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Article

The effects of cochineal and Allura Red AC dyes on *Escherichia coli* and *Bacillus coagulans* growth

Ava Palmatier[1], Tamara Stoner-Sleight[1], Joseph Rasmus[1]
[1] Williamston High School Math and Science Academy, Williamston, Michigan

SUMMARY

As the usage of artificial dyes has increased, so have concerns about their effects. This research aimed to determine if the effects of Allura Red AC dye (FD-&-C Red 40) is more detrimental than the effects of a natural dye, specifically cochineal or carmine red dye, which is made from crushed insects. This research focused on the effects of the respective dyes on the growth of two gut bacteria: *Escherichia coli* and *Bacillus coagulans*, which is also known as *Heyndrickxia coagulans*. Agar plates were streaked with either *E. coli* or *B. coagulans*, followed by application of two sterile disks to each plate, one control and one soaked in dye. The plates were then incubated for 24 hours and 48 hours, respectively. After the allotted growth period, the mean growth coverage rating in the area surrounding each disk was determined for each plate. We hypothesized that the Allura Red AC dye would result in reduced gut bacteria growth, while cochineal dye would have no effect on gut bacterial growth. Using a one-tailed *t*-test, the dye-disk growth means from the trials were compared to their corresponding control disk. Only *E. coli* with Allura Red AC dye had significant results, with the Allura Red AC dye amplifying bacterial growth when compared to the control. This implies that the consumption of Allura Red AC dye may increase the growth of *E. coli* bacteria within the human gut.

INTRODUCTION

In recent years, concerns about the effects of Allura Red AC dye have risen as society has become more reliant on artificial additives. Artificial additives are used to increase consumer attraction toward both edible and non-edible items. While the dyes increase appeal, they have also increased instances of cancer, hyperactivity, and other health issues (1). A study in mice found that the consumption of both Allura Red AC dye and a high-fat diet increased colonic inflammation and DNA damage (2). These side effects have caused people to consider whether the efficient and cheap production of the Allura Red AC dyes outweighs the repercussions of consuming the dye. These concerns have prompted people to look for healthier natural alternatives. The concerns have even influenced the ban of the dye in some countries outside of the United States. However, one study performed on rats using natural dyes including chlorophyll, turmeric and carmine (cochineal) dye concluded that these natural dyes also had negative impacts on the gut (3). The human gut microbiome plays a vital role in overall human health. As one of the major organ systems, a disturbance within the gut impacts other organ systems, as well (4). This is why it is crucial to determine the impacts of natural and artificial dyes on the gut microbiome. Another study negates these findings and states that cochineal dye is safe for consumption. This study found that the mice that consumed the cochineal dye demonstrated similar results to the control group (5). This suggests that more research is needed to determine whether natural dyes, such as cochineal red dye, are a healthier option than artificial dyes, such as Allura Red AC.

This research focused on determining if artificial dyes cause harm to the human gut and if natural dyes are a safe alternative for them. Specifically, we tested the effects of Allura Red AC and cochineal red dye on *Escherichia coli*, a healthy bacteria commonly found in the gut that helps the human body break down and digest food (6). Similarly, we also tested the ramifications of both dyes on *Bacillus coagulans*, which is commonly found in the gut and contributes to creating an anaerobic environment, allowing for the growth of some probiotics (7). *E. coli* and *B. coagulans* are commonly found in the gut and play a crucial role in digestion and illness prevention (7, 8). A decrease in *E. coli* and *B. coagulan* abundance may lead to ramifications throughout the body.

Although there is some research that has observed impacts to the human microbiome from natural and artificial dyes, our research used a different approach to examine these relationships (3). While many *in vivo* experiments on mice or rats have been performed, there has been minimal research done *in vitro* on this topic. An *in vitro* study is advantageous because it allows us to see the direct effects of the dyes on the growth of the bacteria, which are not apparent *in vivo*. There is also limited research in which natural and artificial dyes are compared.

We hypothesized that the Allura Red AC dyes would have a detrimental effect on the growth of the gut bacteria, while cochineal dye would have no effect on gut bacterial growth. We expected that the Allura Red AC dye would inhibit the growth of healthy gut bacteria, while cochineal dye would not, making Allura Red AC more harmful to overall health. Our results determined that cochineal dye has neither a positive or negative effect on *E. coli* and *B. coagulan* growth. It was also determined that Allura Red AC has no effect on *B. coagulans*, but it does have a positive effect on *E. coli* growth. This suggests the possibility that Allura Red AC may positively affect the health within the human gut, while cochineal dye has no impact on gut health.

RESULTS

This study aimed to determine how artificial and natural food dyes affect bacterial growth. To do this we used cochineal dye and Allura Red AC dye and two species of gut bacteria, *E. coli* and *B. coagulans*. Each bacteria species

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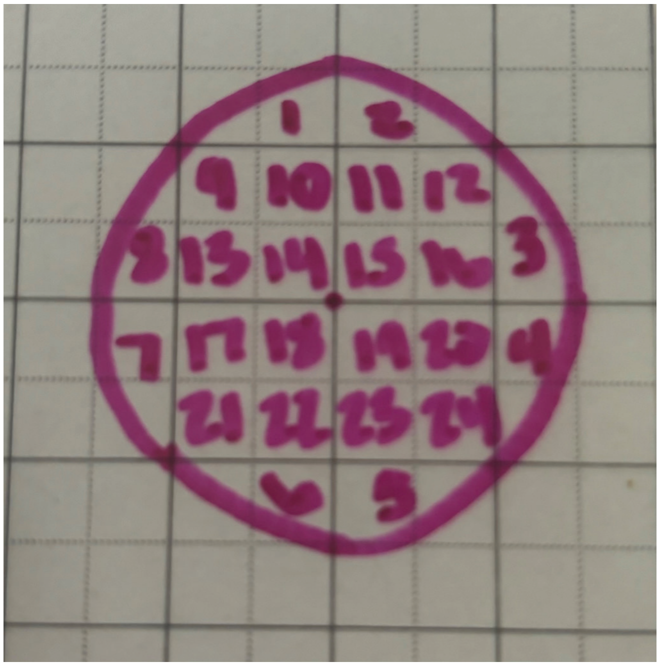

Figure 1: Numbered rating disk for assessing bacterial growth. Translucent grid paper and a compass were used to create this rating grid. Each square within the disk was numbered from 1 to 24. Four of these numbers were randomly selected to rate the growth of bacteria within the square.

grew on separate agar plates, each plate having one blank sterile sensitivity disk (control), one sterile sensitivity disk treated with dye (either cochineal dye or Allura Red AC), and one ampicillin or penicillin-infused antibiotic sensitivity disk. The disk type varied for both species to show growth inhibition was possible in multiple ways. After incubation, we used a modified grid method to quantify the data **(Figure 1)**. Four random boxes from the grid were rated (0–10) based on the bacterial growth in the area and the mean of the ratings was then used to calculate the abundance of growth surrounding treated areas **(Figure 2)**. After data collection was complete, we used a one-tailed *t*-test for two independent means to compare each dye treatment to the control for the corresponding plates (9).

Neither cochineal dye nor Allura Red AC had a statistically significant effect on *B. coagulans* ($p > 0.05$) **(Figure 3)**. Our results showed the mean growth of *B. coagulans* when exposed to cochineal dye was 6.125, which was not significantly different from the mean of the control group (6.367, $p = 0.201$). Similarly, the mean growth of *B. coagulans* when exposed to Allura Red AC was 6.075, which was not significantly different from the mean of the control group (6.133, $p = 0.375$). These results suggest that neither dye has an influence on *B. coagulans* growth.

The Allura Red AC and cochineal dyes differed in their impacts on *E. coli* growth **(Figure 4)**. The mean *E. coli* coverage when treated with cochineal dye was 7.233, which was not significantly different from the mean of the control

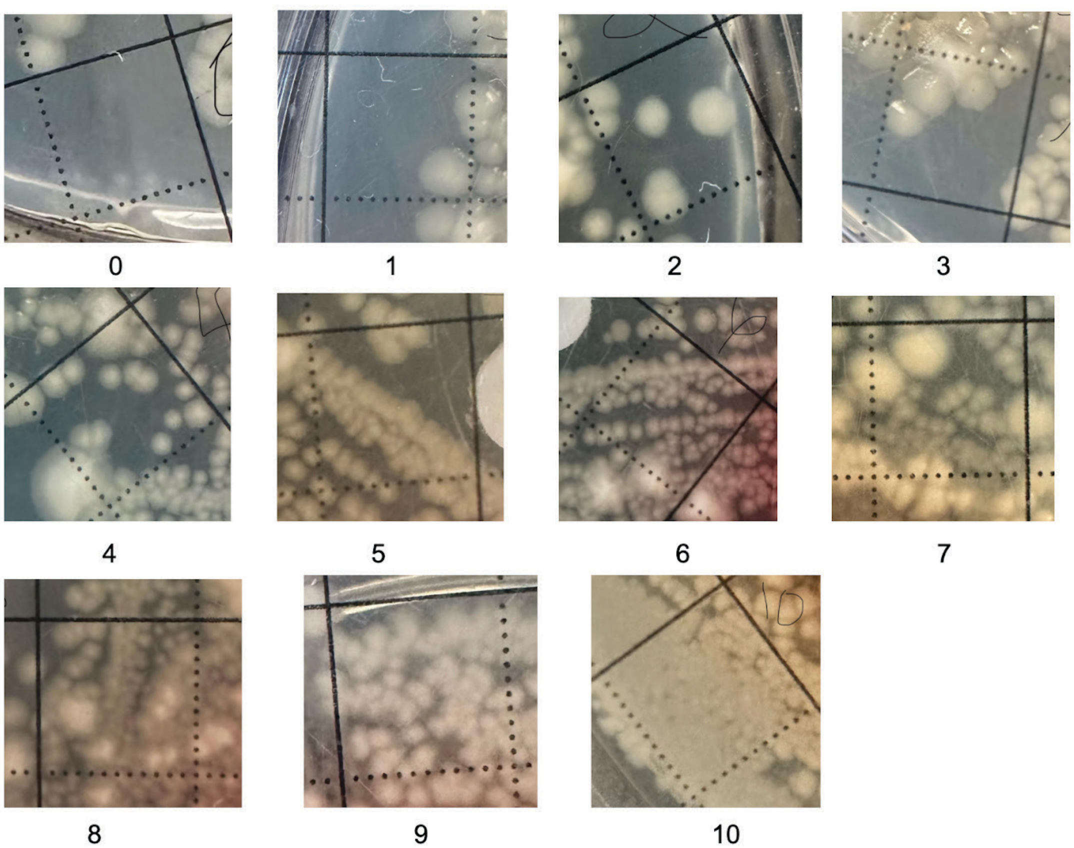

Figure 2: Reference rating scale for bacterial growth (0–10). A control plate of *B. coagulans* was used to create this coverage scale. This scale was consistently used by the researcher who rated the plates with both *E. coli* and *B. coagulans*.

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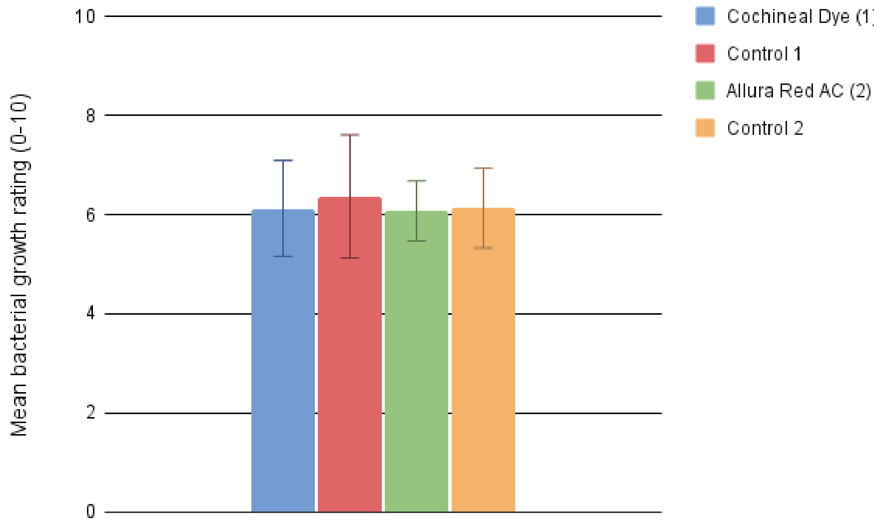

Figure 3: Neither cochineal dye nor Allura Red AC have an effect on *B. coagulans* growth. A bar graph showing the mean square rating (0–10) of *B. coagulans* growth in the treated area after 48 hours of incubation at 37 °C. The mean rating of cochineal dye (blue), control for cochineal dye (red), Allura Red AC (green), and control for Allura Red AC (orange) groups are all shown. Error bars represent the standard deviation of each data set. There is no significant relationship between the dyes and the growth of B. coagulans (*t*-test, $p > 0.05$, n=30).

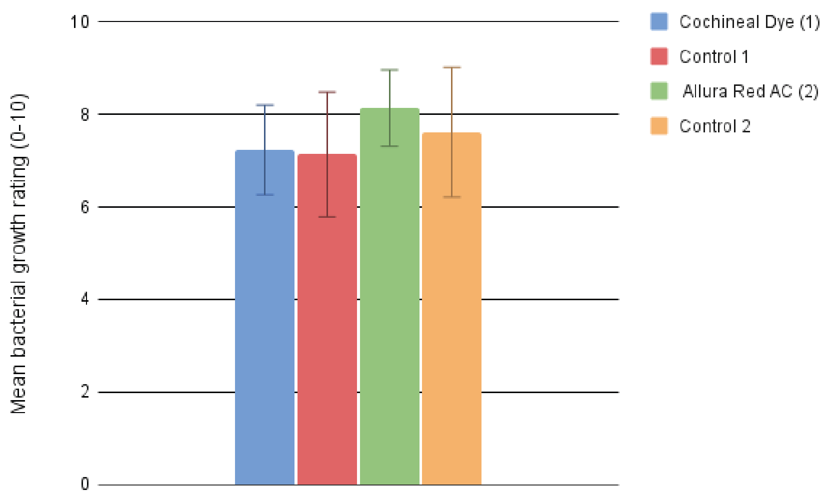

Figure 4: Cochineal dye does not have an effect on *E. coli*, but Allura Red AC does. A bar graph showing the mean square rating (0–10) of *E. coli* growth in the treated area after 24 hours of incubation at 37 °C. The mean rating of cochineal dye (blue), control for cochineal dye (red), Allura Red AC (green), and control for Allura Red AC (orange) groups are all shown. Both control groups were completed at the same time as their corresponding treated group. Error bars represent the standard deviation of each data set. There is no significant relationship between cochineal dye and E. coli growth ($p > 0.05$). In contrast, the results suggest that Allura Red AC dye significantly increases the growth of *E. coli* compared to the control (*t*-test, $p < 0.05$, n=30).

group (7.133, p = 0.371). These results suggest that the cochineal dye did not have any negative or positive effects on *E. coli* growth. In contrast, the mean *E. coli* coverage when treated with Allura Red AC was 8.133, which was significantly higher than the mean of the control for that group (7.608, p = 0.040881) **(Figure 4)**. The results of this treatment suggest that Allura Red AC enhanced the growth of *E. coli.*

Similarly, there was no evidence to suggest cochineal dye had any effects on *E. coli* growth. In contrast, our data showed Allura Red AC enhanced the growth of *E. coli* compared to the control, suggesting our original hypothesis, which was that Allura Red AC would have a negative impact on bacteria growth, cannot be accepted.

DISCUSSION

Our study aimed to determine the effects of cochineal and Allura Red AC dyes on *E. coli* and *B. coagulans* growth. We hypothesized that Allura Red AC would decrease *E. coli*

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and *B. coagulans* growth while cochineal dye would have no effect on the growth of either. The data showed that cochineal dye does not affect the growth of the gut bacteria *E. coli* or *B. coagulans*, so it is reasonable to conclude that our hypothesis is partially supported. The data from the Allura Red AC effect on *B. coagulans* trials also suggested Allura Red AC has no significant effect on *B. coagulans* growth, so our hypothesis for Allura Red AC's effect on *B. coagulans* was not supported by these results. The results for the Allura Red AC on *E. coli* suggested that Allura Red AC actually has a positive effect on *E. coli* growth. These results do not support our hypothesis. Our results show a significant increase in growth of *E. coli* when treated with Allura Red AC dye, the effect of which requires further research. Additionally, this does not correlate with previous research on the effects of Allura Red AC in which it demonstrated a negative effect on gut human health (1). Our findings suggest that neither cochineal dye nor Allura Red AC have a negative effect on these two species of gut bacteria.

We do not yet know if this same effect can be seen with any other gut bacteria, which is why more research with other species is needed. Our study did not investigate other possible effects that Allura Red AC may have, such as cognitive or cancerous effects. Our results do not suggest that Allura Red AC demonstrates an overall positive effect on the human body, as the consumption of the dye may have differing effects on various systems within the body.

The results regarding cochineal dye suggest that the dye demonstrates no effect on *E. coli* or *B. coagulans* growth, which correlates with previous research. These results support our origianal hypothesis about cochineal dye. Cochineal dye did not demonstrate any effect on *E. coli* or *B. coagulans* growth, but this natural dye may affect other species of gut bacteria.

One limitation of our study is that we only used two types of gut bacteria. *E. coli* and *B. coagulans* are not the only types of bacteria that can be used to study gut health. The primary reasons for using these two bacteria were their accessibility, price, and short growth period. For example, an alternate gut bacteria, such as *B. fragilis* (an anaerobic bacteria in the gut), is an accurate representation of gut health but requires more extreme conditions to survive than could be provided in our lab (10). Another limitation of this research was the minimal number of dyes tested, one natural and one artificial. There are several other artificial dyes that have been approved by the FDA, including sunset-yellow, fast green, and annatto (3). Further testing is needed to determine the effects these common dyes have on the human gastrointestinal microbiome. Another limitation to be considered is that we used an *in vitro* method. Other research surrounding this topic used living subjects, such as mice. Using living organisms can better understand how artificial and natural dyes affect the gut and human body. Also, both dyes may interact with *E. coli* or *B. coagulans* differently *in vivo* than they did in this *in vitro* study. More research needs to be done to determine if these dyes are safe to put in food. For example, it is unknown if the dyes used in our experiment may be chemically degraded or modified during digestion; *in vivo* experiments are needed to determine this effect. Despite the limitations of our research, the results provide conclusions that are helpful in determining how synthetic dyes affect the body.

MATERIALS AND METHODS

Experiment Preparation

Two food dyes were tested on nutrient agar plates: one natural (cochineal dye) and one artificial (Allura Red AC). The cochineal dye was Cherry Red from Bake King Liquid Food Colours, and the Allura Red AC dye was Chef-O-Van Red Food Coloring. Four treatments were used: cochineal dye on *E. coli,* Allura Red AC on *E. coli,* cochineal dye on *B. coagulans,* and Allura Red AC on *B. coagulans. The E. coli* (15-5068) and *B. coagulans* (15-4886) were ordered from Carolina Biological (11). Each experimental group had corresponding control data collected from the same set of plates. We also added an antibiotic disk as a positive control. Each group consisted of 30 plates with three sections on each plate, labeled as N or R referring to the dye type (N for cochineal natural dye and R for Allura Red AC dye), C for control, and P for penicillin or AMP for ampicillin.

Dye Application and Bacteria Growth

A sterilized 10 µL inoculating loop was used to streak the plates with *E. coli* or *B. coagulans*. Once streaking was complete, two blank sterile sensitivity disks were placed on each plate. One disk was dipped in either cochineal dye or Allura Red AC (depending on the experimental group) using sterile forceps, and the other disk remained blank as a control. Each disk was then placed onto its respective section of the agar using the forceps. A penicillin- or ampicillin-infused antibiotic sensitivity disk was added to each plate as a positive control **(Figure 5)**. Once this process was complete, the agar plates were placed face down in an aerobic incubator at 37 °C to mimic the temperature of the human gut. The *E. coli* groups were incubated for 24 hours while the *B. coagulans* groups for 48 hours, since *B. coagulans* growth was slower.

Data Collection

To quantify the bacterial growth, a smaller version of the grid method was used (12). Using a compass, we drew a circle on translucent grid paper. This circle enclosed 16 full 0.635 cm^2 squares and 16 partial squares, which were

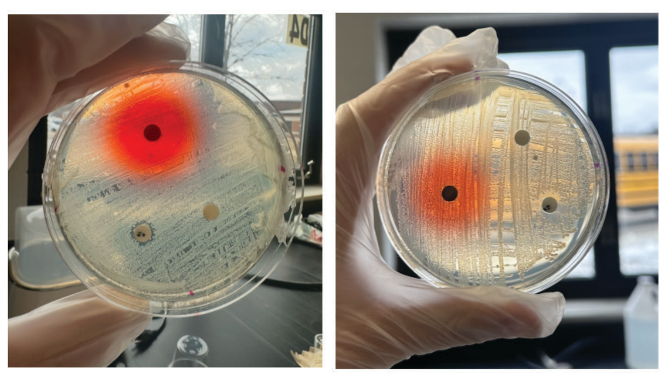

Figure 5: Allura Red AC treated plate (left) versus cochineal treated plate (right). These are example agar plates streaked with *E. coli* using the 90-degree streaking method. Three disks were placed on the agar secondary to streaking: one with the corresponding red dye (red dye halo), one with penicillin (P 10), and one with no treatment (blank). Both of the photos were taken after 24 hours of incubation. These examples are representative of the similarities between the cochineal dye and Allura Red AC trials, which prevented them from being easily distinguished.

counted as eight full squares. Each square on the grid was assigned a number from 1 to 24 **(Figure 1)**. The grid was then placed on the back of the agar plate in line with the center of the treated sensitivity disks. A random number generator was used to select four sections for the treated disks that were then each disk was rated on a scale of zero (no growth) to ten (complete coverage) on the amount of bacterial growth in the section based on the scale we created **(Figure 2)**. The same process was then repeated on the control disk.

The scoring was done by a student in our Math and Science Academy research class at Williamston High School who was randomly selected and was not affiliated with our project. This student rated the plates throughout our study to prevent inconsistent rating. They were told what kind of bacteria was on the plate but not what type of dye each plate was treated with, preventing implicit bias **(Figure 5)**. The scores were recorded on a spreadsheet and the mean growth was calculated for each plate. Then the mean and standard deviation of those means were calculated for the dye and control disks in each group. Next, the p-values were calculated using a one-tailed *t*-test for two independent means to compare each dye treatment to the control for the corresponding plates (9). We collected data on a total of 120 plates, 60 of them with *E. coli*, 60 with *B.coagulans,* 30 from each bacterium for cochineal dye and 30 from each bacterium for Allura Red AC.

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