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Article

Inhibitory effects of captan on growth of *Escherichia coli* and *Bacillus coagulans*

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SUMMARY

As the world's population increases, so does the need for the availability of clean fruits and vegetables. In order to comply with these demands, an increase in the usage of pesticides, such as captan, has also emerged. This study aimed to determine the effect that captan has on the gut microbiome, specifically by using *Bacillus coagulans* and modeling strains of *Escherichia coli* by using *Escherichia coli* K-12. To do this, we mixed a pesticide dilution into melted nutrient agar, while the control groups only contained distilled water. After they had re-hardened, we streaked the plates and placed the *E. coli* and the *B. coagulans* into an incubator. Following incubation, we found a significant difference in bacterial growth between the control and the captan-containing plates. Therefore, we concluded that the pesticide captan has a negative effect on the growth of these bacteria, and, potentially, on the human microbiome. The information found during this experiment could possibly warn regular produce consumers of the potential dangers of not washing their products before consumption.

INTRODUCTION

In order to create a more efficient way to grow fruits and vegetables, many producers have turned to pesticides. As a result, humans and animals are internally exposed to these pesticides through consumption. According to previous research, pesticides affect the growth of gut bacteria in other animals: Mice exposed to benzimidazole carbendazim, a popular fungicide similar to captan, had decreased amounts of Bacteroidetes and Verrucomicrobia in their gut (1). Bees have also experienced negative side effects, such as decreased immune function and increased pathogen colonization, after exposure to pesticides (2). In humans, pesticide exposure in adult twins caused a decrease in the amount of Firmicutes bacterium CAG:110 (3). However, there is no current research available about the pesticide captan and how it influences our microbiota.

We chose the pesticide captan specifically because it is very prevalent in the United States; it is currently used in over 100 products in various forms (4). This study aimed to understand the effect of captan on the growth of bacteria found in the human microbiome as a means to begin to elucidate the effect of captan consumption on human health. Due to their increased prevalence, it is necessary that humans understand the effect that the consumption of produce with traces of pesticides has on their gut bacteria.

The health of the body relies heavily on a balance of bacteria, as difficulties can occur following disparities. An unevenness of healthy and unhealthy bacteria in the gut can lead to a multitude of conditions, such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and, in severe cases, type 2 diabetes, obesity, and atopy (5). Because of these serious possible side effects, we realized that an investigation of captan's effects on the gut was necessary. In order to do this, we chose two bacteria, *Escherichia coli* K-12 and *Bacillus coagulans*, to model the gastrointestinal system of a human. While *E. coli* K-12 is not naturally found in the human gut microbiome, it can be used as a model for these other strains.

Since humans cannot naturally digest some compounds we ingest, some strains of naturally-occurring *E. coli* help break down these compounds and release nutrients that would have previously been unusable (6). *B. coagulans* is a probiotic that also helps aid in the digestion of compounds, specifically carbohydrates and proteins (7). Since some strains of *E. coli* and *B. coagulans* are important to gut health, we chose to model them in our research. Based on results from previous studies, we hypothesized that the captan exposure would inhibit the growth of both *E. coli* and *B. coagulans*. In order to assess this, we exposed the bacteria to the pesticide captan and measured the growth we observed on the plates. In support of our hypothesis, bacterial growth was significantly reduced when exposed to captan.

RESULTS

Previous research has shown that pesticides can have negative effects on bacterial growth. In order to investigate this topic, *E. coli* and *B. coagulans* were exposed to captan, a pesticide commonly used in the United States. These specific bacteria were chosen because an imbalance of these in the gut environment can cause a multitude of medical issues. The results of this experiment could inform frequent produce consumers about the possible dangers of pesticide consumption.

After concluding data collection, we used a one-tailed t-test for two independent means from Social Science Statistics to determine the significance of the data (8). The mean for the captan group of *E. coli* was significantly less than the mean of the control group for the same bacteria ($p = 0.00134$) (**Figure 1**). The significance of the *B. coagulans* data was also determined. The mean for the captan group of *B. coagulans* was significantly less than the mean of the control group for the same bacteria ($p = 0.000284$) (**Figure 2**).

Based on the results of the significance testing, we can conclude that there is suitable evidence to reject our null

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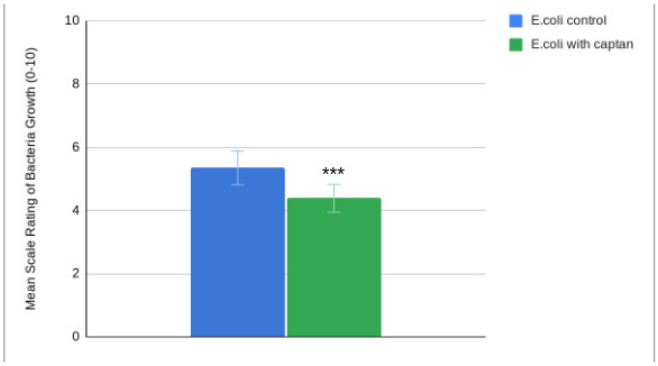

Figure 1. Captan treatment reduces growth of E. coli. Growth was measured on 55 control and 54 captan-containing E. coli plates using a 1-10 rating system. The average rating for the control (blue) and captan-containing (green) groups are shown. The mean scale rating of the control group is 5.35 and the captan group is 4.39. The t-value between these two groups is 3.07411, which corresponds to a p-value of 0.00134. The captan groups are significant compared to the control groups. $p < 0.05$ is shown as ***. Error bars represent the standard deviation in the data.

hypotheses. The primary null hypothesis stated that captan exposure does not have an inhibitory effect on the growth of *E. coli*, and the secondary null hypothesis stated that captan exposure does not have an inhibitory effect on the growth of *B. coagulans*. Since such low p-values were obtained from the data, it is reasonable to assume that these hypotheses are not supported. Evidence suggests that the opposite is the case and captan does inhibit the growth of both bacteria.

DISCUSSION

Our goal for this experiment was to investigate a possible negative relationship between captan exposure and the growth of *E. coli* and *B. coagulans*. Previous research from Giambò and Federica showed that a pesticide similar to captan had a negative impact on the growth of certain gut bacteria not examined in our study (1). Thus, our findings supported these results. Prior to testing, we created two hypotheses: the first stated that the captan would have an inhibitory effect on the growth of *E. coli*, the second stated that the captan would have an inhibitory effect on the growth of *B. coagulans*. The data supported both. Since the final p-values of both significance tests were below 0.05, it is reasonable to assume that our hypotheses are supported and captan does have an inhibitory effect on the growth of both bacteria (**Figure 1, Figure 2**). These p-values suggest that the ingestion of the pesticide captan may have an impact on the human gut microbiome. Although these bacteria were tested outside of the human body, we hypothesize that we would also find similar results if captan was ingested by mice and tested *in vivo*.

Like all experiments, there were limitations to our research. One limitation was that we were only able to model the bacteria found in the human gut. Although *B. coagulans* is a popular gut bacterium, *E. coli* K-12 is not naturally-occurring and can only be used as a model for the naturally-occurring strains. Another limitation was our pesticide dilution. While we found varying information on captan quantity regulations and residue, we used the amount of 0.6 parts per million, which was found on an EPA database. However, this number is within the range for a portion of the regulation quantities for this pesticide, and the level of captan on the surface of fruits and vegetables most likely varies (9). A possible future experiment could involve swabbing the surface of various produce items, calculating the captan levels, and testing the impact that these different levels could have on the microbiome. While we hypothesize that captan would have an inhibitory effect on the growth of bacteria, there could possibly be different results when compared to different levels of the pesticide on fruits and vegetables.

Due to increased usage of pesticides, it is necessary to understand the impact that this could have on daily life. Captan is regularly used on crops such as apples, peaches, strawberries, and almonds. In total, captan is included in approximately 100 products in use today, as well as used on its own (4). We found that exposure to this pesticide can cause a decrease in the growth of both *E. coli* and *B. coagulans*. In order to keep these bacteria safe, some measures must be taken. This includes, according to our data, possibly limiting the exposure to the pesticide captan.

MATERIALS AND METHODS

Preparing bacterial plates

Initially, four groups were created: *E.coli* control, *E.coli* with captan, *B. coagulans* control, and *B. coagulans* with captan. Control *E.coli* and *B. coagulans* were grown on 10mL Carolina Nutrient Agar 1.5% mixed with 5 mL of distilled water. Captan *E. coli* and *B. coagulans* groups were grown on 10mL Carolina Nutrient Agar 1.5% mixed with 5 mL of a captan solution. The captan pesticide was diluted to 0.6 parts per million in distilled water in order for the quantity to be relevant with how much of it is consumed through commercial produce. In order to mix the agar and the water/dilution, the agar was fully melted and then mixed with the water in a petri dish. Plates were left to set for 24 hours before streaking.

Streaking and growing bacteria

After the allotted setting period, the plates were considered firm enough to streak. Bench Paper was placed on the surface of the table to prevent any possible contamination

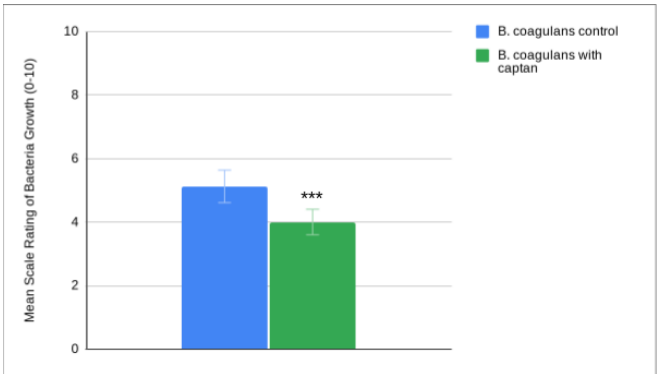

Figure 2. Captan treatment reduces growth of B. coagulans. Growth was measured on 38 control and 37 captan-containing B. coagulans plates using a 1-10 rating system. The average rating for the control (blue) and the average rating for the captan-containing (green) groups are shown. The mean scale rating of the control group is 5.13 and the captan group is 4.01. The t-value between these two groups is 3.6049, which corresponds to a p-value of 0.000284. The captan groups are significant compared to the control groups. $p < 0.05$ is shown as ***. Error bars represent the standard deviation in the data.

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when making the plates. The streaking process was done using an inoculating loop, which measured 10 µl of each bacterial strain. Although the *E.coli* already came in a nutrient broth mixture, *B. coagulans* had to be extracted from on top a solid agar block. In order to use the inoculating loop, which only measured liquids, a small portion of the *B. coagulans* was extracted from the top of the agar block by scraping the loop across the top of the agar. Then, this sample was stirred into Carolina Nutrient Broth. This mixture was given 24 hours to incubate in the vial so more of the bacteria could grow. To streak, a back-and-forth motion was used to ensure that the plates were evenly covered and that individual colonies could be seen. This streaking was done twice; the second time through, the plate was rotated 90 degrees from the original position to ensure all parts of the plate were covered. The inoculating loop was flamed in between each plate to prevent cross-contamination. After streaking, Parafilm was wrapped around the edges of all finished plates to ensure no foreign bacteria affected the data, as suggested by Dr. Ed Robinson. The prepared plates were then placed in an incubator set at 37℃ to mimic the inside temperature of the human gut. Both bacteria were then left to incubate. *E. coli* incubated for 24 hours and *B. coagulans* incubated for 96 hours because of its slower growth (10). After incubation was complete, pictures were taken and the data was recorded.

Counting bacterial growth

We developed a unique counting method using clear grid paper in the shape of an agar plate to quantify the growth. Each 0.8 by 0.8 centimeter square within the grid was numbered from 1-135 and placed in a random number generator. Once a square was selected, a scale rating system was used to determine the amount of growth based on coverage of the plate from a scale of 0 (no growth) to 10 (total growth) (**Figure 3**). A scale rating of 10 is not included in the visuals because any growth observed that seemed to exceed the rating of a 9 was considered a 10. Similarly, a scale rating of 0 is also not included because any square with no visible growth was considered a 0. Five squares were randomly selected per plate and each was matched to the scale rating that most closely represented the amount of growth in that square. While this data was collected with us knowing which group the plates belonged in, the ratings were chosen as accurately as possible with how they related to the references in the appendix.

The ratings were recorded and the mean scale rating was

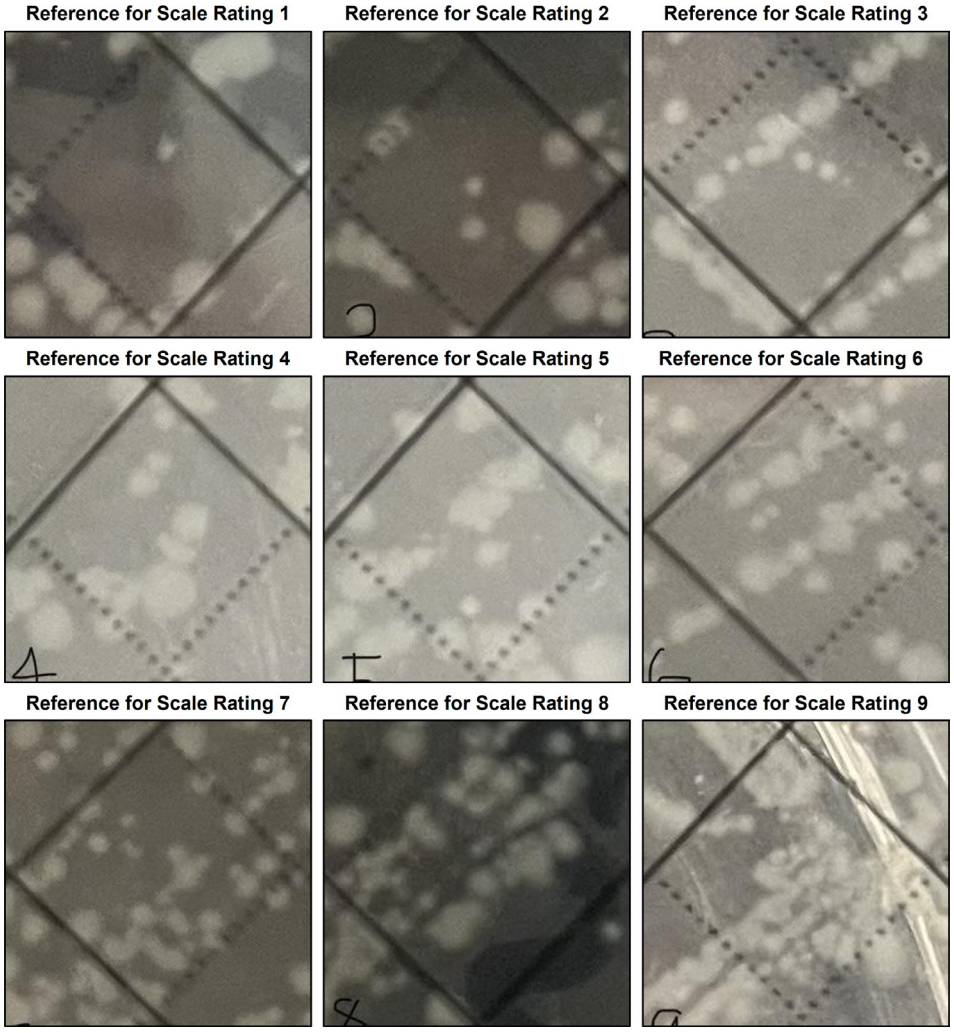

Figure 3: References for scale ratings. These plates picture E. coli grown at the control condition. Several different plates were used to create these references. These scale rating images were the reference for determining the growth on each bacteria plate.

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calculated for each individual plate. Then, all individual means for each plate were combined in a specific group to create an overall mean and standard deviation for each group. All data recorded were compiled into four mean values: *E. coli* control, *E. coli* captan, *B. coagulans* control, and *B. coagulans* captan. These values were used in the significance testing. To ensure a normal distribution, over 30 plates were counted from each group. Overall, 55 plates for *E. coli* control, 54 plates for *E. coli* captan, 38 plates for *B. coagulans* control, and 37 plates for *B. coagulans* captan were produced.

Statistical analysis

After all measurements were taken and mean values were calculated for each of the 4 groups, we used the significance test from Social Science Statistics to determine the significance of the data (8). We did this in order to determine the validity of our null hypotheses, the primary which stated that captan would not have an inhibitory effect on *E. coli* and the secondary which stated that captan would not have an inhibitory effect on *B. coagulans*. Using a one-tailed test for two independent means, we tested each captan group against its corresponding control group for each bacteria. After testing, the means for both captan groups were shown to be significantly less than their corresponding control groups (E. coli: $p = 0.00134$, B. coagulans: $p = 0.000284$). Therefore, since $p < 0.05$, there was suitable statistical evidence to reject our null hypotheses, which stated that the pesticide captan does not have inhibitory effects on the growth of these bacteria. Evidence suggests that the opposite is the case, and captan does inhibit the growth of both *E. coli* and *B. coagulans*.

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