of a darker and dull color from the extract (Fig. 5). I will say that the wool absorbed the green extract from the leaves and ethanol a lot better than the cotton yarn. I do believe that the mordants helped the yarns attain more of the colors compared to the yarn that was only submerged in the extract themselves. Over time the bare yarn in the extracts lost the color in them and didn't last very long.

Figure 4. The dyed wool yarn after washing and air drying. Top left are the roots and solvents product, top right are the leaves and solvents product, and the bottom right are the bark and solvent product.

Figure 5. The dyes cotton yarn after washing and air drying. The top left is the leaves product, the top right is the bark product, and the bottom is the roots product.

DISCUSSION

The reason for this study was to observe what other colors the mulberry can provide with different methods and different solvents used. It was also to compare how different materials could hold the natural dye extract since wool and cotton are made up of different components. As you can see in the figures above, the cotton yarn looks to have a duller appearance compared to the wool yarn which has brighter colors in the picture. Proving my thoughts and questions, the wool material absorbed the colors from the dye better than the cotton yarn did. The wool brought out colors that were closer to the color of the liquid extract, while the cotton absorbed the color, but was duller. Green chemistry is something that is newer to the science world, but with this study scientists and chemists can use multiple parts of plants and natural things to prevent the waste of the materials not used when dyeing textile products. There are some things that

looking back at my experiment I could have altered certain things and done differently to have a different outcome to my experiment, but nonetheless I was able to use conduct an experiment that allowed me to potentially help increase the use of natural dyes over synthetic dyes.

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Evaluation of MCF-7 cancer cell cultures for contamination routes and eradication

Zoe Jerke and Carlos Jacobo

ABSTRACT

A significant challenge in cell culture research is contamination of mammalian cancer cell lines by microorganisms. Microbial contaminants impact experimental results and compromise the integrity of the studies. Sterile samples and aseptic techniques are crucial to prevent the introduction of bacteria, fungi, and mycoplasma, all of which can drastically affect cell viability. A typical cancer cell culture protocol involves maintaining cells in a controlled environment with appropriate media, temperature, and humidity. However, despite strict protocols, contamination can still occur. To address a recurring contamination issue, the existing cell culture protocols were systematically checked to determine the route of contamination. Bacterial growth was observed within the cell tissue wells and were sampled onto a variety of medias to culture the contaminating organism. Cultured isolated bacteria were gram stained and characterized using common laboratory techniques. The main contaminant isolated was then subjected to an antibiotic diffusion assay to determine its susceptibility to a range of antibiotics. Based on the results of these investigations, new and revised protocols were prescribed to clear up the contaminated cell tissue cultures and prevent future occurrences.

Keywords: *Mammalian cell culture contamination, aseptic techniques, Bacillus subtilis, bacterial eradication methods*

INTRODUCTION

Continuous cell lines are germane sources for the investigation of cellular function and understanding pathology. This type of research leads to the development of drug therapies and genetic manipulations. Mammalian cells must be cultured in a high nutrient-rich media with includes Fetal Bovine Serum (FBS) which stimulates the cells to grow at a rapid rate. FBS is derived from a bovine fetus and is widely used as an in vitro cell culture supplement (Weiskirchen et al., 2023). Dulbecco's modified eagle medium (DMEM), a standard media used to maintain and grow mammalian cells, requires 5-20% FBS supplementation for optimal cell growth. Because of its many components such as lipids, hormones, electrolytes, enzymes (proteases) and pH buffering capacity, it is also an optimal environment for contaminates such as bacteria and fungi (Weiskirchen et al., 2023). Contamination in cell tissue cultures could result in a common and fundamental issue of and should be taken into consideration as well (Aseptic Techniques and Safety in Cell Culture, 2025).

A common contaminant that appears in many cells' lines are mycoplasmas. Mycoplasmas have recently gained interest as they tend to contaminate cell lines in research as well as manufacturing bioproducts. Mycoplasmas lack a cell wall making it difficult to detect. Most antibiotics affect bacterial cell wall formation or protein production. Since mycoplasmas do not have a cell wall they express resistance to most beta lactam inhibitors. (Nikfarjam & Farzaneh, 2012). Mycoplasmas can be introduced from multiple sources including humans and animals. Fetal Bovine serum and Trypsin (Swine origin) are usual routes of laboratory contamination. Both FBS and Trypsin are essential to cell tissue culture. In the cell splitting process, trypsin is essential to detach cells from their wells. *M. arginine* and *A. laidlawii* are two common mycoplasmas found in contaminated cell cultures which originate from fetal bovine serum (FBS) or newborn bovine serum (NBS). FBS is a compound used in the cell media before the contamination originated. Human mycoplasmas M. orale, M. fermantans, and M. hominis are all common species that are found in cell culture contaminations. Other origins of contaminations may even include nonsterile supplies because of improper sterilization. misidentification and tainted research findings. This would make it difficult to compare results from various labs, reducing the efficacy of mammalian culture in research. The research affected would include stem cell therapy and the production of therapeutic products (Mahmood & Ali, 2017). Using contaminated cells puts at risk most if not all aspects of cell physiology and can lead to inaccurate results. Because of the issues that come with contaminated cell tissue it is important to use aseptic techniques in sterile environments to prevent the risk of contamination. Good lab practices (GLP) techniques include wearing gloves, long sleeve lab coats, and sterilizing biological safety cabinets (BSC) with ethanol and ultraviolet (UV) light. Although UV light is effective in eradication microorganism it is important to understand its limitations (Division of Occupational Health and Safety, 2025). Ensuring that unused sterile equipment is being utilized is important

Mycoplasmas are not the only organism that cell cultures are at risk of being contaminated with. In a research study by Mahmood & Ali, 19 contaminated cell passages (12%) were found in an examination of 32 stem and feeder cell lines the most common contaminants were gram positive cocci and Mycoplasma species, followed by gram negative and gram-positive rods (Mahmood & Ali, 2017).

There are many ways of detecting contaminations and identifying the contaminant with methods that are instrumental in cell-based therapies and in the manufacturing of recombinant protein therapeutics. Examples include STR profiling in which DNA is profiled through short tandem repeats (STRs), isoenzyme analysis, karyotyping and DNA barcoding. Testing for mycoplasmas is more difficult as they are not visible in an inverted microscope and are not usually detected until they have reached high densities, causing the cell culture to deteriorate. For mycoplasmas the method of detection is usually by testing the cell cultures periodically using fluorescent staining such as Hoechst 33258, ELISA, PCR, immunostaining, autoradiography, and microbiological assays (Mahmood & Ali, 2017).

In our original study, MCF-7 cancer cells were being cultured and prepared to test with different organic substances and cancer treatments to observe the effects of viability and gene expressions. The research was halted due to an ongoing contamination within the cell media. The antibiotic Normocin was used unsuccessfully to eradicate the contaminant. Normocin is an antibiotic designed to eradicate mycoplasma, bacterial and fungal contaminations in cell culture lines (Normocin - Antimicrobial Reagent, 2025). It was apparent that there was a breach in aseptic techniques which led to a reintroduction of contamination. Different areas were tested for possible reintroduction of contamination. The areas tested include the BSC, sterile prepackaged equipment, media filters, incubator, aspirator tubing, and the effectiveness of the UV light used to sterilize the BSC. Multiple samples were taken in each suspected area and observed for growth and further isolated to distinguish the type of bacteria that is causing the contamination. The goal of this research is to identify the source of the contamination and determine the best way to eradicate and prevent recontamination.

MATERIALS AND METHODS

Evaluation of potential sources of contamination

All potential sources of contamination were investigated to find the common infection route of the cancer cell culture. Prospective sources of contamination include the BSC, aspirator, pipette, incubator, and the media. Aseptic techniques were used while testing these sites. The media, broth, and agar plates were incubated at 37 degrees C. All media tested came from the “original” media. The original media refers to the media that was being used to maintain the previous cancer cells. The original media was split into different bottles depending on what treatments they were getting as seen in Table 1.

To test the BSC, it was sprayed down with 70% ethanol as per good lab practices for cell culturing, the UV light was then activated for 10 minutes. The nutrient agar plates were left open in the BSC for 10 minutes, with the blower on and the front glass pulled a quarter of the way up. These NA plates were incubated for 72+ hours and observed for bacterial growth.

Different surfaces that were tested in the BSC to help determine sterility included the BSC work surface, the aspirator, and the pipette. These locations were all tested using the same methods. These locations in the BSC were tested in various spots by utilizing a moist sterile swab. The areas were swabbed after the BSC was wiped down with 70% ethanol and treated with the UV light for 10 minutes. A NA plate was inoculated with the swab to determine growth. The NA plates were evaluated for growth frequently after 48+ hours.

The original media was tested by pouring the media out of the bottle straight into a 6 well plate. This media had been sitting at room temperature for one week. The media was then evaluated for bacterial growth by looking through an inverted microscope right after putting it into wells. It was checked frequently for growth after the 24-hour mark. Pouring straight from a media container typically is not the best aseptic technique. However, this had to be done to eliminate the use of the pipette. By eliminating the use of the pipette, it helped determine if the pipette was causing the contamination. 0.2 mL of the original media was also pipetted into one of the 6 wells and evaluated for growth. The bottle was sprayed down with 70% ethanol before pouring.

To ensure proper techniques, a sample of the original media was sterilized with the autoclave at 121 degrees C and 15 psi for at least 15 minutes (Princeton University, 2025). A two mL sample of media was pipetted into a 6 well plate. While some of the autoclaved media was ran through a 0.2 micrometer filter, which was also put into a 6 well plate. These samples were observed through an inverted microscope for bacterial growth directly after placing into the wells. After 24+ hours, they were evaluated frequently for growth.

A sample of the original media was also run through a single 0.2 micrometer filter. Another sample was double filtered by running the media through a 0.45 micrometer filter and then a 0.2 micrometer filter. These samples were plated into 6 well plates and viewed under an inverted microscope frequently after 24+ hours.

Lastly, a sample of the incubator water pan was

taken. 0.2 mL of the water was pipetted and then placed in nutrient broth. It was observed for growth after 24 hours.

Agar Media

The different agar mediums used to help determine what bacteria was growing in the cell cultures included Nutrient (NA), McConkey (MAC), Mannitol Salt (MSA), and Eosin Methylene Blue (EMB) agar.

NA is a medium that will grow a wide variety of microbes, including gram-positive and gram-negative bacteria. This media is non-specific and non-differential because it contains the essential nutrients to support microbial life and does not contain any inhibitors. The media contains peptone and meat extract, these will provide amino acids, vitamins, and minerals (Millipore Sigma: Nutrient Agar, 2025).

MAC media is both selective and differential. It will only grow gram-negative bacteria because it inhibits the growth of gram-positive bacteria. The gram-positive bacteria are inhibited by the bile salts and crystal violet that is in the media. The media allows differentiation of bacteria by color changes that are produced by lactose fermenting bacteria. Bacteria that can ferment lactose will turn a pink color while the non-lactose fermenters will appear white (Jung & Hoilat, 2024).

MSA media is selective and differential. The media will inhibit the growth of most bacteria that are not halotolerant. A common species that will grow on MSA is the staphylococcus species (Kampf, Lecke, Cimbal, Weist, & Rüden, 1998). A change in agar color helps differentiate bacteria by identifying if the bacteria can ferment mannitol or not. If the agar changes yellow around a colony, it is capable of fermenting mannitol. The bacteria are not capable of fermenting mannitol if the agar stays red (Sharp & Searcy, 2006). MSA has a salt concentration of 7.5% which makes it selective for bacteria that are halotolerant (Kamf et al., 1998).

EMB media is selective and differential. It will only grow gram-negative bacteria. The gram-positive bacteria are inhibited by the presence of eosin and methylene blue in the media. Differences in the color of the colonies can help differentiate the bacteria's ability to ferment lactose or not. If the bacteria can ferment lactose, the colony will appear a metallic green. Weaker lactose fermenters will be pink/purple while non-lactose fermenters will be colorless (Eosin-Metylene Blue (EMB) Agar, 2007).

Tryptic Soy Blood Agar (TSA blood) is used to help differentiate different types of bacteria. This agar is the previous mentioned TSA but with sheep blood added in. The goal of this agar is to help identify what kind of hemolysis the bacteria is capable of. Some bacteria can release hemolysins, an enzyme that partially lyses red blood cells or fully lyses blood cells in the agar around the colonies. There are four types of results that can be observed using TSA blood plates: alpha-hemolysis, beta-hemolysis, gamma-hemolysis, or alpha prime-hemolysis. Alpha-hemolysis is characterized by green/brown discoloration around colonies because of the partial lysis of the red blood cells. Beta-hemolysis will be shown by having a clear zone around the colony representing complete lysis of red blood cells. Gamma-hemolysis is no lysis of the red blood cells so there is no change in color or zone of hemolysis. If the plated bacteria performs alpha prime hemolysis, there will be intact red blood cells right next to the colony but then there will be complete lysis right outside of the intact red blood cells (Sapkota, Blood Agar- Composition, Principle, Preparation, Uses and Hemolysis, 2022).

Isolation Methods

Bacterial communities are difficult to characterize and culture with traditional methods, so the dominant isolate was purified and evaluated. To identify the bacteria present, a 0.2 mL sample was taken from a media well that appeared to have the most bacteria. The 0.2 mL sample was lawn spread on a nutrient agar plate and was placed in the incubator until there was sufficient growth. After 24 hours, a sterile inoculation loop was used to pick up a small sample of bacteria and was placed onto a new nutrient agar plate using the streak plate method and incubated for another 24 hours. After incubation and sufficient growth of the streak plate, a selected individual colony was observed by doing a gram stain. Once the gram stain showed that it was a pure colony, it was put into nutrient broth and was allowed to grow for 24 hours to be used for the following tests.

Analysis of Isolated Colony

The isolated colony that was placed into nutrient broth was then plated onto MSA, EMB, MAC, TSA blood agar. The different plates were analyzed after 48 hours of incubation. This helps identify the characteristics that the bacteria exhibit. The gram stain also helps analyze the isolated colony by showing if it is gram-negative (pink) or gram-positive (purple) and the shape of the bacteria. Another analysis performed was an antimicrobial susceptibility test and a catalase test.

Gram Stain

A gram stain allows to make morphological observations and is often used as a first step to try to identify a bacterium. To perform a gram stain on the bacteria, a procedure from Carolina Biological Supply Company was used. A drop of distilled (DI) water was placed on a clean glass slide and a sterile loop was used to inoculate a small sample of bacteria from the agar plate. The bacteria were then mixed into the water and spread thin. The slide was air dried and then fixed by waving it through the flame of a Bunsen burner three different times. The slide was flooded

with crystal violet for 60 seconds, rinsed with DI water, flooded with iodine solution for 60 seconds, and rinsed again. The slide was then decolorized by using 95% ethanol until the ethanol dripped clear. The slide was flooded with safranin for 60 seconds and rinsed again. Blotting paper was used to dry the slide and then the stained bacteria was viewed under a microscope with oil immersion (Hauser, 1986).

Antimicrobial Susceptibility Testing

To prepare the bacteria that is being tested, a bacterial colony must be isolated. The isolated colony was then put into nutrient broth for 24 hours. A Mueller-Hinton (MH) agar plate was then evenly inoculated with this broth. Antimicrobial disks were then placed evenly apart on the plate, with a maximum of 6 disks per plate. A control plate was done by using three disks saturated with water and three disks saturated with 70% ethanol. The plate was inverted and placed in the incubator for 24 hours. After 24 hours, the zones of inhibition were measured and then interpreted. The zone of inhibition was determined by where no growth was present (Bayot & Bragg, 2024). This was performed to compare the effectiveness of Normocin on the unknown bacteria compared to other common antibiotics used.

Catalase Test

The catalase test was performed to determine if the bacteria can produce catalase. This test was done by taking a sample of the isolated bacteria and dropping 3% hydrogen peroxide onto the sample. If bubbles form, it is oxidase positive and if there is no reaction it is oxidase negative (Reiner, 2010).

UV Susceptibility Testing

A final test to help evaluate the contamination at hand was observing how susceptible the bacteria was to UV light. A 0.2 mL sample of the isolated bacteria was inoculated on NA. Six different exposure times were tested: 0 min (control), 5 min, 10 min, 15 min, 30 min and 60 min. The plates were left in the BSC with the UV light on for the allotted time and then incubated for 24 hours.

RESULTS

Table 1: Sources of Contamination evaluated for growth (+/-) by microbial observation at three trials (T1, T2, T3)

Source tested	**T1**	**T2**	**T3**
Original media (poured)	+		
Original media (pipetted)	+		
Autoclaved media (original) @ room temp	-	-	
Autoclaved and filtered media (original) @ room temp	-	-	
Original media autoclaved (incubated)	+	+	
Original media autoclaved + filter (incubated)	+	+	
Aspirator tubing	-	-	-
Work surface after UV	-	-	-
Pipette	-	-	-
NA plate left in open BSC with blower on	-	-	-
0.45 µm filter original media	+		
0.45 + 0.2 µm filter original media	+		
Incubator water	+		

Table 2: Characterization of Isolated Bacteria Growth on different media

Agar Media	Result (+/-)
NA	+
EMB	-
MSA	+
MAC	-
TSA Blood agar	+

Figure 1: Gram-stain of Isolated Bacteria (1000 TM, bacteria length 4 o.u., width 1 o.u.)

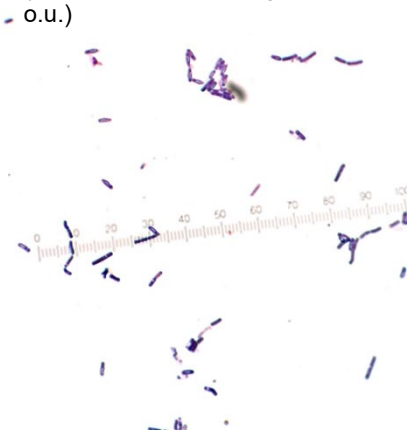


Figure 2: Isolated Bacteria on NA Showing Swarming Growth

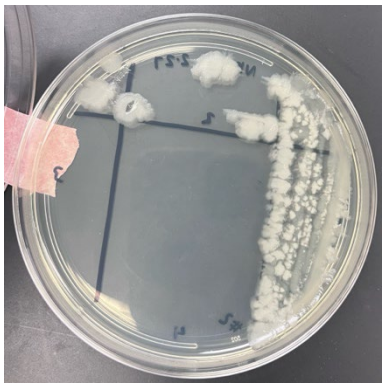


Figure 3: Isolated Bacteria on MSA Showing Negative Mannitol Fermentation but Halotolerant

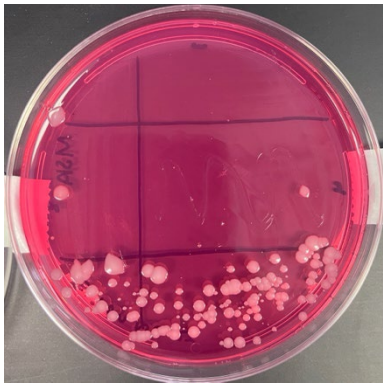


Figure 4: Isolated Bacteria on TSA Blood Demonstrating Hemolysis

Table 3: Antibiotic Disk Assay

Treatment	**Zone of Inhibition (mm)**
(N) Normocin	26
(PS) Penicillin Streptomycin	25
(C) Chloramphenicol	24
(S) Streptomycin	22
(TE) Tetracycline	20
(V) Vancomycin	17
(K) Kanamycin	12
(P) Penicillin	0
(O) Oxacillin	0
(Amp) Ampicillin	0

Figure 5: Antibiotic Disk Assay

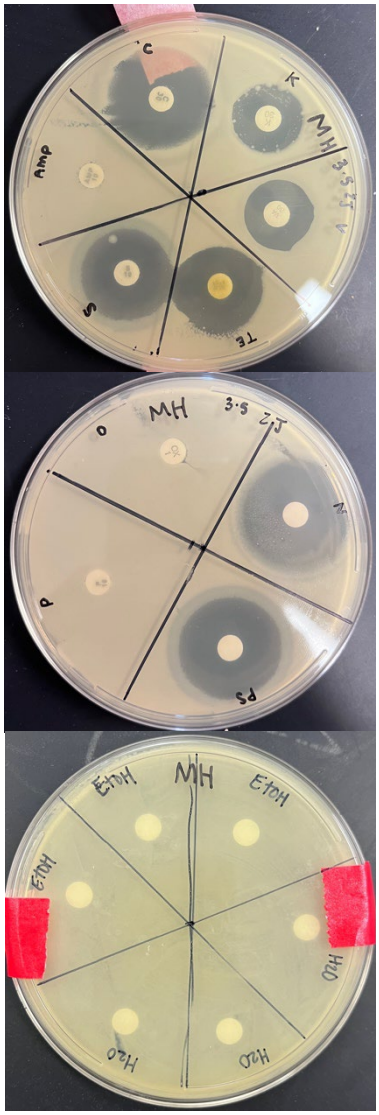
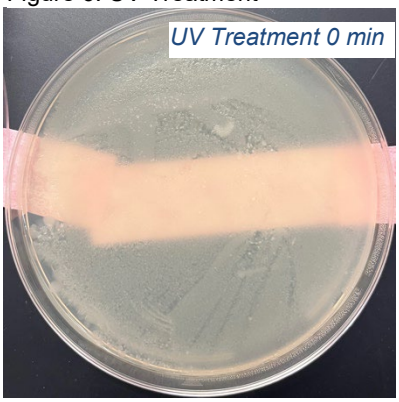
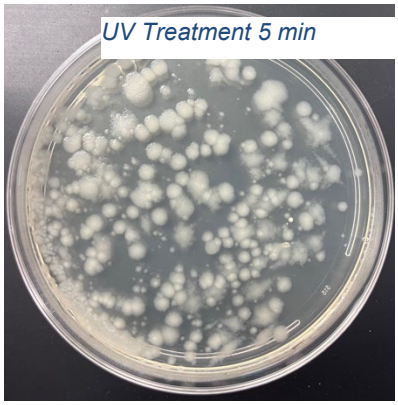
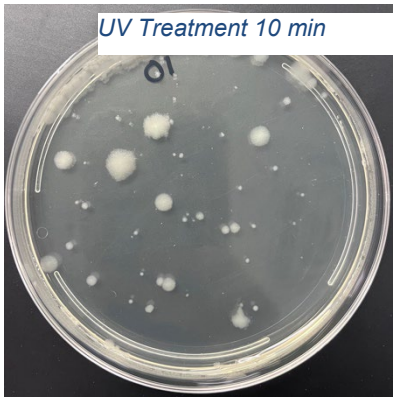
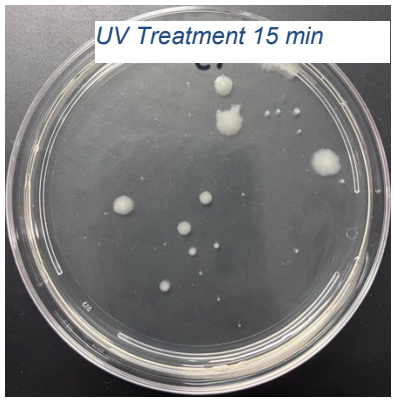


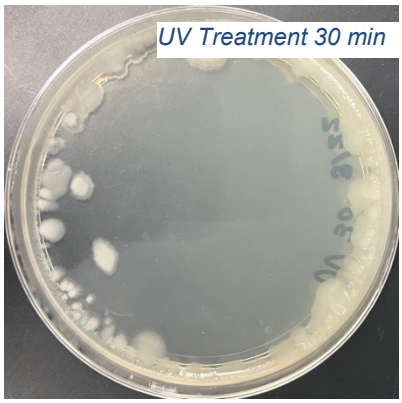
Figure 6: UV Treatment

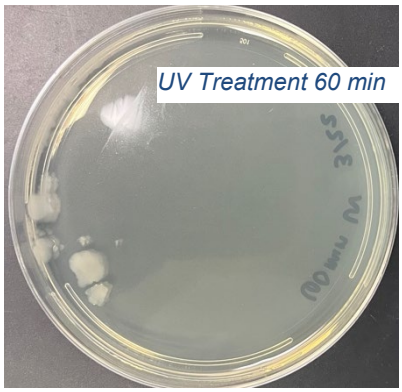












DISCUSSION

Identification of Bacteria and Route of Contamination

Several areas were tested for possible routes of contamination including the media, aspirator, filter, surfaces of the cell culture BSC, incubator water pan, and the electronic pipette. How each possible route was tested can be found in the methods section.

Table 1 shows that the original media that was poured and pipetted into a 6 plate well showed growth. This helps eliminate that the pipette was not contaminating the media when being transferred. The autoclaved media and the autoclaved media + filter showed no growth after viewing with an inverted microscope directly after autoclaving and filtering. After 48 hours of incubation, there was growth in both the autoclaved media and the autoclaved + filter media. In theory, the autoclaved media should have remained sterile, meaning that the bacteria are being introduced in the process of being transferred into the wells or after being transferred into the wells. One of the first thoughts was that the BSC could possibly not be maintaining sterility. To test if the BSC was maintaining a sterile environment, NA plates were left in the open BSC with the blower on. With the three NA plates coming back negative for growth, it helped eliminate the BSC as being the route of contamination. To further test the BSC, surfaces in the BSC were

swabbed. The different surfaces in the BSC includes the pipette, aspirator tubing and the work surface. All these surfaces were negative for growth after 72+ hours of incubation. A double filter was used in hopes to eliminate bacteria in the original media that was not being eliminated with a single filter. With the use of a double filter, the media was still contaminated after being put in the incubator for 72+ hours. Between all these routes, a common route that was not tested yet was the incubator. It would not be a surprise if the incubator had bacteria in it considering it is open to a nonsterile environment every time the door is open. A water sample from the water pan in the incubator was taken and placed into nutrient broth. After 24 hours, there was bacteria growth in the nutrient broth.

The isolated colony is shown in Figure 2. Due to the way the bacteria started to swarm the plate; it helped show that the bacteria were highly motile due to having flagella. The gram stain (Figure 1) showed that the bacteria were rod shaped and gram-positive because the cell wall was stained purple. There were also spores in the isolated colony meaning that the bacteria can produce spores. The bacteria were measured using ocular units during the gram stain. The total magnification was 1,000x which means that one ocular unit is equal to one micrometer. The bacteria length was 1 micrometer, and the width was 4 micrometers. The spore was measured as 0.8 micrometers by 1.5 micrometers. The isolated bacteria were plated onto EMB, MSA, MAC, and TSA blood agar. As suspected, there was no growth on the EMB agar as this media inhibits the growth of gram-positive bacteria (Eosin-Metylene Blue (EMB) Agar, 2007). There was also no growth on the MAC agar because it will also only grow gram-negative bacteria (Jung & Hoilat, 2024). However, there was growth on the TSA blood agar and the MSA agar plate. The growth on the MSA plate (Figure 3) shows that the isolated bacteria can grow in an environment that has a 7% NaCl concentration. The agar around the bacteria remained red which shows that the bacteria is not capable of fermenting mannitol (Sharp & Searcy, 2006). Growth on the TSA blood agar (Figure 4) showed a clear zone around the colonies which shows that the bacteria isolated is capable of beta-hemolysis (Sapkota, Blood Agar- Composition, Principle, Preparation, Uses and Hemolysis, 2022). A catalase test was performed to determine if the bacteria produce catalase enzyme. When the test was performed there was bubbling present which means that it is catalase positive (Sapkota, Catalase Test-Principle, Procedure, Types, Results, Uses, 2022).

The highlighting characteristics of the isolated bacteria include the following: gram-positive, rod shaped, flagellated, spore producing, catalase positive, beta-hemolytic, and halotolerant. The isolated bacteria characterization is consistent with *Bacillus subtilis* (*B. subtilis*) according to multiple sources, including The Bergey's Manual (Breed et al., 1957; Sapkota, *Bacillus subtilis*- 2022; Errington & Aart, 2020). The Bergey's Manual states that *B. subtilis* will grow in a 7% NaCl environment, with limited growth at 10% NaCl (Breed et al., 1957). However, our results did not show that the bacteria were mannitol fermenting. With reading The Bergey's Manual, it was noted that *B. subtilis* has many different variants which gives a controversial aspect to some of the known characteristics of *B. subtilis* (Breed et al., 1957). This could potentially explain why our sample was negative for mannitol fermentation.

The antibiotic disk diffusion assay (Table 3 and Figure 5) was used to help determine if Normocin inhibited growth of this bacteria. It expressed the largest zone of inhibition compared to the other antibiotics tested. This shows that it should have worked to eliminate the growth in the cell culture in the previous research. Additionally, the use of 70% ethanol was not effective against the isolated bacteria.

The results of the UV light exposure times show how beneficial the UV light can be if used for long enough. As seen in Figure 6, bacterial growth is taking control over the plate at 5 minutes of exposure. The growth can be limited when used for 60 minutes. However, this would not be efficient because it is likely to burn out quickly if used for 60 minutes each time the BSC is utilized. As seen in Figure 6, the edges of the plate contain the most growth, this is because the side of the petri dish is blocking the UV rays from treating that area. This shows that the BSC should be decluttered when treated with UV and should be used in combination with another disinfectant (Division of Occupational Health and Safety, 2025).

It is problamatic that the 70% ethanol and the 10 minute treatment time with UV light did not work to eradicate the bacteria. This is concerning because that is what were were doing to sterilize the BSC before working in it each time.

Quality control

While a definitive route was not determined by the research conducted, a definite breach of aseptic technique was determined. There are several precautions that should be taken. This includes the use of personal protective equipment (PPE), keeping a clean environment where research is being conducted, properly disinfecting surfaces, and testing cells for contaminants on a regular basis (Stumpf & Eaton, 2010). The use of PPE will not only help keep the researcher safe from hazardous materials but also helps reduce the risk of contamination to the cell line. PPE that should be implemented while working with mammalian cells should include clean laboratory coats, gloves, face shields, and safety glasses (Aseptic Techniques and Safety in Cell Culture, 2025). The PPE used in the cell culture lab should not be used in other labs.

The cell culture BSC should be in an area where it is free from drafts and through traffic (Aseptic Techniques and Safety in Cell Culture, 2025). Any drafts or traffic can comprise sterility of the BSC by disrupting the laminar flow current. By disrupting the laminar flow, airborne contaminants can enter the cell culture BSC and create routes of contaminations. Another source of draft that could comprise sterility includes talking, laughing, or coughing while working in the BSC. These actions should be kept to a minimum. If one must do any of those, they should turn away from the BSC (Aseptic Techniques and Safety in Cell Culture, 2025). As of spring of 2025, the cell culture BSC that is used at McPherson College is set up in a lab room that is not used by many students. However, there is through traffic due to a door that leads to the green house that is in this research lab. There is another door that leads to the green house in the biology lab that could be used as well. A suggestion would be to only use the door in the biology lab to access the green house. This would help eliminate unnecessary traffic through the research lab. Another suggestion to help reduce drafts would be to keep the door closed in between the microbiology room and the research lab and limit the use of the door to go in between the two. The door leading from the hallway into the research lab could be used instead to access either of these labs.

To keep the BSC a sterile environment, it must be kept clean and free of clutter. The only items that should be kept in the BSC include the materials needed for your research. The materials that are in there should be kept clear of the vents in the back and front of the BSC to promote good airflow (Aseptic Techniques and Safety in Cell Culture, 2025). Before conducting research in the BSC, sterilization of the BSC should be done. It is suggested to spray the BSC surfaces down with 70% ethanol and then the UV light should be left on for 20+ minutes. The BSC should be left open with the blower on for 20 minutes before putting anything in the BSC. All materials entering the BSC should be sprayed with 70% ethanol and wiped down with lint free wipes (Cell Culture Contamination Troubleshooting, 2025) (Division of Occupational Health and Safety, 2025). Keeping the lab clean that contains the BSC is crucial as well. This would include sweeping the floors, disinfecting counters, and keeping it clutter free. Cleaning the lab should occur one to two times a week. In addition, refrigerators and incubators should be cleaned frequently (Life Technologies, 2025). Incubators are not sterile environments due to the door being opened which exposes it to the outside environment. A typical room has 100-1,000 microorganisms per cubic meter (Bates, 2016). However, our incubator is in the microbiology room which is highly likely to have a greater number in diversity of microorganisms per cubic meter. To help keep contamination out of the cell cultures, the incubator should be cleaned once a week with a disinfectant that won't cause harm to the cells. 70% ethanol can be used to clean the humidity pan, chamber, and shelves. When it is cleaned, the humidity pan water should be changed out each time. The outside of the incubator should also be wiped down regularly (Bates, 2016). Another suggestion that could be helpful in maintaining the sterility of the cell cultures would be to move the cell culture incubator out of the microbiology lab and into the same room as the cell culture BSC.

Bacillus subtilis

When Gram-positive *Bacillales* and other closely related genera begin to slow their growth rate because of depletion of nutrients or other environmental factors, they initiate the process of sporulation. Sporulation is the morphological process that gram-positive bacteria partake in ultimately turning themselves into a dormant cell that is resistant to severe environmental conditions (Rodriguez et al., 2023). Because of the spore's lack of metabolic activity, it allows itself to survive in these harsh environments for a prolonged amount of time. Endospores can live in this state for years or even decades. The spores' layers and core characteristics allow extensive resistance to a large range of temperatures, pressure, ultraviolet radiation, and many harmful chemical substances such as hypochlorite and aldehydes. In addition, bacteria with motility by flagella can lead to biofilm formation and antibiotic resistance promotion. The stimuli that initiate bacteria to partake in sporulation is unknown but some characterized signals that initiate sporulation include starvation, a viral attack, or abrupt oxygen change (Rodriguez et al., 2023).

DMEM media used for tissue culturing is full of proteins and nutrients needed for rapid cell growth. This in turn, also makes it a prominent media for bacteria and their spores (Mahmood & Ali, 2017). Filtering is a technique used to sterilize any media that may be contaminated. It is an alternative to autoclaving, which is not recommended for cell media. Autoclaving cell media can degrade its biological components making it ineffective. (Sterile Lab Media Filtration and Cultureware, 2025). Although this technique should theoretically filter all organisms and spores, there have been instances noted where spores and other organisms are identified even after filtering. In an online discussion board, Lyna M'Rabet from the University of Veterinary Medicine Hannover, states that after filtering her cell-free supernatant bacterial culture in a 0.2 micrometer filter, she is still not able to get rid of the spore contamination (Research Gate, 2022). Similar results have been observed in this discussion. After double filtering with a 0.45 and 0.2 micrometer filter, contamination persisted in the media.

Although spores can build resistance to common sterilization techniques, they can still be eradicated with extreme conditions. The most popular techniques being UV radiation, gamma-radiation, dry heat, wet heat, chemicals (oxidizing agents, aldehydes, alkali disinfectants), plasma, and germinants which is the induction of germination with enzymes, nutrients, or heat and pressure. These sterilization techniques can damage different components of the spore including DNA, inner membrane, and proteins within the spore core. Although these are all effective treatments for spore eradication, they only work at extremely high and harsh conditions (Rodriguez et al., 2023). These methods may not be beneficial in sterilizing mammalian cell cultures as they will also be damaged.

Nonculturable bacteria

Growth of bacterial colonies on nutrient agars is expected to be observed within 24-72 hours of when the sample is first inoculated. During this process, growth was not observed on the nutrient agars when samples were taken from the cell media even though there were clear signs of viable bacteria living within it. When put under stress many bacteria including human pathogens respond back to their environmental cues by entering a state where the cells remain viable but no longer culturable on standard laboratory media (Oliver, 2010). Viable but nonculturable (VBNC) bacteria can be induced in many ways including: nutrient starvation, incubation outside the normal temperature range of growth, elevated or lowered osmotic concentrations, oxygen concentrations, and even exposure to white light (Oliver, 2010). After several weeks of working with these bacteria, the media samples stopped growing on agar plates. It was concluded that the bacteria could have entered a VBNC state. In addition to not being culturable, VBNC bacteria can also be non-responsive to antibiotics. Research has shown a threefold increase in DAP-DAP cross-linking, an increase in mucopeptides bearing a covalently bound lipoprotein, and a shortening of the average length of glycan strands (Signoretto et al., 2002). These results are abnormal when compared to exponentially growing cells. In a study by Del Mar Lleo et al., the use of antibiotics acting on peptidoglycan or protein synthesis was evaluated on *Enterococcus faecalis* in the VBNC state and showed that many beta-lactams can inhibit the resuscitation of VBNC cells. Other antibiotics, such as Vancomycin, was completely ineffective in regards of blocking the resuscitation of bacteria in the VBNC state. This insensitivity to antibiotics occurs even when Vancomycin is used at 100 times its minimum inhibitory concentration (MIC). The authors believe this insensitivity may be because of the cell's insufficient synthesis of D-ala-D-ala, which is the specific target of Vancomycin (Lleò et al., 2007). Vancomycin showed to only be effective when used at 500 times the MIC (Oliver, 2010). Although antibiotics may be effective at excessively high doses, this is not a solution to mammalian cell culture contaminations. Excessive treatment of tissue culture with a standard dose of antibiotics may have unwanted effects to mammalian cell cultures, causing cytotoxic and cytostatic activity, even altering the biological patterns of cultured mammalian cells (Nazihahasma and Ahmad, 2020).

Final thoughts

The final origin of contamination was not able to be determined. The different sources that were suspected include: the BSC, incubator, filters, UV light malfunction, pipette gun, aspirator tubing, and poor sterile techniques. It can be ruled out that the contamination did not come from the BSC as it was recently serviced. When autoclaved and filtered the media appears to be sterile with no debris or contamination until it is incubated. Once incubated a contamination appears within the media. It is unknown whether the contamination is because of the incubator, the incubator water or from a faulty carbon dioxide filter which may also carry contaminants into the media. It can be determined that neither of those sites could be sources of contamination since a sealed bottle filled with autoclaved and autoclaved/filtered media still appear with a contamination after being incubated. The samples taken from the aspirator tubing all showed to be negative when plated on NA, as well as the BSC when treated with UV for 10 minutes and the pipette gun is also negative on NA. This leaves sterile techniques and placement of equipment such as the incubator and the BSC as the possible route of contamination. It was determined that the bacteria most likely contaminated the media outside of the BSC and was able to get through the filter. Being able to go through the filter was most likely attributed to the spores that *B. subtilis* produce. With these spores, they are also known to be resistant to multiple treatment options. Eradication and a definitive route were not concluded due to time limitations. Progress was made in developing a plan for future cell studies at McPherson College.

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Analyzing the estrogenicity of common ingredients in chemical hair relaxers using *Caenorhabditis elegans*

Jayla Moore

ABSTRACT

Exposure to endocrine-disrupting chemicals (EDC) causes overstimulation of estrogen receptors, which over time, may increase the risk of cancer. *Caenorhabditis elegans,* who have been established as a model organism for reproductive toxicity studies, possess a nuclear hormone receptor that functions similarly to the human estrogen receptor. In this study, C. *Elegans* were exposed to three suspected EDCs found in hair straightening products, methyl paraben, diethyl phthalate, and octamethylcyclotetrasiloxane, for 72 hours to assess their effects on reproductive gene expression. mRNA was extracted using the Trizol method, converted to cDNA, and then analyzed via RT-qPCR to quantify differences in expression relative to the controls. Understanding how these chemicals influence hormonal signaling pathways may provide broader insight into cancer disparities, as Black women have higher exposure to EDCs and a high prevalence of malignant endometrial cancers.

Keywords: *Endocrine disruption, methyl paraben, diethyl phthalate, octamethylcyclotetrasiloxane, RT-qPCR*

INTRODUCTION

Cancer is characterized by rapid uncontrolled cell growth by genetic mutations in various cellular pathways. Hanahan summarized these characteristics as the 6 hallmarks of cancer (Hanahan, 2000). These hallmarks include self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptotic pathways, limitless replicative potential, angiogenesis, and metastasis. The physiology of the female reproductive system provides an environment that makes it relatively easy for cells to develop the hallmarks. Increased estrogen levels during certain phases of the menstrual cycle induce rapid cell growth, which can mimic cancerous growth. Both breast and uterine cancer are considered to be estrogen-dependent because these tissues are the primary target for estrogen signaling pathways (Rutkowska *et al*., 2016).

Uterine cancer, specifically, accounts for 4% of all cancers in women worldwide (Allen *et al.*, 2008) and is the most common type of gynecological cancer in the U.S. (Chatterjee *et al.*, 2016). The Center for Disease Control (CDC) reports that both incidence and mortality rates in the United States are increasing annually at 0.7% and 1.1%, respectively. These statistics are decreasing for almost every other type of cancer (Henley *et al.,* 2018). It is evident that non-Hispanic Black women are disproportionately affected by more invasive forms of uterine cancers. When compared to their White counterparts, Black women were 1.9 times more likely to be diagnosed with clear cell tumors, 2.4 times more likely to be diagnosed with malignant mixed Mullerian tumors, and 2.19 times as likely to be diagnosed with serous tumors. The mortality rate for Black women with these tumor types were all at least twice the rate for White women with the same diagnosis. Incidence and mortality rates for Hispanic and Asian women were found to be equal or less than those of White women (Cote *et al.*, 2015). While studies investigating potential genetic causes are important, we must also analyze epidemiologic factors. Black women tend to have curlier hair that requires more maintenance. Some women may opt to straighten their curls, which may be a style preference or simply more convenient for their lifestyle. Hair can be straightened by heat with a flat iron or by alkaline chemical treatments. Flat irons only temporarily straighten the hair, and the strands usually revert to their original state after being exposed to water. Chemical hair relaxers, on the other hand, provide a semi-permanent alternative that can be exposed to water. They work by breaking the disulfide bonds of the keratin fibers in hair strands. The more disulfide bonds in someone's hair, the curlier their hair is. Thus, the goal of a chemical relaxer is to denature the keratin by breaking the disulfide bonds with a high pH solution, causing the hair to lose its curl. The industry for chemical hair relaxers is valued at 719.88 million USD as of 2024. Despite growing concerns about the safety of these products, the value is expected to reach 889.83 million USD by 2029 (AMA Research, 2024).

These relaxers contain numerous compounds that are either known or suspected endocrine-disrupting chemicals (EDC's) (Helm *et al.,* 2018). Hair relaxers contain octamethylcyclotetrasiloxane (D4), which has been shown to increase estrogen-receptor alpha (ERα) activity in mice (He *et al.,* 2003). Rats exposed to methyl paraben and ethyl paraben exhibited abnormally high expression of estrogen-responsive genes (Sun *et al.,* 2016). Diethyl phthalate doesn't bind directly to ERα, but still causes phosphorylation of the receptor that triggers the same response as if estrogen was bound to the receptor (Fiocchetti *et al.,* 2021). All of these compounds can be found in

chemical relaxers, commonly disguised in the ingredients list as just fragrance. The main ingredients of the relaxers, such as petroleum, mineral oil, calcium hydroxide, sodium hydroxide, and guanidine carbonate, have little to no evidence suggesting they may be endocrine disruptors. However, since estrogen levels range from only 30-400 pg/mL throughout the life of the average premenopausal female, miniscule amounts of EDC's can have large impacts. A meta-analysis performed by the National Institute of Environmental Health Science found that women who reported frequent use of straightening products were more than twice as likely to develop uterine cancer (Chang *et al.,* 2022). This study raises concern about the role of hair relaxers in the onset of hormone-dependent cancers, such as uterine cancer.

The hormonal activity of other hair products marketed towards Black women, such as leave-in conditioners, hair oils, root stimulators, and hair lotions have already been examined and shown to be hormonally active in some way (James-Todd *et al.,* 2021). However, these studies haven't included relaxers. The ingredients of these products have the ability to enter the body relatively easily because they are often placed on the scalp, which has a higher absorption than skin on the rest of the body, due to the larger amount of hair follicles that allow penetration. Additionally, relaxers must be extremely alkaline in order to serve their purpose, having pH values over 12 (Sishi, van Wyk, and Khumalo, 2019). This corrosive nature often causes scalp lesions that provide additional entry into the body for EDC's.

As previously mentioned, uterine cancer is associated with high levels of unopposed estrogen. ERα is a ligand-gated transcription factor that, once activated, interacts directly with the genome to stimulate cell division (Rodriguez *et al,.* 2019). Estrogen levels peak during the follicular phase of the menstrual cycle, causing the endometrial cells to proliferate and thicken. Progesterone is responsible for counteracting the effects of the estrogen spike, preventing endometrial hyperplasia. The mechanism of these two hormones is a delicate balancing act. When EDC's trigger proliferation signals, the body has no way of differentiating between ERα caused by estrogen and ERα activity caused by EDC's. The body will only produce enough progesterone to counteract the estrogen being synthesized internally and won't account for potential EDC interference. When ERα is being expressed at abnormally high levels, there's not enough progesterone to counteract the rapid proliferation of the endometrium, which can turn into a breeding ground for cancer.

Caenorhabditis elegans are small organisms that have several nuclear hormone receptors (NHRs). NHR-14, in particular, has been shown to be estrogenic and behave similarly to ERα when exposed to EDC's (Mimoto *et al.,* 2007) (Garcia-Espineira, Tejeda-Benitez, and Olivero-Verbel, 2018). Stimulation of NHR-14 induces the production of vitellogenins, the primary protein precursor for egg yolk (Jones *et al.,* 2000). Since some EDCs are able to activate ERα without binding directly to active site, it was decided that it is necessary to conduct this study in-vivo, using C. *Elegans* as a model organism.

The goal of this study is to identify the ingredients in hair relaxers that may be responsible for increased expression of ERα, and in turn, an increased risk for uterine cancer. Additionally, this study will investigate if different combinations of the identified chemicals are more estrogenic than others.

MATERIALS AND METHODS

Worm Maintenance

Wildtype *Caenorhabditis elegans* strain were obtained from Carolina Biological Supply. The worms were incubated at 25°C, and OP50 E. coli were used as a feeder organism. All C. *elegans* were cultured on nematode growth media (NGM). The E. *coli* and NGM were also obtained from Carolina Biological Supply.

Age Synchronization

To account for natural age-related fluctuations in vitellogenin levels, the cultures used in this experiment were all subcultured on the same day from the same original culture.

Exposure to Endocrine-Disrupting Chemicals

The reagents purchased from Fischer Scientifc were estradiol, methyl paraben, diethyl phthalate, and octamethylcyclotetrasiloxane (D4). The concentrations of the compounds were determined by the concentrations in commercially available hair relaxers, obtained in previous research through gas chromatography mass spectroscopy. Due to the hydrophobic nature of these compounds, they were first dissolved in corn oil purchased from Carolina Biological Supply. Through several serial dilutions, D4 was diluted to 24 µg/g, methyl paraben to 796 µg/g, and diethyl phthalate to 52 µg/g. Estradiol will be diluted to 20pg/mL. Then, 2-3mL of oil solution was spread on top of the NGM. The worms lived between the medium and oil layer.

The negative control group was exposed to only corn oil, while the positive control group was exposed to the estradiol. The remaining groups were exposed to a singular EDC or a combination of EDCs. Each group was exposed to their assigned oil solution for 72 hours. Each group was repeated three times.

Lysis of Worms

The lysis and RNA isolation protocol was taken from Washington University School of Medicine's Greer Lab. The worms from each culture were suspended in M9 buffer and pipetted into centrifuge

tubes. The samples were centrifuged at 8,000rpm for 5 minutes. The worms were washed 2-3 more times with M9 buffer and ampicillin purchased from Carolina Biological Supply to minimize residual bacterial contamination. The mass of each pellet of worms was recorded. The worms were transferred to larger test tubes and suspended in 2mL of Trizol Buffer. The tubes were vortexed, centrifuged at 4°C, and then thawed at 37°C. This was repeated 6 more times to break open the worm membranes.

RNA Isolation

2mL of chloroform were added for every 1mL of packed worms. Tubes were inverted for 15 seconds and then allowed to sit at room temperature for 3 minutes. The samples were centrifuged for 15 minutes at 4500rpm at 4°C. Aqueous supernatant, containing RNA, was transferred to a new tube.

0.7 volumes of isopropanol were added to each tube containing the aqueous supernatant. The tubes sat at room temperature for 10 minutes, then were centrifuged at 8250 rpm for 10 minutes. RNA did not precipitate, as expected, after centrifugation. An ethanol precipitation was done to increase the yield of RNA. 2.5 volumes of 95% ethanol was added, and then the samples were left overnight at -80°C. The samples were centrifuged for 15 minutes, and then an RNA pellet was visualized. The supernatant was carefully discarded, and the pellet was washed with cold 75% ethanol. The tubes were left open to allow the ethanol to evaporate. The purified RNA pellet was then resuspended in RNase free water and stored at -80°C until time for quantification.

Converting RNA to cDNA

Since PCR only amplifies DNA sequences, the RNA samples had to first be converted to DNA. This was completed using a polymerase master mix from New England BioLabs. The samples incubated in a thermocycler at 25°C for two minutes, 55°C for 10 minutes, and 95°C for one minute.

The samples were placed into a UV spectrophotometer, using a nanocell accessory. Absorbance was observed at 210nm, 220nm, 260nm, 280nm, and 320nm. Absorbance was not detected at any of these wavelengths. The new cDNA samples were stored at -20°C for 5 days before being quantified.

Quantifying Vitellogenin Production

Differences in vitellogenin production were quantified using real-time quantitative PCR (RT-qPCR). Primers for Fisher Scientific were designed to amplify the vit-6 gene. SYBR Green qPCR Master Mix was used for the reaction. Samples were ran in duplicate and included 18S and no reverse transcriptase controls.

RESULTS

Once the *C. elegans* have been treated with their assigned reagent, I suspect there will be significant differences in vitellogenin levels. The negative control, which is just corn oil, serves as a baseline vitellogenin level. The positive control, which contains estradiol, provides a definitive demonstration of how estradiol affects *C. elegans*. The RT-qPCR fluorescently tag each newly synthesized DNA molecule. The machine measures how many cycles it takes for the fluorescent signal to reach a detectable threshold, also known as the cycle threshold. This indirectly measures how much cDNA was placed in the machine, which corresponds to the amount of mRNA that was in the worm.

Table 1: qPCR results relative to mass of worm pellets

Group	Mass of Pellet (mg)	Cycle Threshold (Ct)	Ct per mg of pellet
Corn Oil 1	31.3	No Ct	N/A
Corn Oil 2	43.2	14.54	0.3366
Corn Oil 3	37.1	No Ct	N/A
Estradiol 1	17.0	No Ct	N/A
Estradiol 2	21.9	36.54	N/A
Estradiol 3	29.4	No Ct	N/A
D4 1	26.7	No Ct	N/A
D4 2	24.2	No Ct	N/A
D4 3	40.8	No Ct	N/A
MP 1	36.3	No Ct	N/A
MP 2	27.6	No Ct	N/A
MP 3	15.6	No Ct	N/A
DP 1	39.5	15.96	0.404
DP 2	63.3	No Ct	N/A
DP 3	50.8	No Ct	N/A
MP+DP 1	37.0	33.43	0.9035
MP+DP 2	35.9	23.68	0.6596
MP+DP 3	13.4	No Ct	N/A
MP+D4 1	51.2	No Ct	N/A
MP+D4 2	35.1	No Ct	N/A
MP+D4 3	13.4	No Ct	N/A
DP+D4 1	38.8	No Ct	N/A
DP+D4 2	17.2	29.62	0.5807
DP+D4 3	26.0	No Ct	N/A
DP+D4+MP 1	24.7	No Ct	N/A
DP+D4+MP 2	25.5	39.46	N/A

DP+D4+MP 3	29.6	15.24	0.5149

Smaller Ct values means less PCR cycles were needed to reach the fluorescent threshold, which in turn, means higher expression of the targeted vitellogenin gene. Ct values were not detected for most of the samples, indicating that they never met the fluorescence threshold. Only Ct values lower than 35 are considered valid as values outside this range tend to be inaccurate. Ct values above 35 were disregarded, as fluorescence detection is more likely to be due to background influence than actual DNA amplification.

The only samples that gave valid Ct values were corn oil group 2, DP group 1, MP+DP groups 1 and 2, DP+D4 group 2, and MP+DP+D4 group 3. The samples were ran in duplicate, however, not a single sample gave a Ct result from both reaction wells. The results in Table 1 show the Ct numbers for the 6 out of 58 reaction wells.

DISCUSSION

The purpose of this study was to analyze the effects of the chemical interactions between methyl paraben, diethyl phthalate, and D4 on gene expression. However, significant challenges arose during the experimental process, especially during RNA extraction, that compromised the results of this study and contributed to low detection of the target gene. The results were overwhelmingly inconclusive with only 5 out of 58 samples showing detectable Ct values and there being no detectable cDNA concentration via UV spectrophotometry.

Previous studies have successfully shown that *C. elegans* can be used as model organisms for endocrine toxicity studies. For instance, research by Tseng *et al.* (2013) employed similar methods of isolating RNA with Trizol and then quantifying with qPCR to assess neurotoxicity of phthalates in *C. elegans.* While research such as Tseng's demonstrates the potential of *C. elegans* in toxicity studies, they ultimately fail to emphasize delicate nature of extracting, storing, and handling RNA.

The failure to obtain RNA pellets after isopropanol precipitation indicates that RNA yield was much lower than in previous studies. Any manipulation to a precipitation protocol, especially one that adds large volumes of ethanol, can further reduce RNA yield. Additionally, the RNA that was extracted had a high risk for degradation. The samples were frozen after being suspended in Trizol, again after being suspended in isopropanol, again after being suspended in ethanol, and then once more after being suspended in RNase-free water. Freeze-thaw cycles should be avoided as much as possible as formation of ice crystals can cause mechanical damage to RNA. This was unavoidable in this study due to difficulty obtaining pellets.

There is no observable pattern in the samples that did yield Ct values versus those that did not. There's no way to decipher whether those samples had a detectable reading because they had higher levels of gene expression or simply because their RNA extraction was more successful.

If this study were to be reproduced, liquid *C. elegan* cultures may be beneficial for increasing sample sizes. The only caveat would be finding a solvent that the EDCs are soluble in that isn't toxic to the worms. Next, freeze-thaw cycles should be minimized to maintain RNA integrity. For the best results, RNA should be extracted and synthesized to cDNA as quickly as possible. Lastly, performing a qPCR inhibition test would confirm that the sample hadn't been contaminated with reagents that would inhibit amplification.

The findings of this study provide insights into the technical difficulties in analyzing gene expression in *C. elegans.* This study chose to use *C. elegans* for cost and convenience. However, it may now be concluded that this experiment is better suited on human cell lines, which have higher RNA yields and more standardized extraction protocols. Human cell lines are significantly more expensive than *C. elegans* cultures, but the price may be worth the convenience and lab time. Transitioning to human tissues would also be more relevant to the greater purpose of this study in investigating the effects of these chemicals on hormone-dependent cancer prevalence. Addressing these challenges would lead to a better analysis of EDCs, their interactions with one another, and their molecular effects. This would ultimately improve our understanding of their potential health effects.

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Isolation and Characterization of *Escherichia coli* from Manure of Grass-Fed Cattle

Carly Pomrenke

ABSTRACT

The increasing prevalence of antimicrobial resistance (AMR) in human and animal populations presents a significant public health challenge. This study aimed to isolate and characterize *Escherichia coli* (*E. coli*) strains from the manure samples of grass-fed cattle and assess their antibiotic resistance profiles. Although grass-fed cattle were not treated with antibiotics, the results suggest that environmental factors play a significant role in the presence of antibiotic-resistant bacteria. The study found that there were strains of *E. coli* resistant to commonly used antibiotics, including penicillin, tetracycline, and erythromycin, but no resistance to quinolones, third-generation cephalosporins, and kanamycin were detected. These findings indicate that despite the absence of direct antibiotic use in the cattle, resistance can still emerge, likely due to bacterial transfer from neighboring farms or the presence of naturally resistant strains. The study highlights the potential risks of AMR spreading through manure, which is commonly used as fertilizer, contributing to environmental contamination and the transmission of resistant bacteria to humans. This research demonstrates the importance of monitoring antibiotic resistance in all livestock systems and calls for more comprehensive strategies to mitigate AMR in agriculture.

Keywords: Antimicrobial resistance (AMR), *Escherichia coli* (*E. coli*), Indole Resistance, Grass-fed cattle, Cattle Manure, Public Health

INTRODUCTION

In the United States, the average person consumes 57 pounds of beef annually (Shahbandeh, 2024). This is a testament to the nation's large-scale beef consumption and the vital role cattle play in the American agricultural industry. Cattle, known scientifically as *Bos taurus*, belong to the Bovidae family, a group of hooved mammals that includes sheep, goats, and bison. As ruminants, cattle possess a unique digestive system with one stomach that is divided into four different compartments. These compartments house various types of bacteria that aid in the breakdown of plant material. This allows cattle to efficiently process plant matter which makes up the majority of their diet. This digestive process is critical to their ability to extract nutrients from grasses and other diets.

The diets of cattle can vary widely, with two major feeding systems: grass-fed and grain-fed. Grass-fed cattle are exclusively fed grass and forage, with unrestricted access to pasture (American Grassfed Association, 2022). This feeding system is often associated with a leaner meat composition, as the animals' diets are lower in fat compared to those of grain-fed cattle. In fact, research have found that 100 grams of grass-fed beef contains 2,773 mg fewer total saturated fatty acids than the same amount of grain-fed beef (Nogoy, 2022). In contrast, grain-fed cattle are typically raised in confined spaces and are fed a diet primarily made up of corn and soy, which promotes faster growth. Grain-fed cattle are also more likely to be treated with antibiotics and hormones to prevent disease and encourage rapid weight gain (Piedmont, n.d.). This practice is typically used in feedlots, where the cattle are being prepared for slaughter. Antibiotics work by reducing bacterial metabolites and suppressing infections, thus allowing the cattle to absorb nutrients more efficiently and grow at an accelerated rate (DeVuyst, 2017). However, the overuse and misuse of antibiotics in agriculture has led to the global issue of antimicrobial resistance (AMR), which is now considered one of the greatest threats to public health worldwide.

Antimicrobial resistance is the ability of a bacteria to resist the effects of drugs that once killed or inhibited them. Antimicrobial resistance has been identified as a major global health crisis, causing an estimated 1.27 million deaths in 2019 (Pabst, 2023). This number has only grown as the overuse of antibiotics in both human and animal populations continue to increase. Livestock farming, in particular, is a significant contributor to the development and spread of AMR, with the global size of the livestock population exceeding that of the human population (Plumer, 2015). The crowded conditions in which many cattle are raised provide fertile ground for the spread of infections, and the use of antibiotics as a preventative measure is common practice. As a result, antibiotic-resistant bacteria are often present in the livestock, and these bacteria can be transmitted to humans through direct contact with animals, consumption of contaminated meat, or environmental contamination via manure.

Escherichia coli (*E. coli*), a gram-negative bacterium commonly found in the gastrointestinal tract of warm-blooded animals, including cattle, serves as a model organism in studies of antibiotic resistance (CDC, 2014). *E. coli* is a glucose, lactose, and sucrose fermenting bacteria, which means that it can metabolize sugar and produce acid. While many strains of *E. coli* are harmless and are naturally present in the gut microbiota, certain strains can be pathogenic and cause serious foodborne illnesses in humans. The ability of *E. coli* to acquire resistance to antibiotics adds an additional layer of concern for

public health. Antibiotic resistance in *E. coli* can arise through genetic mutations or horizontal gene transfer, which allows the bacterium to adapt to the presence of antibiotics in its environment. This makes *E. coli* a critical focal point in the study of AMR, particularly in the context of livestock farming, where antibiotics are routinely used to promote growth and prevent disease. Some *E. coli* strains, such as those resistant to quinolones and third-generation cephalosporins, pose a particular threat, as these antibiotics are commonly used to treat severe infections in humans. *E. coli* resistant to quinolones can be isolated by exposing it to ciprofloxacin, and *E. coli* resistant to third-generation cephalosporins can be isolated by exposing them to cefotaxime (Webber, 2001; Park, 2014).

While the use of antibiotics in cattle farming is necessary for disease control and growth promotion, it also contributes to the emergence of antibiotic-resistant bacteria in both grain-fed and grass-fed cattle systems. Although grass-fed cattle are not typically treated with antibiotics, studies have suggested that antibiotic-resistant bacteria can still be present in these animals. This may be due to a variety of factors, including environmental contamination from neighboring farms, bacterial transfer from other livestock, or the presence of naturally resistant strains within the bacterial population (DeFrancesco, 2021; Poudel et al., 2020). Even without direct antibiotic treatment, grass-fed cattle may harbor antibiotic-resistant bacteria, which can then be spread into the environment, particularly through manure (Kirk et al., 2017; Subbiah et al., 2019). In *Figure One*, it describes the mechanism for how antibiotic resistance can pass throughout the environment into the human population. Given that this is an extremely intricate system, the main focus of this experiment is on how the animal feeds affect antibiotic resistance.

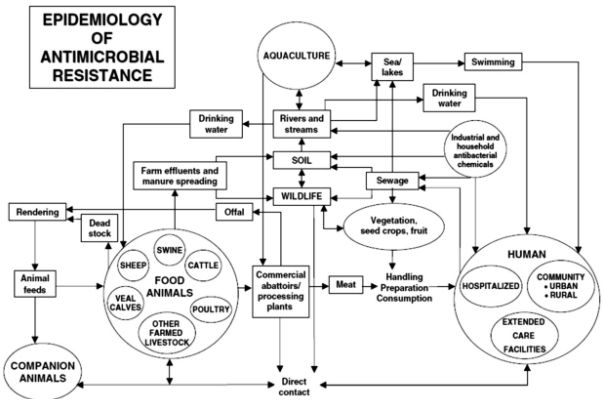

***Figure 5**. Epidemiology of Antimicrobial Resistance. Specifically in this experiment, the antibiotic resistance channel of animal feeds to food animals is examined. Food animals can then spread antibiotic resistance to humans through multiple different channels (Landers, 2012).*

Despite the growing recognition of antibiotic resistance as a global health threat, there remains a significant gap in research on the role of grass-fed cattle in the development and spread of AMR. Most studies have focused on cattle raised in intensive, antibiotic-treated environments, leaving a lack of understanding regarding how manure from grass-fed cattle may contribute to the spread of antibiotic-resistant bacteria. This research aims to isolate and characterize *E. coli* strains from the manure of grass-fed cattle to provide valuable insights into the mechanisms of antibiotic resistance in agricultural ecosystems. By understanding the prevalence of antibiotic-resistant *E. coli* in manure from grass-fed cattle, this study will contribute to broader efforts to mitigate the risks of AMR in agriculture and inform future practices that promote both animal health and environmental sustainability. Ultimately, this research will help fill critical gaps in knowledge and support efforts to safeguard both public health and the integrity of food production systems.

MATERIALS AND METHODS

Collection of Samples

Using a sterile scoop approximately 50 g of a fresh fecal sample were taken and transferred into a labeled sterile specimen container. A total of ten individual samples were taken from the site. The container was placed in a cooler with wet ice and transported back to the lab for further processing.

Preparation of Samples

Using a sterile wooden scoopula 10g of each fecal sample were placed in a separate 710 mL labeled sterile sample bag with 90 mL of buffered peptone water and massaged for 2 minutes. The bags were then placed in an incubator at 37°C overnight.

Plating – Isolation of *E. coli* strains

Using a sterile 10 microliter loop, three different plates of MacConkey agar were streaked with the sample from the buffered peptone water bag. MacConkey agar is specifically used to isolate a lactose fermenting bacteria. For the control, one plate does not contain antibiotics, one plate has cefotaxime at the ratio of 1 mg/L, and the other plate has ciprofloxacin at the ratio of 0.5 mg/L. These plates were incubated at 37°C overnight. All further plates will be using the same materials.

From each type of MacConkey agar, a colony with the morphology of *E. coli* was selected using a 1 microliter loop and was streaked onto a new plate of the same plate type. These were then incubated at 37°C overnight.

Confirmation of *E. coli*

One colony from each of the plates were transferred onto filter paper and 1 droplet of indole spot test onto the colony. If the colony turns blue, that indicates that it is positive for *E. coli*. To compare, a confirmed colony of *E. coli* was tested.

After a negative indole test, more tests were run to determine what bacteria was isolated. A motility test was inoculated and incubated at 37°C overnight. A Methylene Red test was inoculated and incubated at 37°C. Then tested at 24 hours and 48 hours. A colony was streaked onto EMB agar and incubated at 37°C overnight. Then a Gram stain was performed on an isolated colony.

Culturing *E. coli*

From the confirmed colony plates, a sterile microliter loop was used to transfer one isolated colony to a tryptic soy broth culture tube. Each tube was vortexed and then incubated at 37°C overnight.

Kirby-Bauer Disk Diffusion Test

From the broth samples, 0.2mL were transferred onto Muller Hinton agar and was spread evenly. The antibiotic disks were placed on the agar and incubated overnight at 37°C overnight. The diameters of inhibition zones were recorded in mm using a caliper. These results were compared to the CLSI standards to determine if they are resistant or not (CLSI, 2023).

I adapted the methods outlined in Manishimwe (2021) to isolate and characterize antibiotic-resistant E. coli strains from manure samples of grass-fed cattle. All further testing and techniques were followed with the procedures from the American Society of Microbiology (American Society for Microbiology, 2020).

RESULTS

The experiment did not yield any strains of *E. coli* resistant to quinolones and third-generation cephalosporins. In *Figure 2*, it shows the MacConkey agar plate with cefotaxime and in *Figure 3* it shows the MacConkey agar plate with ciprofloxacin.

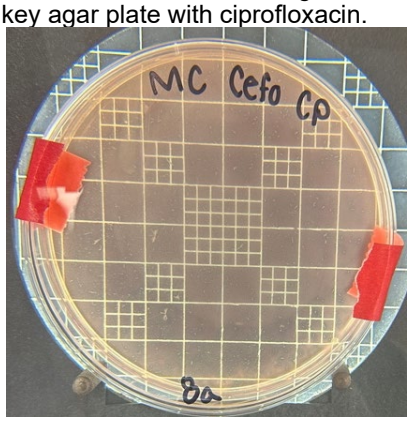

***Figure 6**. MacConkey Agar with Cefotaxime after 24 hours. There were no colonies of any bacteria isolated from this type of agar.*

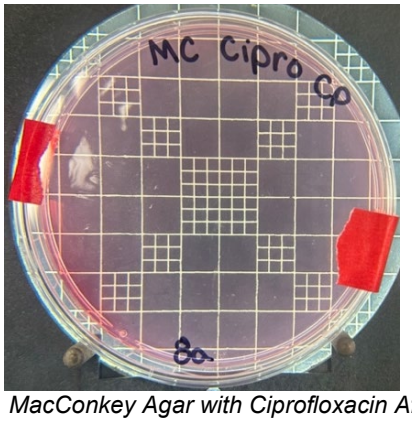

***Figure 7**. MacConkey Agar with Ciprofloxacin After 24 hours. There were no colonies of any bacteria isolated from this type of agar.*

On the MacConkey agar without antibiotics, there were colonies with *E.* coli type morphology. They were circular, pink colonies. The pink color demonstrated that this colony is a lactose-fermenting bacteria, which is a characteristic of *E. coli* and is shown in *Figure 4*.

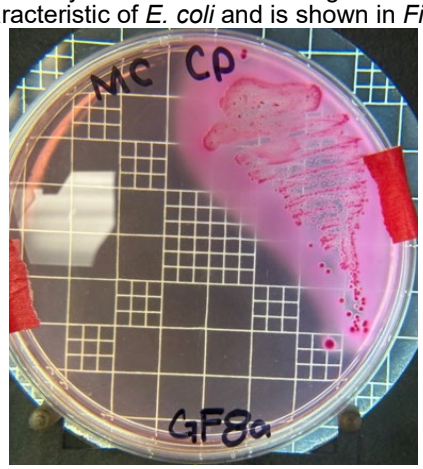

***Figure 8.** MacConkey Agar after 24 hours. The pink colonies represent a lactose fermenting bacteria.*

All colonies isolated from indicated to be indole negative. As a comparison, a known colony was tested to be indole positive. Given this information, it was predicted that *E. coli* was not isolated, so more tests were required before antibiotic resistance was tested. In terms of motility, the motility test was positive, shown in *Figure 5*.

***Figure 9**. Motility test after 24 hours. The pink specs throughout the agar, confirms that the bacteria are motile.*

A methyl red (MR) test was then performed at 24 and 48 hours, and the culture turned a bright orange-red color, shown in *Figure 6*. This confirms that it is a glucose fermenter, the methyl red indicators turned red, which means the product of the fermentation is acidic (American Society for Microbiology, 2021).

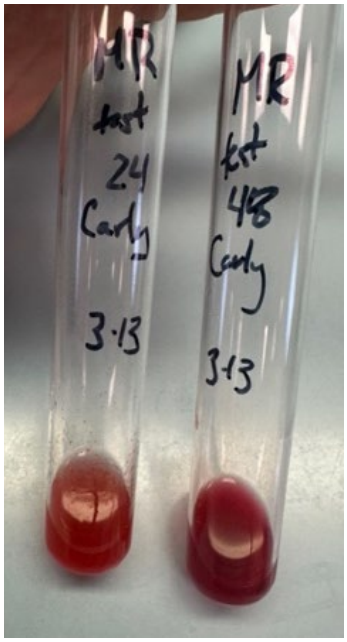

***Figure 10**. MR test – 24 hours versus 48 hours. The red results confirms that the bacteria are a glucose fermenter.*

To confirm that the bacteria isolated was *E. coli*, a colony from each sample was plated onto Eosin Methylene Blue (EMB) agar. The indicator in the agar will confirm if the bacteria is able to ferment lactose. *E. coli* is able to quickly ferment lactose, producing a green sheen. After 24 hours, the bacteria presented with a green sheen, confirming that it is *E. coli,* shown in *Figure 7*.

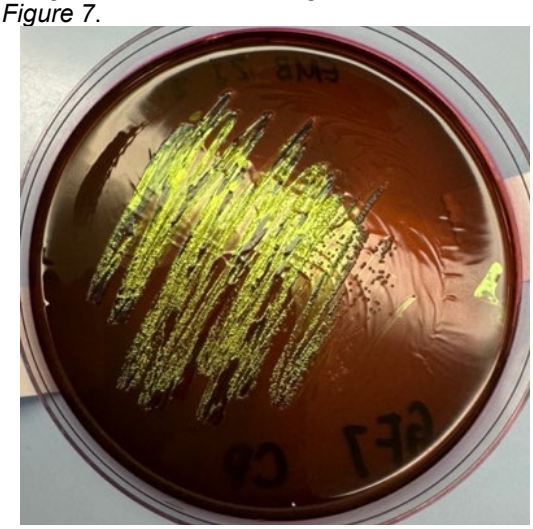

***Figure 11.** EMB Agar after 24 hours. The green sheen determined that the bacteria isolated was E. coli.*

After doing a gram-stain, the bacteria presented to be gram-negative, with a small rod-shaped structure, shown in *Figure 8*.

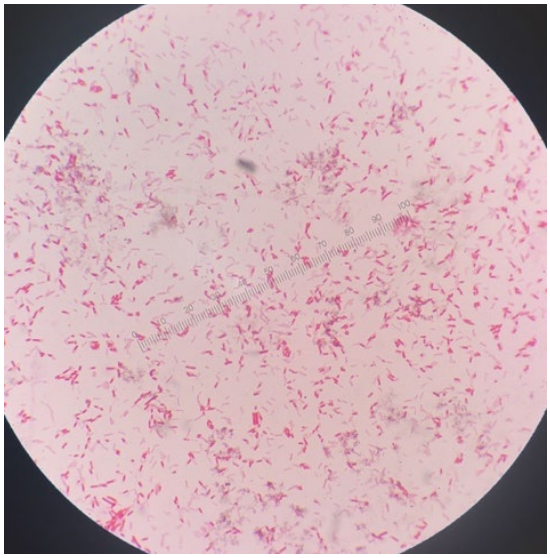

***Figure 12.** E. coli* gram stain at 100x. The result is indicative of *E. coli* as it is a gram-negative bacterium and is rod-shaped.

Once the bacteria were confirmed to be *E. coli,* the isolated were then tested for antibiotic resistance using the Kirby-Bauer Disk Diffusion Test. In *Table 1*, the results of each sample are shown in the form of an averages and standard deviations.

	Average ZOI (mm)	Standard Deviation	Resistance Status
Penicillin	7.7	4.4	Resistant
Tetracycline	11.2	9.2	Resistant
Bacitracin	0	0.0	Not Effective
Kanamycin	21	2.3	Sensitive
Erythromycin	6.2	5.5	Resistant

***Table 2**. Antibiotic Resistance Results. ZOI - zone of inhibition. Resistance status was determined by the CLSI guidelines (Clinical and Laboratory Standards Institute, 2023).*

DISCUSSION

The primary aim of this study was to isolate and characterize different strains of *Escherichia coli* from manure samples of grass-fed cattle and to assess their antibiotic resistance. While antimicrobial resistance (AMR) continues to be a significant global public health threat, particularly due to certain agricultural practices, understanding the role of grass-fed cattle in the spread of resistant bacteria is crucial. The results of this study indicate that grass-fed cattle can harbor E. coli strains resistant to commonly used antibiotics, such as penicillin, tetracycline, and erythromycin. Although no resistance to quinolones and third-generation cephalosporins was observed, these findings are significant, as penicillin and tetracycline are widely used in both human and veterinary medicine (Van Boeckel et al., 2015). Given that the cattle sampled were never treated with antibiotics, it suggests that environmental factors may

be the primary contributors to the presence of antibiotic-resistant bacteria. These could include bacterial transfer from neighboring farms or the presence of naturally resistant strains (Poudel et al., 2020). Furthermore, resistant bacteria can be spread through manure, which is often used as fertilizer, facilitating environmental contamination and the potential transmission to humans (Van Boeckel et al., 2015).

The initial step in confirming *E. coli* involved a series of morphological, biochemical, and molecular tests. The first test was the indole test, a biochemical assay used to identify *E. coli*. Indole production is a distinctive characteristic of *E. coli*, as the bacterium produces the enzyme tryptophanase, which degrades tryptophan into indole (Brenner et al., 2005). However, in this study, all isolated colonies tested negative for indole production. This was unexpected, as *E. coli* typically tests indole-positive, leading to further investigation. It is important that some strains of *E. coli* may lack tryptophanase activity, and other environmental factors or mutations might contribute to indole-negative phenotypes, suggesting that the bacterial strains isolated could be atypical in their behavior (Wong et al., 2018). These findings are consistent with studies that have shown *E. coli* strains from diverse environmental sources may exhibit varying biochemical properties (Montenegro et al., 2021).

The lack of quinolone and third-generation cephalosporin resistance is reassuring, as these antibiotics are critical for treating severe human infections. However, the resistance to other antibiotics highlights the complexity of AMR in agriculture and the necessity for further research. In particular, the study raises important questions about the role of non-antibiotic-treated livestock systems in the development and spread of resistant bacteria. Although grass-fed systems may limit direct antibiotic use, they are not immune to the problem of AMR, especially when considering the interconnectedness of agricultural ecosystems and the potential for cross-contamination between farms (Poudel et al., 2020). It is possible that resistant bacteria may be spread through shared water sources or via animals, equipment, or workers moving between farms (Tadesse et al., 2017). The findings of this study suggest that environmental contamination plays a more prominent role in the transmission of antibiotic-resistant bacteria than previously thought.

The results of the antibiotic susceptibility testing reveal the varying levels of resistance and sensitivity from the isolated *E. coli*. Of the antibiotics that were tested, bacitracin was used as the control because it is only effective against gram-positive bacteria. This not only served as a control but also another confirmation of isolating a gram-negative bacterium. It was found that the isolated *E. coli* was resistant to penicillin, tetracycline, and erythromycin. Penicillin and tetracyclines are commonly used in human and veterinary medicine. Penicillin is a broad-spectrum antibiotic and is classified as a β-lactam antibiotic. The antibiotic works by inhibiting the cross-linking of the peptidoglycan within the cell wall. As the bacteria's cell wall weakens, water floods in due to osmotic pressure and kills the cell (Nguyen, 2018). Tetracycline is a broad-spectrum antibiotic and is classified as a protein synthesis inhibitor. Tetracyclines are able to disrupt protein synthesis by halting the ribosomal process of turning mRNA into a functioning protein. Without this process, the cell becomes bacteriostatic and can no longer function (Shutter, 2023). Erythromycin is also a protein synthesis inhibitor and works in the same pathway. It has been typically used in medicine for various respiratory infections (Farzam, 2023). Kanamycin showed a significant zone of inhibition, which indicates that the bacterium was sensitive to this antibiotic. Kanamycin is a broad spectrum antibiotic and is classified as an aminoglycoside. An aminoglycoside is also a protein synthesis inhibitor (Block, 2023). Bacitracin, erythromycin, and tetracycline are the only antibiotics approved for subtherapeutic in cattle. However, tetracyclines and bacitracin are the ones most commonly used (National Center for Biotechnology Information, 2013). Given that the cattle sampled were grass-fed, and the *E. coli* isolated was resistant to tetracycline, it points to environmental factors of resistance rather than the animals diet. Due to the high variability in the data range of tetracycline, it is important that more research is done to confirm these finding. As well as compare these findings to the manure samples of grain-fed cattle.

These results underscore the importance of comprehensive monitoring of antibiotic resistance in all types of livestock systems, including grass-fed cattle. Efforts to mitigate the spread of AMR must go beyond simply reducing antibiotic use in farming. Improved waste management practices, regular surveillance for resistant strains, and the development of alternative methods for disease prevention and growth promotion are critical components of a broader strategy to combat AMR in agriculture (Aarestrup et al., 2012). In addition, addressing the population density of livestock relative to the enclosure area could further help in reducing the likelihood of bacterial spread and contamination.

In conclusion, this study contributes to the growing body of evidence suggesting that antibiotic-resistant bacteria can be found in grass-fed cattle, raising concerns about the role of these systems in the development and spread of AMR. While grass-fed systems may be less reliant on antibiotics, they are not exempt from the global AMR crisis. Further research is needed to fully understand the mechanisms of resistance in these systems and to develop sustainable practices that protect both public health and the integrity of food production systems. It is also important to investigate the population density of the herd relative to the enclosure area, as this could impact the prevalence of resistant strains in the environment.

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Research Awards in the Natural Sciences

The awards are sponsored by the Natural Sciences of McPherson College and Midwest Oilseeds of Adel, Iowa.

The **Burkholder Research Award**, the highest award, is presented in recognition of outstanding achievement in student research. The **Merit Research Award** is presented in recognition of achievement in student research.

Each student completing a senior research project in the Natural Sciences is a candidate for an award. The Natural Science Faculty select the winners of the awards. Three criteria are used to judge the quality of the research and in selection of student award winners: (1) Selection and planning of a research project; (2) Quality of the research work, including techniques, observations made, and the analysis of data; and (3) Reporting the research, consisting of preparation of the research paper, and a poster or oral presentation to students and faculty.

Each student receiving an award will receive a Certificate of Award. Those receiving the Burkholder Award will have their name inscribed on a plaque, and will receive a year membership in the American Association for the Advancement of Science and a subscription to the journal *Science*.

Year	Burkholder Award	Merit Award
2025		Zoe Jerke, Jayla Moore
2024		Taylor Bohannon, Maggie Brown, Madison Turley
2023	Olivia Smith	Bethany Masters Ice, Nathan Saffer, Ann Marie Weesner
2022		Brionnah Fessler, Erica Paradise, Emma Singleton
2021		Sydney Burton, Jessica James, Choucranie N. Kayembe, Aaron Leck, Allison Pamela Penalva Vanegas
2020		Sebia Kalambayi Kabedi, Garrett Owen
2019	Parkes Wolters	Micaila Curtis, Samantha Nelson
2018		Nora Grosbach, Amy Makovec
2017	Nathan Finch	Sheryl Evans, Lucas Giesey
2016	Tiffany Fraser, Ashley Long	Alia Khalidi, Kaley Kinnamon
2015	Nathaniel Schowengerdt	Shannon Coldren, Sydney Lipton, Jordan Stewart
2014	Yi Qun Chai, Sean DeYoung	Lori Crain, Alejandro Esparza, Christian Rodriguez
2013	Amanda Baxter, Emily James, Taylor Roop	Torey Fry, Kasey Miller
2012	Audrey McTaggart	Savannah Sievers, Andrew Skinner
2011		Karissa Ferrell, Kelley Green, Ashley Zodrow
2010	Ashlee Jost, David Miller	
2009	Adam Horinek	Amanda Pangburn, Nicole Sampson, Lezli Warkentin
2008	Joel Grosbach, Landon Snell, W. Brett Whitenack	Alan Grosbach
2007	Callie Crist	Rhonda Hoffert, Jamie Rodriguez
2006	Travis Allen	Lisa Sader
2005	Joseph Blas	David Cockriel, Jenny Harper, Danielle Lucore
2004	Robert Ullom	
2003	Michelle Schulz	Adeline Cripe
2002	Elizabeth Stover	Renata Lichty
2001	Genelle Wine	Jonas Lichty
2000	Nathan McLaughlin	Jeffrey McPherson
1999	Roy Johnson, Jr.	Jennifer M. Amiot, Janet Bowen, Eric D. Putnam, Anna Katharina Schenk
1998		Rebecca Standafer, Cameron Mahler
1997	Kerri Kobbeman	Rod Samuelson
1996		Chris Owens, Wes Seckler, Stasi Zirkel
1995	Monica Embers, Heather Hughbanks	Erik Harmon
1994	Adam Smith	Susan Blubaugh, Sherry Coopple, Adeola Grillo,

		Paula Worley
1993	Tyson Burden	Robin Morgan
1992	Pete Hanson	
1991		Thomas Champion, Shannon Hull
1990	James Dechand	David Maxey
1989		Michelle Roesch
1988	David Lehmen	Cynthia Aeschacher, Sandra Ashbaugh
1987	David Krehbiel, Marla Ullom	Cassandra Clark, Marsha Morley, Jay Nicholson

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