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Article

Effect of heme vs. non-heme iron supplements on gut microbiome fitness

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SUMMARY

About 80% of people around the world suffer from iron deficiency, and doctors suggest taking over-the-counter supplements. However, dietary iron comes in two main forms: heme and non-heme. Both affect the carefully held balance of bacteria in our gut differently. This project studied the influence of heme and non-heme iron on the growth of pathogenic *Serratia marcescens* and non-pathogenic *Escherichia coli* bacteria. We hypothesized that the growth of bacteria would increase as the iron concentration rose, with the influence of heme iron being stronger than that of non-heme iron, as numerous previous studies have found that heme iron is more readily absorbed. In the growth medium, we grew *E. coli* and *S. marcescens* separately in no-iron control plates, increasing concentrations of heme or non-heme iron, or 0.5 µg/mL Ciprofloxacin, an antibiotic that kills bacteria. Then, we grew the bacteria together in a moderate concentration of non-heme or heme iron to test species competition. The results largely confirmed the hypothesis. In higher iron concentrations, both heme and non-heme iron elevated bacterial growth, suggesting that a greater quantity of free iron promotes bacterial growth. However, in the highest iron concentration of heme or non-heme iron, bacterial growth decreased, as overabsorption caused cytotoxicity. Heme iron plates had a higher bacterial growth than non-heme iron plates likely due to better iron absorption. The large magnitude by which the growth of *S. marcescens* was increased by heme iron in comparison to *E. coli* demonstrates the potential dangers of overconsumption of heme iron and suggests that some pathogenic bacteria can conduct iron absorption more efficiently. As ingesting too much iron can lead to dysbiosis and intestinal diseases, the conclusion of this study makes an important contribution to the relative safeties of heme and non-heme iron consumption.

INTRODUCTION

Iron is a nutrient essential to most forms of life. In humans, iron is a major component of hemoglobin, the protein in red blood cells used to carry oxygen from human lungs to other parts of the body. Iron also plays a key role in brain and muscle function (1). Iron deficiency, which impacts 80% of people around the world, can lead to anemia, a blood condition characterized by symptoms like fatigue, pale skin color, and decreased immunity (2). Doctors often instruct those who are iron deficient to take over-the-counter iron supplements, but there are many different options to choose from (3).

Digesting iron is essential for the growth of a complex ecosystem of Coliform bacteria, most of which are beneficial and non-pathogenic, inside the human gut. Coliform bacteria are rod-shaped bacteria located in the human intestines (4). A condition known as dysbiosis refers to the state of imbalance in the gut microbiome, which is associated with reduced diversity of bacterial strains. This may lead to certain intestinal diseases (4). Several other diseases such as cancer, autoimmune, and neurodegenerative disorders are now also thought to be influenced by the gut microbiome (5).

Escherichia coli (*E. coli)* and *Serratia marcescens* (*S. marcescens*) are both Gram-negative bacteria, meaning they have an outer lipid membrane and a thin peptidoglycan layer underneath. *E. coli* are found in the lower intestine of people and are typically harmless. They are an important part of a healthy human intestinal tract and prevent the uncontrolled growth of harmful bacteria (6). *S. marcescens*, on the other hand, is an opportunistic pathogen often found in the natural environment and can reach the gut through contaminated food or water. It is associated with hospital-acquired infections and high antimicrobial resistance (7). The gastrointestinal tract is recognized as the predominant location for *S. marcescens* in the human body, as is the case for most members of the Enterobacteriaceae family (8). In bacteria, iron is crucial to the function of proteins controlling DNA replication, RNA synthesis, and cellular respiration (9).

Dietary iron comes in two main forms: heme, found in animal flesh like meat, poultry, and seafood, and non-heme, found in plant foods like whole grains, nuts, seeds, legumes, and leafy greens (10). Heme iron is more bioavailable than non-heme iron and is absorbed more readily into the bloodstream than non-heme iron since it has a more direct absorption pathway through enterocytes, cells lining the intestines (11). The bioavailability of iron in food refers to how easy it is for the body to absorb the iron (10). Heme iron is characterized by the heme protein attached to a lone iron atom (11). The human body absorbs around 15 - 35% of heme iron consumed. Around 2 - 20% of non-heme iron, with no heme protein, is absorbed by the human body, indicating a more controlled absorption rate (10).

Iron in our gut can either be bound to special molecules,

like heme, or unbound (free iron) (12). Pathogenic bacteria, including *S. marcescens*, prefer heme iron. This reliance on heme acquisition has not been thoroughly studied and cannot be clearly explained. Heme iron is also a part of hemoproteins which are responsible for the electron transport chain and cellular respiration in bacteria (13). After iron is absorbed into the bloodstream through our small intestine, the remaining, unabsorbed iron moves on to the colon and interacts with the host-microbiota interface (13). However, since iron is limited, the bacteria compete to sequester the iron that is available. Different bacteria develop different mechanisms to collect iron from their host environment and take the iron stored in other molecules (12). Many bacteria secrete iron-chelating compounds called siderophores to collect free iron from the extracellular environment (14).

Beneficial bacteria are often at a disadvantage when acquiring iron since pathogenic bacteria frequently have many more ways to sequester iron for themselves (4). Usually, bacteria require 0.02 to 0.50 µg/mL of iron in the environment to grow, though not all the iron can be absorbed due to the limited solubility of inorganic iron in a non-acidic microenvironment (15). Since iron is usually found in ferric form (Fe^{3+}) in foods, it must be dissociated by gastric acid into Fe^{2+} before it can be transported and absorbed by the body (15).

Though iron is essential for the biological processes bacteria are involved in, an excess of this element promotes the formation of free radicals, leading to damaging oxidative and nitrosative stress (16). An excess amount of iron can lead to iron toxicity, an accumulation of iron in the large intestine (10). Cells have mechanisms to limit iron toxicity. The ferric uptake regulator (Fur) protein regulates the cellular iron level by restricting the intake of free iron. The iron citrate efflux transporter (IceT) protein reduces levels of cellular iron and protects cells from death from iron overload. While bacteria can compensate for elevated free iron levels, these responses are likely less rapid than the immediate increase in free iron, causing a cytotoxic effect in some cells (16).

To understand the relative safeties of heme and non-heme iron, our study investigated the effect of different concentrations of heme and non-heme iron on *E. coli* and *S. marcescens*. We hypothesized that the growth of both non-pathogenic *E. coli* and pathogenic *S. marcescens* would increase with increasing concentration of iron but would increase more with heme than non-heme iron. We also hypothesized that an iron overload environment would be toxic to both *E. coli* and *S. marcescens*.

Our results supported our hypotheses. We concluded that both heme and non-heme iron promote bacteria growth in a dose-dependent manner, though an excess of either type of iron in the environment resulted in the death of both pathogenic and non-pathogenic bacteria. Additionally, heme iron promotes the growth of pathogenic bacteria better than non-pathogenic bacteria. Furthermore, heme iron was more efficient at promoting growth than non-heme iron. This imbalance of pathogenic and non-pathogenic bacteria is shown to be harmful for our gut and causes various diseases (4).

RESULTS

We sought to understand the effect of heme versus non-heme iron on the gut microbiome by exposing pathogenic *S. marcescens* and non-pathogenic *E. coli* gut bacteria to different concentrations of heme and non-heme iron. We grew the bacteria individually in 1, 5, and 10 µg/mL of heme or non-heme iron, and then we grew the bacteria together in

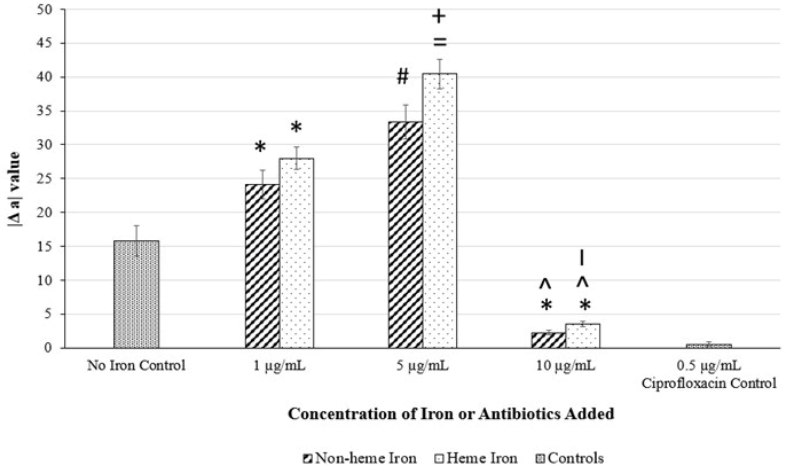

Figure 1: Effect of non-heme and heme iron on *E. coli* growth. We plated E. coli in the no-iron control, 1, 5, or 10 µg/mL concentrations of heme or non-heme iron, and the 0.5 µg/mL Ciprofloxacin control. The change in growth (|Δ*a*|) of *E. coli* between 0 and 48 hours was calculated. We averaged the data from 3 trials and calculated the mean ± standard deviation of each reading, as shown. We conducted one-way ANOVA tests and Tukey tests. **p* < 0.05 compared with no-iron control. #*p* < 0.05 compared with 1µg/mL non-heme iron plate. =*p* < 0.05 compared with 1 µg/mL heme iron plate. +p < 0.05 compared with 5 µg/mL non-heme iron plate. |*p* < 0.05 compared with 10 µg/mL non-heme iron plate. ^ *p*< 0.05 compared with the 0.5 µg/mL Ciprofloxacin control.

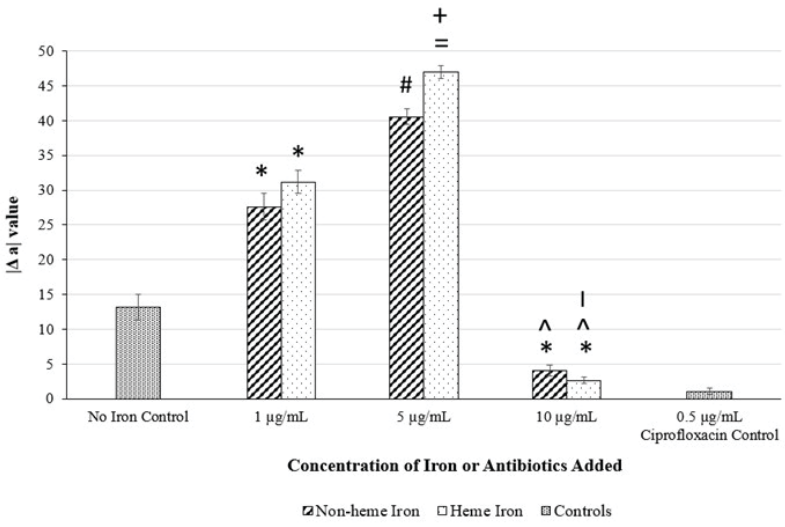

Figure 2: Effect of non-heme and heme iron on *S. marcescens* growth. We plated *S. marcescens* in the no-iron control, 1, 5, or 10 µg/mL concentrations of heme or non-heme iron, and the 0.5 µg/mL Ciprofloxacin control. The change in growth (|Δ*a*|) of *S. marcescens* between 0 and 48 hours was calculated. We averaged the data from 3 trials and calculated the mean ± standard deviation of each reading, as shown. We conducted one-way ANOVA tests and Tukey tests. **p* < 0.05 compared with no-iron control. #*p* < 0.05 compared with 1 µg/mL non-heme iron plate. =*p* < 0.05 compared with 1µg/mL heme iron plate. +*p* < 0.05 compared with 5 µg/mL non-heme iron plate. |*p* < 0.05 compared with 10 µg/mL non-heme iron plate. ^*p* < 0.05 compared with the 0.5 µg/mL Ciprofloxacin control.

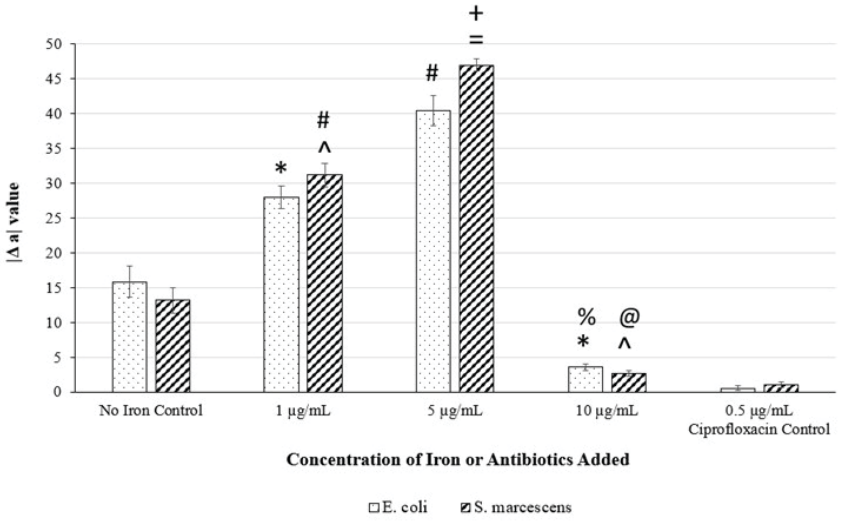

Figure 3: Growth of *E. coli* and *S. marcescens* in heme iron. We plated either *E. coli* or *S. marcescens* in the no-iron control, 1, 5, or 10 µg/mL concentrations of heme iron and the 0.5 µg/mL Ciprofloxacin control. The change in growth (|Δ*a*|) of bacteria between 0 and 48 hours was calculated. We averaged the data from 3 trials and calculated the mean ± standard deviation of each reading, as shown. We conducted one-way ANOVA tests and Tukey tests. **p* < 0.05 compared with the *E. coli* no-iron control plate. ^*p* < 0.05 compared with the *S. marcescens* no-iron control plate. #*p* < 0.05 compared with the *E. coli* 1 µg/mL iron plate. =*p* < 0.05 compared with the *S. marcescens* 1 µg/mL iron plate. +*p* < 0.05 compared with the *E. coli* 5 µg/mL iron plate. %*p* < 0.05 compared with the *E. coli* 0.5 µg/mL Ciprofloxacin control plate. @*p* < 0.05 compared with the *S. marcescens* 0.5 µg/mL Ciprofloxacin control plate.

the same Petri dish in 5 µg/mL of heme or non-heme iron to test species competition. We added no iron for one negative control and added 1 µg/mL Ciprofloxacin, an antibiotic, for a second control. We used the a reading, which measured the green and red hue of the Petri dish, on the CIELAB color scale to quantify bacterial growth. We report the Δ*a* and |Δ*a* values|, which represents the change in bacterial growth from 0 hours to 48 hours, in the experimental conditions. A positive Δ*a* value represents an overall pink-red hue, the color of the *S. marcescens* colonies in Coliscan Easygel, indicating more *S. marcescens* growth. A negative Δ*a* value represents an overall blue-green hue, the color of *E. coli* colonies in Coliscan Easygel, indicating more *E. coli* growth. We conducted one-way ANOVA statistical tests and Tukey HSD

post-hoc tests to compare the bacterial growth of different species.

The |Δ*a*| value of both the *E. coli* plate and *S. marcescens* plate in 1 µg/mL heme iron was greater than the |Δ*a*| of their respective no-iron controls ($p < 0.01$). The |Δ*a*| values increased further in 5 µg/mL of heme iron ($p < 0.01$). Non-heme iron plates followed the same pattern, with both species having a greater |Δ*a*| value in their 1 µg/mL iron plates than in their no-iron controls ($p < 0.05$), and an even greater |Δ*a*| value in their 1 µg/mL iron plates than their 5 µg/mL iron plates ($p < 0.01$) **(Figure 1)**. The |Δ*a*| value in the 5 µg/mL non-heme Petri dishes was less than the |Δ*a*| value in the 5 µg/mL heme Petri for both *E. coli* and *S. marcescens* ($p < 0.05$). In 10 µg/mL of heme or non-heme iron, the |Δ*a*| value was lower than that in the no-iron control or other iron concentrations ($p < 0.01$) **(Figure 2)**. These data indicate that the species grow in the presence of both heme and non-heme iron, though more so with heme iron, until toxic iron levels are reached.

In the 1 µg/mL and 5 µg/mL heme iron Petri dishes, the |Δ*a*| value of *S. marcescens* was greater than the |Δ*a*| value of *E. coli* ($p < 0.05$) **(Figure 3)**. There was also greater *S. marcescens* growth than *E. coli* growth in 5 µg/mL non-

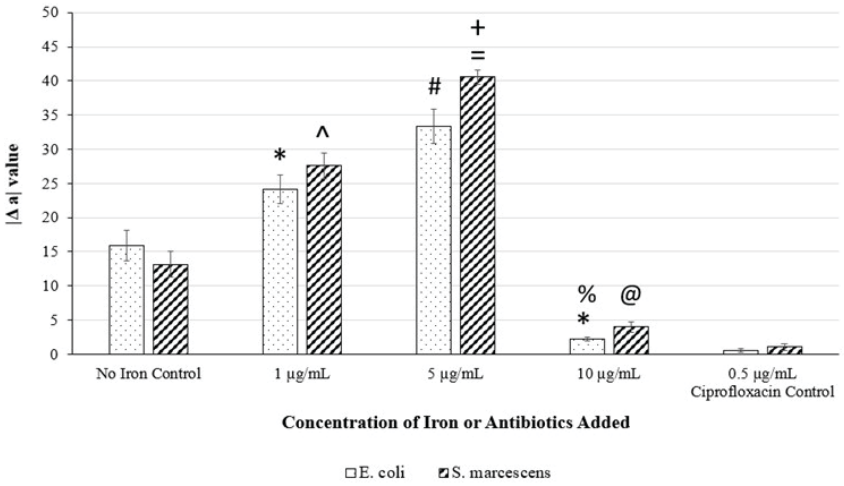

Figure 4: Growth of *E. coli* and *S. marcescens* in non-heme iron. We plated either *E. coli* or *S. marcescens* in the no-iron control, 1, 5, or 10 µg/mL concentrations of non-heme iron, and the 0.5 µg/mL Ciprofloxacin control. The change in growth (|Δ*a*|) of bacteria between 0 and 48 hours was calculated. We averaged the data from 3 trials and calculated the mean ± standard deviation of each reading, as shown. We conducted one-way ANOVA tests and Tukey tests. *$p < 0.05$ compared with the *E. coli* no-iron control plate. ^$p < 0.05$ compared with the *S. marcescens* no-iron control plate. #$p < 0.05$ compared with the *E. coli* 1 µg/mL iron plate. =$p < 0.05$ compared with the *S. marcescens* 1 µg/mL iron plate. +$p < 0.05$ compared with the *E. coli* 5 µg/mL iron plate. %$p < 0.05$ compared with the *E. coli* 0.5 µg/mL Ciprofloxacin control plate. @$p < 0.05$ compared with the *S. marcescens* 0.5 µg/mL Ciprofloxacin control plate.

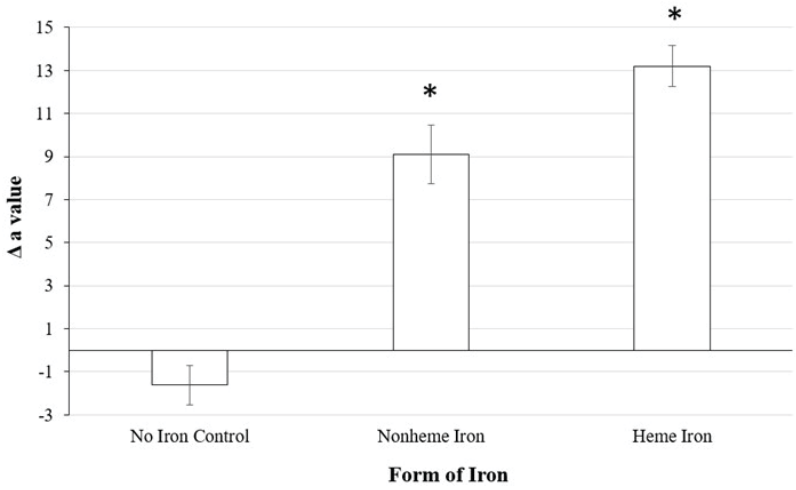

Figure 5: Growth of *E. coli* and *S. marcescens* in 5 µg/mL heme and non-heme iron. We plated both *E. coli* and *S. marcescens* together in the no-iron control and 5 µg/mL of heme or non-heme-iron and measured the Δ*a* value reading of the Petri dishes after 48 hours. A negative Δ*a* value means there was greater *E. coli* growth than *S. marcescens* growth. A positive Δ*a* value means there was greater *S. marcescens* growth than *E. coli* growth. We averaged the data from 3 trials and calculated the mean ± standard deviation of each reading, as shown. We conducted one-way ANOVA tests and Tukey tests. *$p < 0.05$ compared with no-iron control. #$p < 0.05$ compared with 5 µg/mL non-heme plate.

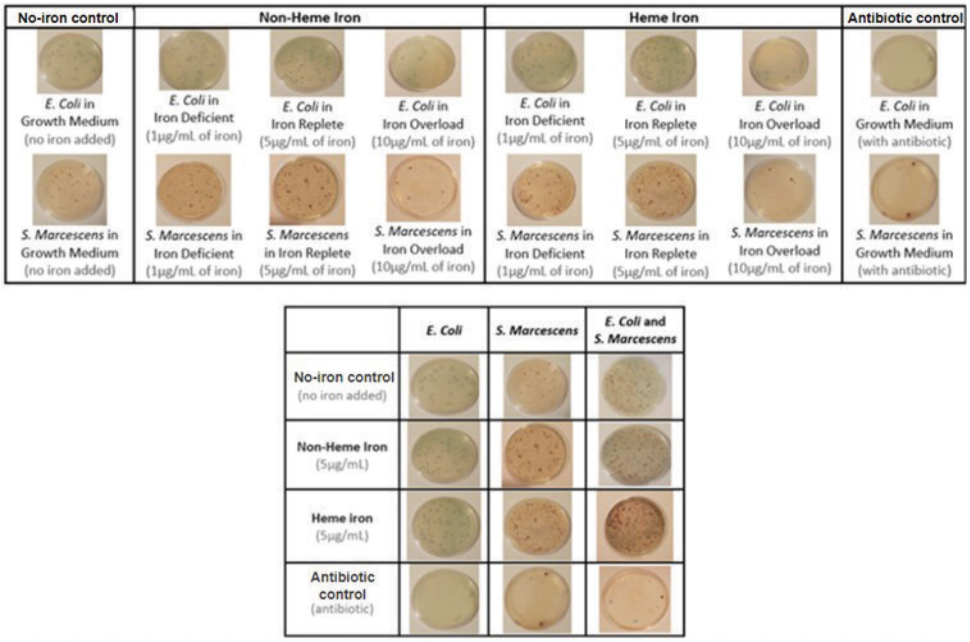

Figure 6: Bacterial growth on Petri dishes after 48 hours. We plated the bacteria in the no-iron control, 1, 5, or 10 µg concentrations of heme or non-heme iron, and the 0.5 µg/mL Ciprofloxacin control. The bacterial growth on the Petri dishes after 48 hours qualitatively confirms the data obtained using the colorimeter.

heme iron (p-value < 0.01), though the growth was similar in 1 µg/mL non-heme iron (p > 0.05) **(Figure 4)**. When *E. coli* and *S. marcescens* were grown in the same Petri dish, the Δ*a* value was -1.6 in the no-iron control, 9.1 in 5 µg/mL of non-heme iron, and 13.2 in 5 µg/mL of heme iron. This implies more *E. coli* growth occurred in the no-iron control and more *S. marcescens* growth occurred in the 5 µg/mL heme iron plate and 5 µg/mL non-heme iron plate. The ratio of *S. marcescens* growth to *E. coli* growth in the plates with both *E. coli* and *S. marcescens* was greater in the non-heme group than the no-iron control (p < 0.01). It also increased from the non-heme plate to heme plate (p < 0.01) **(Figure 5)**. The data has been qualitatively confirmed by the Petri dishes after 48 hours **(Figure 6)**.

DISCUSSION

This research studied the effect of different concentrations of heme and non-heme iron on the growth of pathogenic and non-pathogenic bacteria. We predicted that the growth of both *E. coli* and *S. marcescens* would increase with increasing concentrations of iron but would increase more with heme than non-heme iron. We also hypothesized that an iron overload environment would be toxic to both *E. coli* and *S. marcescens*. The results largely support this hypothesis. We saw that, when grown in 1 µg/mL of heme iron, both the *E. coli* growth and *S. marcescens* growth was greater than when grown without iron, and when grown in 5 µg/mL heme iron, the growth increased further. *S. marcescens* and *E. coli* growth in non-heme iron followed the same pattern, with increased growth in 1 µg/mL and 5 µg/mL non-heme iron. The bacterial growth in 5 µg/mL of heme iron was greater than the bacterial growth in 5 µg/mL of non-heme iron for both bacterium types. This agrees with the fact that *E. coli* and *S. marcescens* grow faster with more iron but absorb heme iron better than non-heme iron (13). We can see that in certain iron concentrations heme iron is better for the growth of these species.

When grown in 10 µg/mL of heme or non-heme iron, the bacterial growth was less than that in the no-iron control or other iron concentrations, but they still had a larger |Δ*a*| value than the Ciprofloxacin control, meaning that this iron concentration did not kill as many bacteria as the antibiotic. The data shows that too much iron can lead to cytotoxicity and prevent the growth of the bacteria. Though bacteria have mechanisms such as Fur or IceT protein to limit the iron uptake, these responses are not as prompt as the iron absorption, resulting in bacterial death (16).

In 1 µg/mL or 5 µg/mL of heme and 5 µg/mL of non-heme iron, *S. marcescens* grew more than *E. coli*. This data shows that the pathogenic bacteria, *S. marcescens*, absorbs more iron than the non-pathogenic bacteria, *E. coli*, since pathogenic bacteria generally have more mechanisms to sequester iron (4). To confirm these results, an experiment with both bacteria in 5 µg/mL of iron was conducted, and a similar outcome was found. The Δ*a* value for the growth of *E. coli* and *S. marcescens* in the same Petri dish with no iron present was less than zero. This indicates that more *E. coli* grew than *S. marcescens* on that plate. In 5 µg/mL of non-heme iron, the average Δ*a* value was larger than the Δ*a* value of the no-iron control, indicating that the amount of *S. marcescens* significantly increased from the control when some iron was added. In 5 µg/mL of heme iron the average Δ*a*

value was even larger than the Δ*a* value of plate with 5 µg/mL of non-heme iron. This suggests that heme iron promotes the growth of pathogenic bacteria more than non-heme iron since it is absorbed better, and the pathogenic bacteria can sequester more iron.

Overall, this experiment demonstrates that heme iron promotes bacterial growth, especially the growth of *S. marcescens*, the pathogenic bacteria, more than non-heme iron. In general, greater iron concentrations lead to more bacterial growth of both species. However, too much iron can cause bacterial death, due to iron toxicity, and often leads to the dysbiosis.

If the dosage is not monitored correctly, taking too many iron supplements could potentially cause a similar effect, which in turn is associated with increased risk for developing several other diseases (15). Though heme iron supplements may seem better due to greater absorption by our intestine, non-heme iron is generally safer since it has a more controlled absorption rate (4). An overconsumption of red meat, abundant with heme iron, may also promote pathogenic gut bacteria. Iron deficiency is a wide-spread issue, but direct blood infusions, rather than ingesting iron supplements, can be used as an alternative treatment (2). Likewise, a temporary reduction of iron consumption could be used to inhibit the pathogenic bacterial growth in the gut in particularly harmful bacterial infections. Those who do take over-the-counter dietary iron supplements should use them in moderate quantities and regularly monitor their iron levels through blood tests to reduce the risk of intestinal diseases.

The limited availability of resources impacted the data collected in this study. For greater accuracy in the Δ*a* measurements, we would have preferred a spectrophotometer over a colorimeter, and we could have conducted more trials. Lab sterility and incubator temperature fluctuations could have caused errors in the data collected. For further research, we can test various other pathogenic and non-pathogenic bacteria with heme and non-heme iron. This research may not represent all gut bacteria due to the various methods for iron absorption. Rather than using Gram-negative bacteria, we could use Gram-positive bacteria, which have different absorption mechanisms, growth rates, and more vulnerability to antibodies (6). These differences could possibly impact the results of the experiment. Additionally, we could conduct studies on mice, by altering their diet, to confirm how types of iron affect their gut microbiome. In order to apply the conclusions from this research in food and health industries, we must study the effect of long-term human intake of heme or non-heme iron. This research makes important contributions to understanding the relative safeties of heme and non-heme iron supplementation, since 80% of people around the world suffer from iron-deficiency (2).

MATERIALS AND METHODS

We prepared three trials of plates for the following experimental groups: no-iron control *E. coli*; no-iron control *S. marcescens*; 1 µg/mL Ciprofloxacin *E. coli*; 1 µg/mL Ciprofloxacin *S. marcescens*; 1 µg/mL, 5 µg/mL, and 10 µg/mL non-heme iron and heme iron *E. coli*; 1 µg/mL, 5 µg/mL, and 10 µg/mL non-heme iron and heme iron *S. marcescens*; no-iron control *S. marcescens* and *E. coli*; 1 µg/mL Ciprofloxacin *S. marcescens* and *E. coli*; and 5 µg/mL non-heme iron and heme iron *S. marcescens* and *E. coli*. We used a non-pathogenic *E. coli* strain and a pathogenic *S. marcescens* strain.

Easygel and Petri Dish Preparation

To plate bacteria in petri dishes, we used a sterile inoculating loop to add 1 BactoBead of the respective bacteria (Edvotek) to 15 mL of Coliscan Easygel (Micrology Laboratories), and we swirled the Easygel solution. For the non-heme Easygel plates, we added 2.2, 10.8, or 21.5 mg of non-heme iron to 135 mL of Coliscan Easygel with bacteria cells to create 1, 5, or 10 µg/mL iron medium stock in a 250 mL flask, respectively. For the heme Easygel plates, we added 4.3, 21.7, or 43.5 mg of heme iron to 135 mL of Coliscan Easygel with bacteria cells to create 1, 5, or 10 ug/mL iron medium stock in a 250 mL flask, respectively. For the antibiotic Easygel plates, we added 20 mg of Ciprofloxacin powder to 125 mL of distilled water. From the antibiotic solution, we added 1 mL into 15 mL of Coliscan Easygel solution containing bacteria cells. For each plate, we poured 15 mL of Easygel into the fresh calcium-treated Petri dish, and we gently swirled the plate. We placed the plate in 35°C incubator for 90 minutes to allow gel to solidify, before we inverted the plate and allowed the bacteria to grow.

Data Collection

We removed the Petri dishes from the incubator at 0 hours, 12 hours, 36 hours, and 48 hours post-inoculation. The Coliscan Easygel growth medium allowed differentiation of *E. coli* as green-blue and *S. marcescens* as pink-red colonies. We used a colorimeter to find the L*a*b* reading of each plate using the CIELAB color space. The *a* reading, which measured the green and red hue of the Petri dish, was collected to determine bacteria growth. A positive *a* value represents an overall pink-red hue and the growth of *S. marcescens* colonies. A negative *a* value represents an overall blue-green hue and the growth of E. coli colonies. We calculated the Δ*a* value by subtracting the base value of a (0-hour reading) from the value of *a* at 48 hours. This showed the change in color, and therefore the bacterial growth, that occurred over 48 hours. A negative Δ*a* value indicated *E. coli* growth and a positive Δ*a* value indicated *S. marcescens* growth. The greater the |Δ*a*| value was, the stronger the color was, so more bacterial growth occurred. Since the pink-red and blue-green are opposite colors, they balance each other out. In the Petri dishes with *E. coli* and *S. marcescens* grown together, an overall negative Δ*a* value indicated more *E. coli* growth, and an overall positive Δ*a* indicated more *S. marcescens* growth. We conducted one-way ANOVA

statistical tests and Tukey tests using VassarStats, where the data points were inserted to calculate the *p* value. First the data from all of the *E. coli* plates were compared, then the data from the *S. marcescens* plates, followed by the data from the heme iron plates, and then the non-heme iron plates. Each group was compared not only with the data from each other's plates, but also the data from the appropriate controls. If the *p*-values in the tests were less than 0.05, the results were statistically significant.

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